Determining the Effects and Signaling Properties of β-Blockers in House Dust Mite-Driven Murine Models of Asthma

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Determining the Effects and Signaling Properties of β-Blockers in House Dust Mite-Driven Murine Models of Asthma

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I dedicate this dissertation to mom, dad,

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for their unconditional love and support

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Abstract

Asthma is a chronic inflammatory disease of the airways affecting more than 300 million people worldwide. Some characteristic features of asthma include airway inflammation, mucus hypersecretion, airway hyper-responsiveness and remodeling causing clinical symptoms such as recurrent coughing, wheezing, chest tightness, and shortness of breath. As often patients require life-long management of these symptoms, health care costs associated with asthma management are continually increasing, particularly in developed countries such as the United States, where asthma is highly prevalent.

The gold-standard drugs for asthma therapy include inhaled corticosteroids (ICS) and β_2-adrenoceptor (β_2AR) agonists. However, chronic monotherapy with β_2AR agonists has been associated with worsening of asthma control and an increase in asthma-related mortality. Previous studies in our laboratory discovered that agonist activation of the β_2AR is actually required for developing the asthma phenotype in ovalbumin-driven murine models of asthma. Furthermore, our studies suggested that chronic treatment with certain β-blockers such as nadolol and ICI 118, 551 was able to attenuate the asthma phenotype in murine models. However, not all β-blockers seemed to work as carvedilol and propranolol did not reduce the asthma phenotype in mice. Further studies in our laboratory suggested a correlation between the ability of

β-blockers to reduce the asthma phenotype in murine models and their inhibition of the β_2AR- associated β-arrestin-2-ERK1/2 pathway.

All our previous studies were conducted in ovalbumin-driven murine models of asthma. However, ovalbumin models have been criticized for lacking clinical relevance. To address the limitations of the ovalbumin models, we tested the β-blockers carvedilol and nadolol in more clinically relevant models of asthma using the allergens from house dust mites (HDM). To further increase the clinical relevance of our present studies, we designed protocols to study the 'prophylactic' and 'therapeutic' effects of β-blockers by initiating drug treatment pre- and post- the development of asthma phenotype. Lastly, we investigated the role of β-arrestin-2 and ERK1/2 signaling in mediating the effects of β-blockers in murine asthma models.

The major findings of our project are: 1. that nadolol, but not carvedilol attenuated the asthma phenotype in the HDM-driven asthma model in both the prophylactic and therapeutic treatment protocols; and 2. that the effect of β-blockers dissociated ERK1/2 phosphorylation from the asthma phenotype suggesting ERK1/2 activation in lungs may be required during the initial development of asthma, but may have a limited role in perpetuation of the asthma phenotype.

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1. Introduction and Statement of Problem

Asthma is a complex, heterogeneous disease of the airways characterized by chronic inflammation, mucous hypersecretion and airway hyper-responsiveness (AHR). Approximately, 334 million people worldwide suffer from asthma making it a major global public health problem (Global Asthma Network 2014). Within the United States, nearly 24 million people including 6 million children are struggling with asthma, making it a serious health and economic burden. These numbers continue to rise, particularly in the low and middle income countries, which previously had lower prevalence of asthma. Therefore, it is becoming increasingly important to design new and improved therapeutic strategies for better management of asthma.

The most commonly prescribed drugs for asthma therapy include inhaled corticosteroids (ICS) for treating the underlying inflammation, along with β_2 adrenoceptor (β_2AR) agonists as bronchodilators. The β_2AR agonists have been continuously researched for better receptor selectivity and duration of action. Whereas the acute use of these drugs produces bronchodilation and can be lifesaving during asthma exacerbations, their chronic use has been shown to increase airway inflammation, worsen asthma control and increase asthma related morbidity and mortality (Abramson, Walters et al. 2003, Shore and Drazen 2003, Nelson, Weiss et al. 2006, Salpeter, Buckley et al. 2006, Johnston

and Edwards 2009, Xia, Kelton et al. 2013). In fact, according to the latest guidelines on asthma management listed in the GINA 2017 report, the long acting β_2AR agonists (LABAs) are never to be used without ICS. However, chronic use of high dose ICS therapy is also associated with serious systemic adverse effects including suppressed growth in children, reduced bone density and increased potential for fractures, osteoporosis and cataracts (Dahl 2006, Hossny, Rosario et al. 2016). The potential risk associated with the current drugs for treating sustained asthma underscores the need to investigate alternative therapies.

β-adrenoceptor antagonists (β-blockers) are contraindicated in the treatment of asthma because their acute use can cause bronchoconstriction. However, previous research from our laboratory has shown that chronic treatment with certain β-blockers such as nadolol and ICI-118,551 attenuates the asthma phenotype in ovalbumin-driven murine asthma models (Callaerts-Vegh, Evans et al. 2004, Nguyen, Omoluabi et al. 2008). Our original idea to investigate these drugs for asthma therapy was based on the analogy with congestive heart failure, where despite initially being contraindicated, certain β-blockers became first line drugs in chronic therapy for CHF. Furthermore, pilot clinical trials have also shown a dose dependent decrease in airway hyper-responsiveness by nadolol in mild asthma (Hanania, Singh et al. 2008). However, not all β-blockers were found to be beneficial. Our previous results have shown that chronic

treatment with β-blockers such as carvedilol and propranolol did not attenuate the asthma phenotype in murine asthma models (Thanawala, Valdez et al. 2015). Also, another clinical study showed chronic propranolol treatment in a subset of asthmatics did not reduce AHR (Short, Williamson et al. 2013). These discrepancies in the therapeutic efficacy of different β-blockers in asthma suggest mechanistic differences between these drugs.

A possible explanation for the differential effects of β-blockers in asthma lies in their 'biased signaling' profiles at the β_2AR. *Biased signaling* is a phenomenon, where ligands can preferentially activate one of the several pathways linked to a receptor relative to a reference ligand. The β_2AR can signal through at least two pathways, the canonical Gs-cAMP (cyclic-adenosine monophosphate) pathway and the β-arrestin-2-ERK1/2 (extracellular-signal regulated kinases) pathway. Several *in vitro* studies have shown that β-blockers differ in their effects at the β-arrestin-2-ERK1/2 pathway. For example, nadolol and ICI-118,551 inhibit the ERK1/2 pathway, while carvedilol and propranolol activate the ERK1/2 pathway (Galandrin and Bouvier 2006, Wisler, DeWire et al. 2007, van der Westhuizen, Breton et al. 2014). Based on these *in vitro* reports, our *in vivo* results suggest the differential effects of β-blockers in asthma are correlated with their signaling behavior at the ERK1/2 pathway (Thanawala, Valdez et al. 2015).

All of our previous studies in murine models of asthma used ovalbumin allergen for inducing the phenotype. The traditional ovalbumin-driven models have been criticized for their lack of clinical relevance for things such as: a) the ovalbumin allergens driving the phenotype; b) the systemic route of delivery for sensitizing mice to the allergens (Cates, Fattouh et al. 2004); and c), continued respiratory exposure to ovalbumin develops immune tolerance and reduces inflammation in mice (Swirski, Sajic et al. 2002). In light of these limitations, many researchers have begun using dust mites (HDM), rather than ovalbumin, as a more clinically relevant allergen for asthma research in murine models. House dust mites are a common source of aero-allergens implicated in causing allergic asthma. Nearly 85 % asthmatics worldwide are allergic to HDM (Gregory and Lloyd 2011). The whole body extract from the species of HDM, *Dermatophagoides pteronyssinus* containing the Derp family of allergens, is being increasingly used to develop the asthma phenotype in murine models (Cates, Fattouh et al. 2004, Post, Nawijn et al. 2012).

The current dissertation was aimed to study the effects of the prototype β-blockers- nadolol and carvedilol in murine models of asthma utilizing the clinically relevant allergens in HDM extract. These drugs were selected based on our previous studies showing their differential effects in ovalbumin-driven asthma models and their different signaling profiles at the ERK1/2 pathway. To further add clinical relevance to our studies, we designed novel protocols to investigate

the effects of these β-blockers both pre- and post-development of the asthma phenotypes. The present study also contributes to our understanding of the signaling mechanisms causing the differential effects of β-blockers in asthma by studying the ERK1/2 pathway.

2. Literature Review

2.1 Asthma

2.1.1 General Introduction

Obstructive airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) are some of the highly prevalent chronic diseases worldwide, characterized by chronic airway inflammation, airway obstruction and airway hyper-responsiveness (AHR). Although asthma and COPD have some overlapping characteristics, these diseases differ in the type of inflammation, symptoms, pathophysiology and the degree of airway obstruction and AHR. The present dissertation is focused on asthma and the therapeutic potential of β-blockers in treating asthma.

Asthma is a chronic disease of the airways characterized by several features including chronic airway inflammation, mucous hypersecretion, airway remodeling, and airway hyper-responsiveness (AHR). It is a heterogeneous disease that involves a complex interplay of airway structural cells such as, epithelial and smooth muscle cells, and inflammatory cells with multiple susceptibility genes, environmental factors and lifestyle choices. The disease presents itself in several clinical symptoms such as, recurrent coughing, wheezing, chest tightness, shortness of breath, and airway obstruction causing a limited airflow. Asthma can develop at any stage of life from childhood to adulthood. The

symptoms however may vary over time, and may be precipitated by exposure to allergens or irritants, respiratory infections or exercise and other stresses (Global Initiative for Asthma 2017).

Asthma is one of the highly prevalent chronic diseases worldwide with greatest occurrence rates in young children and the elderly population. Approximately, 334 million people in the world suffer from asthma with the highest prevalence observed in Australia, Brazil and countries in Northern and Western Europe (Global Asthma Network 2014). The global prevalence of asthma has been difficult to estimate and the number presented in the Global Asthma Report is based on the best data available from epidemiological studies carried out in different countries. The World Health Organization predicts an approximate increase of 100 million cases of asthma by the year 2025 (World Health Orgaization 2007).

In the United Sates, nearly 8 % of the population (~ 25 million people) has asthma. It is one of the highest ranked chronic diseases causing an economic burden to the country, with a staggering annual cost of $56 billion in 2011 (Loftus and Wise 2015). Furthermore, asthma exacerbations have a significant effect on patients in terms of increased visits to the emergency room and hospitalization, missed school and work days, limitations on their daily activities, and an overall diminished quality

of life. Despite being a manageable disease, the current mortality rate is quite high, with approximately 3600 deaths in the year 2015 (Centers for Disease Control and Prevention 2015).

The ever increasing prevalence of asthma and the associated high socio-economic burden call for an improvement in the current diagnosis of the disease; better management of the symptoms; and an improvement in the current therapeutic strategies.

2.1.2 Asthma Phenotypes and Pathogenesis

Asthma is a chronic heterogeneous disease of the airways characterized by a multitude of phenotypes orchestrated by diverse genetic and environmental factors. Historically, asthma has been considered a T-Helper-2 (Th2) mediated allergic inflammatory disease. However, recent advances in understanding the pathogenesis of asthma have identified more phenotypes of the disease. A molecular phenotyping analysis of mild asthmatics identified two major phenotypes with clinical implications based on responsiveness to corticosteroids (Wenzel 2012):

a) Th2 high phenotype, which occurs in nearly 50 % of asthmatics, is characterized by an up-regulation of Th2 cells and mediators such as, IL-4, IL-5 and IL-13; increased number of eosinophils, and specific immunoglobulin IgEs. TH-2 high patients show allergic symptoms and

respond well to inhaled corticosteroids. The importance of Th2 phenotype is further elaborated later in this section to highlight why we chose a Th2 model.

b) Th2 low phenotype may be present in 50 % of the asthmatic population, but the underlying pathophysiology is less understood compared to Th2 high disease. Th2 low phenotype does not present with allergic symptoms and shows less airway obstruction. It may be mediated by TH17 cytokines and neutrophils. Patients with Th2 low phenotype are often non-responsive to treatment with corticosteroids.

Other than the Th2 phenotype, asthma exists in several different forms exhibiting diverse array of characteristics, pathophysiology and therapeutic response. These include late-onset eosinophilic asthma, exercise-induced asthma, obesity-related asthma, steroid-resistant asthma, and neutrophilic asthma, among others (Wenzel 2012).

Among the various asthma phenotypes, allergic asthma is most common, which occurs in nearly 50 % of the asthmatic population. In most cases, it develops at a younger age in response to specific allergens and often co-exists with other conditions such as, atopic rhinitis, and atopic dermatitis (Schatz and Rosenwasser 2014). The pathogenesis involves a complex interaction of the innate and adaptive immune systems, orchestrating a Th2 mediated immune response (Fig. 1). The airway

epithelial cells are the first barrier to entry of airborne allergens and regulate the development of the Th2 phenotype (Fahy and Locksley 2011). The allergen derived pathogen-associated molecular patterns (PAMPs) activate the various pattern recognition receptors (PRRs) present on the epithelium. These PRRs include protease activated receptors (PAR-2) and Toll-like receptors (TLRs), and their activation releases epithelial derived cytokines and growth factors such as, thymic stromal lymphopoietin (TSLP), granulocyte macrophage-colony stimulating factor (GM-CSF), IL-25 and IL-33 (Jacquet 2013). TSLP induces the recruitment and maturation of dendritic cells (DCs) to skew the differentiation of T helper cells into Th2 phenotype. The Th2 cells release several inflammatory cytokines and chemokines such as,

- IL-4 and IL-13, which induce allergen specific IgE production from B cells. IgE in turn, binds to the specific receptors on mast cells and basophils, stimulating the release of inflammatory mediators-histamine, cytokines and leukotrienes (Ishmael 2011). IL-13 promotes development of the features of asthma including, AHR and airway remodeling, through its effects on the effector cells such as, epithelium, smooth muscle and fibroblasts (Olin and Wechsler 2014).

- IL-5 promoting recruitment and survival of eosinophils, a characteristic feature of asthma (Ishmael 2011).
- IL-9 promoting survival of mast cells, eosinophils and other cytokines (Ishmael 2011).
- CCL11 (Eotaxin), a chemokine ligand, which promotes recruitment of eosinophils (Ishmael 2011).
- CCL5 (RANTES), a chemokine ligand, which recruits T cells, eosinophils and basophils

The epithelium derived cytokines- IL-25 and IL-33 activate the innate immune system by releasing the recently identified innate lymphoid cells (ILC2), also known as nuocytes (Fahy and Locksley 2011). Nuocytes release large amounts of the inflammatory Th2 cytokines-IL-5 and IL-13 (Fahy and Locksley 2011), and serve as a link between the innate and adaptive immune responses mediating Th2 inflammation (Martinez and Vercelli 2013).

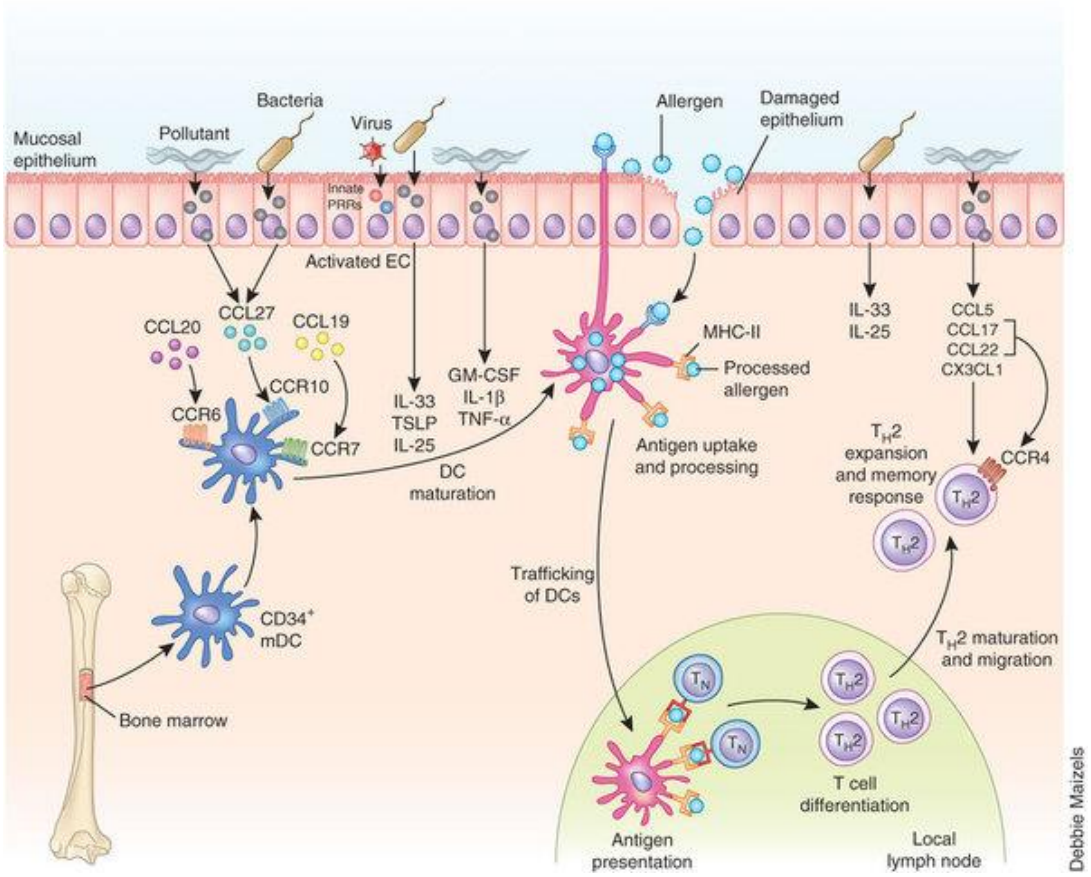

Figure 1. Pathogenesis of asthma Allergens and other environmental toxins disrupt the epithelial barrier to gain entry into the mucosa. This interaction releases epithelial derived cytokines and chemokines, and promotes dendritic cell recruitment and maturation. Activated dendritic cells migrate to the lymph nodes, where they cause differentiation of naïve T cells to Th2 phenotype. The activated Th2 cells release various cytokines that act on effector cells to produce the asthma phenotype. Figure adapted from (Holgate 2012).

The Th2 allergic response in asthma produces several characteristic pathological features of the disease causing airway obstruction (Fig. 2) and limiting the flow of air into the lungs. The various features of asthma include:

a) Mucous metaplasia

Airway mucus forms an important defense against airborne pathogenic stimuli. However, the over production and secretion of mucus (mucous metaplasia) coupled with its diminished clearance in pathologies like asthma can result in airway obstruction. The predominant types of mucins produced by the airways are MUC5AC and MUC5B (Piccotti, Dickey et al. 2012). In allergic asthma, the Th2 inflammatory cytokine IL-13 stimulates mucin MUC5AC overproduction and hyper-secretion, resulting in mucous metaplasia via a pathway mediated by signal transducer and activator of transcription 6 (STAT6).

b) Airway Hyper-responsiveness (AHR)

Airway hyper-responsiveness is the increased sensitivity of the airways to constricting agents such as, histamine and methacholine. AHR varies in different asthmatic subsets with a higher presence in allergic asthma showing high eosinophil numbers in the airways (Chapman and Irvin 2015).

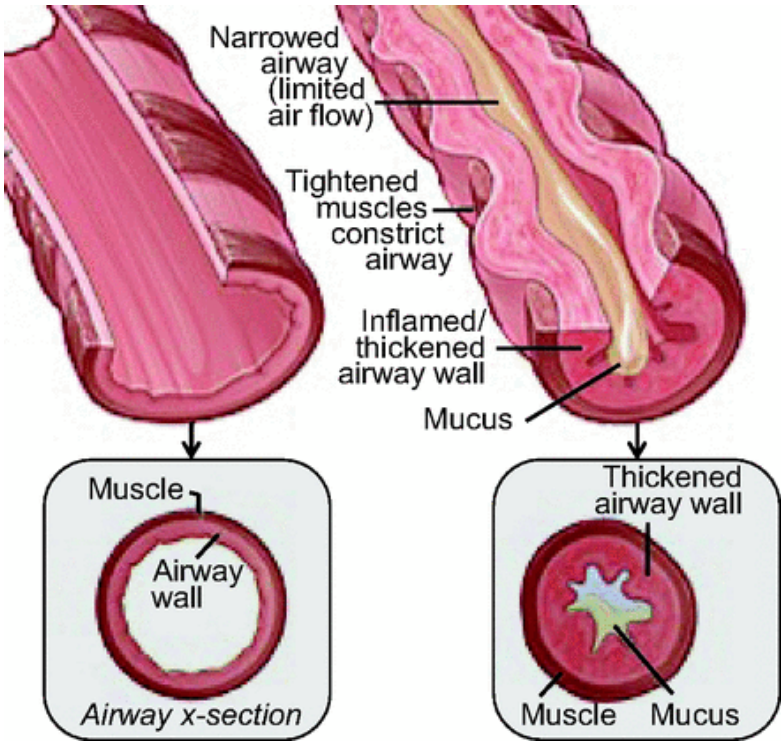

Figure 2. Airway Obstruction in Asthma The figure represents characteristic narrowing of the airways in asthma caused by inflammation, mucous metaplasia, and bronchial constriction. Image adapted from (Doeing and Solway 2013).

AHR is mainly caused by an increased contractility of airway smooth muscle (ASM) cells. The rate of contraction normally depends on the rate of formation of actin-myosin cross-bridges, which is regulated by a balance between the activities of myosin light chain kinase (MLCK, which phosphorylates and activates myosin) and myosin light chain phosphatase (MLCP, which dephosphorylates myosin). An increase in intracellular calcium (Ca2+) directly phosphorylates MLCK, thereby increasing smooth muscle contraction. A small GTPase protein, RhoA phosphorylates and inactivates MLCP in parallel and further contributes to smooth muscle contraction (Erle and Sheppard 2014). The Th2 inflammatory cytokines- IL-4 and IL-13 promotes AHR by increasing smooth muscle contractility via their effects on calcium signaling and RhoA expression (Erle and Sheppard 2014, Chapman and Irvin 2015).

Factors contributing to AHR can arbitrarily be divided into two components- persistent and variable (Busse 2010). The persistent component of AHR is mainly attributed to the structural changes associated with airway remodeling such as thickening of the basement membrane, smooth muscle hypertrophy, extracellular matrix deposition and vascular changes. The variable component is caused by the underlying inflammation in response to environmental allergens (Fig. 3). However, these processes do not act in isolation and are interdependent.

For example, persistent inflammation in asthma can further promote structural changes in the airways thereby contributing to the more persistent component of AHR (Busse 2010).

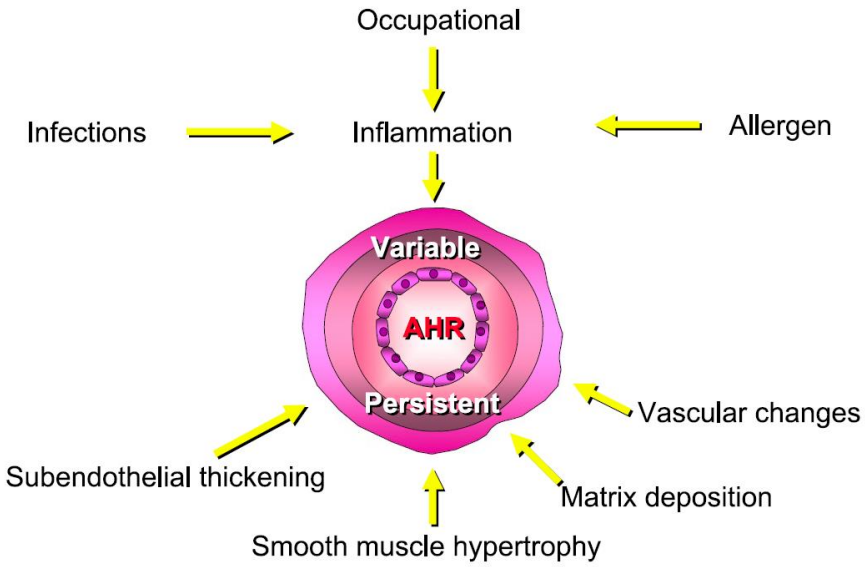

Figure 3. Factors Affecting Airway Hyper-responsiveness (AHR) The figure represents the arbitrary categorization of the factors contributing to AHR into 'persistent' and 'variable' components. Figure adapted from (Busse 2010).

c) Airway Remodeling

Structural changes in the airways associated with the progression of asthma constitute airway remodeling. These structural changes further contribute to declining lung function by increasing airway thickening and narrowing and reducing airway compliance (Busse 2010). Pathologically,

airway remodeling is characterized by an increased deposition of extracellular matrix proteins (ECMs) such as collagen and glycoproteins, resulting in subepithelial and subbasement membrane thickening; increased ASM hypertrophy and hyperplasia; increased airway fibrosis caused by deposition of connective tissues and fibroblasts; and angiogenesis (Martinez and Vercelli 2013). Airway remodeling may be associated with Th2 inflammation, but in many cases, remodeling develops independent of inflammation and does not respond to anti-inflammatory treatment (Manuyakorn, Howarth et al. 2013).

2.1.3 Therapeutic Strategies

Inhaled corticosteroids (ICS) and long acting β_2-adrenoceptor agonists (LABAs) are the mainstays for asthma therapy as they target chronic airway inflammation and bronchoconstriction, the major targets for the management of asthma. The ICS/LABA combination therapy has been very successful in controlling mild to moderate asthma in most patients. Yet, a large population of asthmatics remains refractory to this therapy. The complex heterogeneity of asthma also makes it challenging to design a standard therapeutic strategy to treat its various clinical phenotypes. All these factors have led to an extensive research on improving the currently approved drugs and finding

novel therapies for asthma. The global initiative for asthma (GINA) recommends a step-wise therapeutic strategy for asthma as detailed in Fig. 4

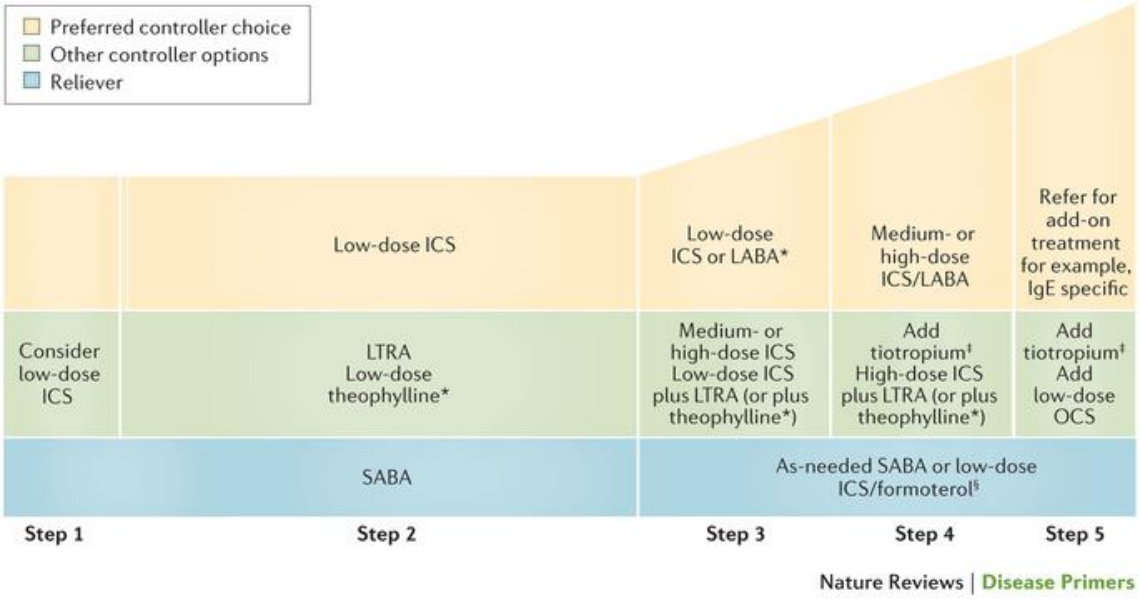

Figure 4. Step-wise Therapeutic Strategy for Asthma The figure represents a schematic of the step-wise therapeutic strategy for different stages of asthma as recommended by the Global Initiative for Asthma (GINA). The strategy classifies asthma based on the severity of symptoms and recommends treatment accordingly. The relative width of each step in the figure represents the number of patients receiving the treatments, and the height represents severity of the disease. * Theophylline is not recommended for treating children 6-11 years of age. ‡ Tiotropium is not recommended for treating patients below 18 years of age. ICS, inhaled corticosteroids; SABA short acting β_2 adrenoceptor-agonist; LTRA, leukotriene receptor antagonist; LABA, long acting β_2 adrenoceptor-agonist; OCS, oral corticosteroid. Figure adapted from (Holgate, Wenzel et al. 2015).

A number of drug classes are currently approved and/or under development for the treatment of asthma. Many of them are included in the therapeutic program laid out by GINA (Fig. 4). The efficacies and limitations of the various drugs approved and/or under development for treating asthma are discussed below.

a) Inhaled corticosteroids (ICS)

Therapeutic efficacy: ICS are the most effective anti-inflammatory drugs and constitute the first-line therapy for controlling asthma. The currently used ICS include different formulations of budesonide, ciclesonide, beclomethasone, fluticasone, mometasone, and triamcinolone (Global Initiative for Asthma 2017).

Mechanism of action: Corticosteroids bind to and activate glucocorticoid receptors (GR) causing their nuclear translocation. The ligand bound GRs then bind to the specific DNA sequences in the promoter region of the glucocorticoid response elements (GREs). The predominant mechanism of the anti-inflammatory effect of ICS is the suppression pro-inflammatory gene transcription including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). This process known as ‘trans-repression’ does not require direct binding to DNA (non-genomic effect), and involves recruitment of histone deacetylase-2

(HDAC-2) to the transcription complex to repress gene transcription (Barnes 2006). In addition, higher concentrations of glucocorticoids also induce transcription of anti-inflammatory genes such as, interleukin-10 (IL-10), glucocorticoid-induced leucine zipper protein (GILZ), inhibitor of NF-κB (i-κB), dual specificity protein phosphatase-1 (DUSP-1) etc. (Barnes 2006, Newton and Giembycz 2016).

Limitations: Chronic use of ICS carries several limitations. Most of the currently used ICS are absorbed from the lungs and can potentially cause systemic side effects, such as adrenal insufficiency, suppressed growth in children, reduced bone density, osteoporosis, glaucoma etc. (Dahl 2006). These side effects may result from gene activation, especially by high doses of the steroids (Barnes 2006). Furthermore, treatment with corticosteroids is not curative and the symptoms return once they are discontinued (Olin and Wechsler 2014). A subset of asthmatics are also refractory to the effects of corticosteroids, particularly patients with Th2-low or neutrophilic asthma, making if necessary to find alternate therapies.

New developments: Extensive research on ICS in recent years has resulted in development of improved drugs and combinations to address the limitations associated with the use of ICS for asthma

therapy. For example, ciclesonide is a pro-drug that is converted to its active compound, dis-ciclesonide in the lungs and therefore has a reduced potential for side-effects (Chini, Monteferrario et al. 2014). Another attempt to reduce the side-effects of high dose ICS therapy is through the development of 'dissociated' steroids where, the trans-activation mechanisms with a potential for causing side-effects were separated from the anti-inflammatory trans-repression mechanisms (Barnes 2010). The current guidelines for asthma therapy recommend using ICS in combination with LABAs for better therapeutic efficacy in persistent asthma. The ICS/LABA combination therapy is superior to increasing the dose of ICS alone and has several synergistic benefits (Newton and Giembycz 2016).

b) **Bronchodilators**

Therapeutic efficacy: Bronchodilators are used to prevent and relieve bronchial constriction in asthma. β_2-adrenoceptor (β_2AR) agonists are the most frequently used bronchodilators, both as rescue medication in acute asthma exacerbations, and as a controller therapy with ICS.

Mechanism of action: The β_2ARs are highly expressed in the airway smooth muscle cells (Johnson 2001). Agonist activation of β_2ARs relaxes smooth muscles by activating adenylate cyclase and

increasing the intracellular levels of cyclic adenosine monophosphate (cAMP). Increased level of cAMP activates protein kinase A (pKA), which phosphorylates and inactivates proteins required for muscle contraction. Higher concentration of cAMP also reduces intracellular calcium concentration ultimately causing relaxation of smooth muscles (Johnson 2001). In addition to the adrenergic system, parasympathetic system comprising of activation of muscarinic receptors by acetylcholine, plays an important role in maintaining smooth muscle tone by causing smooth muscle contraction. Anticholinergic agents, therefore, have a potential for use as bronchodilators in asthma therapy (Wasilewski, Lougheed et al. 2014).

Limitations: The short acting β_2AR agonists (SABAs) were initially used as controller therapy in asthma. However, several studies found an association between the chronic use of β_2-selective agonists fenoterol and albuterol and an increased risk of asthma-related mortality, which led to their switch from controller therapy to rescue medication to relieve acute bronchospasms in asthmatic emergencies (Wasilewski, Lougheed et al. 2014). Also, several studies in the past including clinical trials have shown that chronic treatment with LABAs, such as salmeterol, when used as a monotherapy, can worsen airway inflammation, and cause a significant increase in asthma related

deaths (Abramson, Walters et al. 2003, Shore and Drazen 2003, Nelson, Weiss et al. 2006, Salpeter, Buckley et al. 2006, Johnston and Edwards 2009). In light of these limitations, the current guidelines on asthma management have recommended that LABAs are never to be used without ICS and should not be used for a long term even with ICS (Global Initiative for Asthma 2017). The beneficial and adverse effects of β_2AR agonists will be discussed in detail in the following sections.

New developents: Ultra-long acting LABAs such as, indacaterol, velanterol, olodaterol, carmoterol etc. have been recently developed for use in combination with ICS requiring once-daily dosing (Charriot, Vachier et al. 2016). Additionally, certain long acting muscarinic antagonists including tiotropium bromide are now being used as an added therapy in severe asthma poorly controlled by ICS/LABA combination therapy (Barnes 2010, Charriot, Vachier et al. 2016). Recent investigations are also targeting analogs of vasoactive intestinal peptide (VIP) and potassium channel modulators as alternate classes of bronchodilators.

c) Lipid mediator blockers

Therapeutic efficacy: The pathophysiology of asthma is driven by several inflammatory lipid mediators which have recently been targeted for asthma therapy. These include cysteinyl leukotrienes (CysLTs)

which are synthesized through the 5-lipoxigenase (5-LO) pathway, and are activated in several inflammatory cells during asthma (Montuschi 2010). The currently approved drugs in this class are the leukotriene receptor antagonists (LTRAs) that include montelukast, zafirlukast and pranlukast; and the 5-LO inhibitor zileuton (Montuschi 2010, Global Initiative for Asthma 2017).

Mechanism of action: The CysLTs, through their activation of CysLT1 receptors, are involved in airway smooth muscle contraction, mucus hypersecretion, recruitment and activation of eosinophils, airway fibrosis and remodeling, and may play a role in mediating the neutrophilic phenotype of asthma (Montuschi 2010). LTRAs such as monteleukast, inhibit these effects, and improve lung function. They are used as a monotherapy in mild asthma, and in combination with ICS and LABAs to control moderate to severe asthma (Montuschi 2010). They can be particularly effective in exercise induced asthma, and may decrease the need for rescue β2AR agonists (Montuschi 2010).

Limitations: LTRAs are less effective than corticosteroids in controlling severe asthma and have limited efficacy against AHR (Barnes 2010, Olin and Wechsler 2014). They may cause severe adverse effects in

some patients such as, Churg-Strawss Syndrome and increased risk for suicides (Montuschi 2010).

New Developments: Several other lipid mediators including prostaglandin D2 (PGD2) are released from inflammatory cells, and are involved in driving the Th2 phenotype in asthma. PGD2 receptor (also known as chemoattractant homologous receptor expressed on Th2, CRTH2) antagonists, such as AMG-853 and OC000459 are under development for asthma therapy (Barnes 2010).

d) Anti-Immunoglobulin (Ig) E therapy

IgE is a critical mediator of atopic (allergic) asthma. It is produced by activated B lymphocytes upon stimulation by interleukins, IL-4 and IL-13. Allergens crosslink with the specific IgEs bound to the high affinity Fc epsilon receptors on mast cells, basophils and dendritic cells causing their degranulation and releasing their mediators (Olin and Wechsler 2014). Anti-IgE antibodies are currently being developed as a therapeutic option in severe asthma.

Therapeutic Efficacy and mechanism of action: The only approved anti-IgE monoclonal antibody for asthma therapy is omalizumab. It prevents the activation of IgE receptors and the release of inflammatory mediators from mast cells and basophils (Chini, Monteferrario et al. 2014). The current guidelines recommend adding

omalizumab as an add-on therapy in patients with moderate to severe uncontrolled allergic asthma (Global Initiative for Asthma 2017).

Limitations: Treatment with omalizumab is very expensive and may show limited clinical efficacy (Barnes 2010). Patients have to be continually monitored for bio-markers to determine its therapeutic efficacy, and its continued use may cause some moderate to severe side effects (Chini, Monteferrario et al. 2014).

New developments: Several new drugs targeting the inhibition of the release of inflammatory mediators from mast cells and other immune cells are being developed. These include inhibitors of the stem cell factor (SCF) receptor c-KIT and the spleen tyrosine kinase (Syk) (Barnes 2010, Chini, Monteferrario et al. 2014).

e) Cytokine modulators

Cytokines play a major role during asthma pathogenesis. They are involved in mediating chronic inflammation, airway remodeling, mucous metaplasia, airway obstruction, and airway hyper-responsiveness, among other effects. Hence, they have become significant targets in controlling asthma. Numerous randomized clinical trials have tested drugs blocking the Th2 cytokines- IL-4, IL-13 and IL-5 in recent years with some promising results (Barnes 2010, Chini, Monteferrario et al. 2014, Charriot, Vachier et al. 2016). In fact, recent

guidelines on asthma management recommend an add-on treatment with anti-IL5 drugs for patients that have severe eosinophilic asthma uncontrolled with high-dose ICS (Global Initiative for Asthma 2017).

Therapeutic Efficacy and mechanism of action:

i) Anti-IL5 Drugs

IL-5 is produced by several Th2 immune cells, and promotes maturation and recruitment of eosinophils in asthma. Several monoclonal antibodies such as mepolizumab, reslizumab and benralizumab have been developed to block the effects of IL-5 in eosinophilic asthma (Charriot, Vachier et al. 2016). Their efficacies are briefly listed in Table 1.

ii) Anti-IL-4 and IL-13 Drugs

IL-4 and IL-13 are also produced during Th2 immune response in allergic asthma. They promote the synthesis and release of IgE from basophils, and directly activate the IL-4Rα/IL-13Rα receptor complex on effector cells to produce mucus hypersecretion and AHR (Chini, Monteferrario et al. 2014). Recent studies have focused on developing drugs that block the effects of both these cytokines at the receptor complex (listed in Table 1).

Limitations: Despite showing promising results in pre-clinical studies, many of the cytokine blocking therapies have failed to produce

significant improvement in patient symptoms, lung function or asthma exacerbations (Barnes 2010). Of the promising ones, many show limited efficacy in only a subset of asthmatics, therefore requiring careful selection of patients and assessment of their biomarkers. For example, the anti-IL-5 monoclonal antibody mepolizumab reduced exacerbations in asthmatics showing severe and persistent sputum eosinophilia (Barnes 2010).

New developments: Besides the Th2 cytokines, some Th1 and Th17 cytokines are also being investigated as important therapeutic targets, particularly in Th2 low asthma phenotypes refractory to ICS therapy. Brodalumab, a monoclonal antibody against IL-17, was recently tested in patients with moderate to severe asthma, but did not seem beneficial (Olin and Wechsler 2014). Other cytokine targets that are currently being investigated in pre-clinical models include IL-25, IL-33, GM-CSF, tumor necrosis factor (TNF)-α, and some chemokine receptor antagonists against CCR3, CCR2, CXCR2, among others (Barnes 2010).

Table 1. Cytokine modulators for asthma therapy				
Drug	**Target Cytokine**	**Responder Phenotype**	**Stage of Development**	**Clinical Efficacy**
Reslizumab (Castro et al. Lancet Respir Med. 2015)	IL-5, mAb	Moderate to severe asthma	phase III trials completed	Effective in patients with elevated eosinophils in blood and sputum
Mepolizumab (Pavord et al. Lancet, 2012)	IL-5, mAb	Hyper-eosinophilic, severe asthma	Phase II completed, phase III, IV ongoing	Effective and well tolerated in severe eosinophilic asthma
Benralizumab ((CALIMA) FitzGerald et al. Lancet, 2016; (SIROCCO) Bleecker et al.	IL-5, mAb	Severe asthma	Phase III trials completed	Reduced annual exacerbations, well tolerated in severe eosinophilic asthma

Lancet, 2016)				
Pascolizumab (Chini et al. 2014)	IL-4, mAb	Symptomatic steroid naïve asthma	Phase II trials completed	Unfavorable data
Pitrakinra (Wenzel et al. Lancet, 2007)	IL-4/IL-13 antagonist	Atopic asthma	Phase II trials completed	Reduced asthma exacerbations, improved FEV1
Lebrikizumab (Hanania et al. Thorax, 2015; Hanania et al. Lancet Respir Med. 2016)	IL-13, mAb	Moderate to severe uncontrolled asthma	Phase II, III trials completed	Improved lung function and reduced exacerbations, Phase III trials did not show improvement
Tralokinumab (Piper et al. Eur Respir J., 2013)	IL-13, mAb	Moderate to severe asthma	Phase II trial completed	Improved lung function
mAb: monoclonal antibody; FEV1: forced expiratory volume in 1 s				

2.1.4 Rationale for developing new drugs for treating asthma

The current therapeutic strategy for asthma has been very effective in controlling symptoms, improving lung function and reducing exacerbations in a majority of asthmatics. However, none of the available treatments are preventive, curative or provide long-term benefits. The prevalence of asthma is constantly rising, and has become a global economic burden, most notably in lower income nations. Despite the availability of numerous drugs, a significant number of asthmatics remain uncontrolled. Many patients with severe, persistent symptoms are refractory to the current therapeutic options, while others show poor adherence to regular inhaler therapy (Barnes 2010). One of the major challenges is to find effective drugs for treating the corticosteroid resistant population of asthmatics. There is also a need to develop orally active drugs for treating mild to moderate asthma to avoid regular inhaler use, but this may increase the potential for side effects (Barnes 2010). The currently approved drugs also suffer from some severe adverse effects, which often make them intolerable to many patients.

All these reasons make it necessary to pursue new therapeutic options for treating asthma. However, the complicated pathophysiology of asthma makes it very challenging to determine therapeutic targets that can be utilized across its numerous subsets. Needless to say, there is an unmet need to improve

the currently available drugs and to design new therapies that are both safe and effective in providing long-term control of asthma.

2.2 β_2 Adrenoceptor Signaling in Asthma

The short-acting β_2-adrenoceptor (β_2AR) agonists are the cornerstone drugs for treating acute asthma. These drugs are the most effective bronchodilators and are inhaled to provide quick relief during acute bronchoconstriction. Furthermore, the long acting β_2AR agonists (LABAs) in combination with corticosteroids constitute the gold-standard controller therapy for asthma. The β_2AR agonists bind to the Gαs protein coupled-β_2ARs on the airway smooth muscle (ASM) cells, and activate adenylate cyclase, resulting in cyclic adenosine monophosphate (cAMP) production. Subsequently, cAMP phosphorylates protein kinase A (PKA), which phosphorylates and activates multiple targets involved in reducing intracellular calcium concentrations and causing smooth muscle relaxation (Fig. 5) (Shore and Drazen 2003). However, previous studies from our laboratory using *in vivo* models of asthma showed that mice lacking the β_2AR did not develop the asthma phenotype, suggesting a requirement of the β_2AR signaling in the development of asthma (Nguyen, Lin et al. 2009). Furthermore, chronic use of β_2AR agonists has been associated with a loss of asthma control, worsening of asthma exacerbations, and even an increase in airway hyper-responsiveness (Abramson, Walters et al. 2003, Shore and Drazen 2003, Nelson, Weiss et al. 2006, Salpeter, Buckley et al. 2006,

Johnston and Edwards 2009). The following sections discuss both the beneficial and adverse effects of β_2AR agonism in asthma.

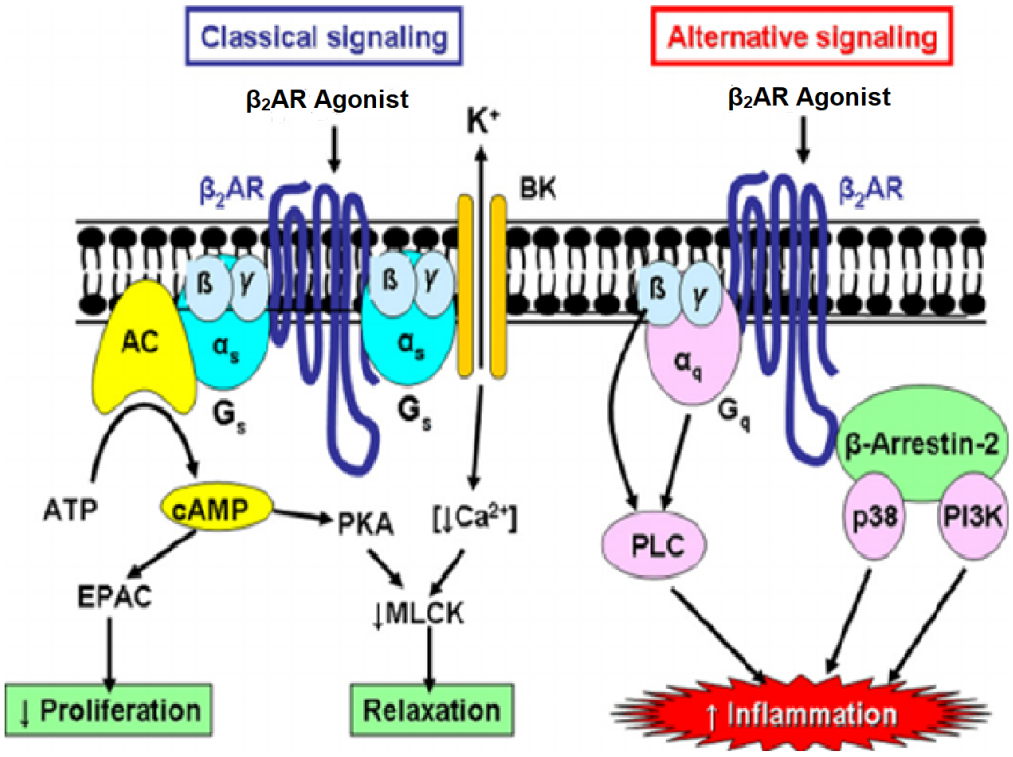

Figure 5. β_2-Adrenoceptor Signaling Pathways The figure represents a schematic of the signaling pathways downstream of the β_2-Adrenoceptor (β_2AR). The canonical Gs-mediated pathway promotes cAMP accumulation that is known to reduce smooth muscle proliferation and cause smooth muscle relaxation. β_2AR activation also stimulates an alternative signaling pathway mediated by β-arrestin-2 stimulation of downstream signaling components p38 and PI3K that are known to increase airway inflammation. β_2AR can also act through $G_{\beta\gamma}$ sub-units to activate PLC and promote airway inflammation. cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; EPAC, exchange proteins directly activated by cAMP; MLCK, myosin light chain kinase; PLC, phospholipase C; PI3K, phosphoinositide 3 kinase. Figure adapted from (Barnes 2011).

2.2.1 Beneficial Effects of β_2 Adrenoceptor Agonists in Asthma

The dysregulated functioning of the airway smooth muscle cells in asthma results in excessive airway narrowing and hyper-responsiveness to contractile mediators, such as acetylcholine, histamine, cysteinyl-leukotrienes, thromboxanes, and bradykinin (Brusasco, Crimi et al. 2006). These mediators activate $G_{\alpha q}$-coupled receptors; resulting in a phospholipase C (PLC) mediated phosphoinositide (PI) hydrolysis and production of inositol 1, 4, 5-triphosphate (IP_3). This increases intracellular calcium (Ca^{2+}) concentration, and promotes myosin light chain kinase (MLCK) activity, leading to smooth muscle contraction (Billington, Ojo et al. 2013).

The β_2AR are highly expressed on the airway smooth muscle (ASM) cells. Agonist stimulation of the β_2AR causes ASM relaxation (bronchodilation), and decreases the sensitivity of the ASM cells to pro-contractile stimuli (bronchoprotecton) in asthma (Shore and Drazen 2003). The primary mechanism of β_2AR agonist induced ASM relaxation is by producing cAMP, and activating the downstream signaling pathways through PKA and exchange proteins directly activated by cAMP (Epac). These pathways antagonize the Gq-PLC-mediated contraction of ASM by several mechanisms that limit Ca^{2+} mobilization, and modulate Ca^{2+} homeostasis and sensitivity within the ASM cells (Billington, Ojo et al. 2013). Another recently recognized mechanism suggests that β_2AR agonists upregulate the expression of

regulator of G-protein signaling 2 (RGS2), which reduces signaling from Gαq-coupled receptors (Holden, Bell et al. 2011).

Besides the contractile function, airway smooth muscle cells assume proliferative and secretory functions in asthma, which results in increased ASM mass and the release of pro-inflammatory cytokines and chemokines from the ASM (Billington, Ojo et al. 2013). These processes promote airway remodeling and perpetuate the inflammatory process associated with asthma pathogenesis. The β_2AR-mediated increase in cAMP inhibits ASM cell proliferation through anti-mitogenic effects involving the inhibition of extracellular-signal regulated kinases (ERK1/2) and phosphoinositide 3'-kinase (PI3K). The β_2AR agonists also inhibit the release of pro-inflammatory mediators, Eotaxin and RANTES from the ASM cells, thus producing an anti-inflammatory effect in asthma (Billington, Ojo et al. 2013).

2.2.2 β2-Adrenoceptor Dysfunction in Asthma

Several studies have shown that the β_2ARs are less responsive to agonists in the airways of asthmatics (Brusasco, Crimi et al. 2006). However, it is unclear whether the dysfunctional signaling is a direct result of asthma or a result of excessive use of β_2AR agonists (Barisione, Baroffio et al. 2010). There are several mechanisms leading to β_2AR dysfunction in asthma.

a) Receptor desensitization

The reduced responsiveness of a receptor to a specific agonist is broadly known as desensitization. In case of the β_2AR, desensitization can occur in response to prolonged use of specific agonists (homologous desensitization) or in response to other inflammatory mediators (heterologous desensitization). Homologous desensitization is induced by PKA and G-protein receptor kinase (GRK)- mediated phosphorylation of the receptor, and the subsequent recruitment of β-arrestins, terminating the receptor signaling. On the other hand, heterologous desensitization is mediated by protein kinase C (PKC) phosphorylation of the receptor. The phosphorylated receptor may then undergo internalization, where it may get dephosphorylated and recycled back to the membrane or degraded by lysosomes (Brusasco, Crimi et al. 2006). Desensitization of the β_2ARs occurs more readily in inflammatory cells, such as mast cells, as compared to the airway smooth muscle cells, resulting in a marked decrease in the anti-inflammatory and bronchoprotective effects, but not in the bronchodilator effects of agonists (Barnes 1999).

b) Genetic Polymorphisms

Receptor desensitization, as regulated by endogenous catecholamines or by prolonged administration of β_2AR agonists, may be influenced by genetic

polymorphisms in the β_2AR gene. The most commonly studied nucleotide polymorphisms occur in amino acids at positions 16 and 27 of the β_2AR gene (Brusasco, Crimi et al. 2006). Several studies have suggested that the β_2AR polymorphisms may be associated with the heterogeneity of asthma, and also the variable response to agonists. For example, some studies found that asthmatics homozygous for Arg16 showed higher frequency of exacerbations during chronic daily use of albuterol, compared with patients using albuterol less regularly (Israel, Drazen et al. 2000). Some *in vitro* studies also suggested that the Gly16 polymorphism showed increased β_2AR desensitization, and the Glu16 polymorphism was less prone to desensitization in the airway smooth muscle cells (Brusasco, Crimi et al. 2006). However, there have been inconsistent results on the effects of β_2AR polymorphisms on both the receptor regulation, and the bronchodilator response to agonists (Brusasco, Crimi et al. 2006). Although, these polymorphisms are not directly associated with the pathogenesis of asthma, there is a degree of correlation between the β_2AR polymorphisms and asthma severity, which may be useful in predicting the course of the disease. However, to date the studies on β_2AR polymorphisms have not been conclusive enough to affect treatment guidelines (Global Initiative for Asthma 2017).

2.2.3 Adverse Effects of β_2 Adrenoceptor Agonists in Asthma

Several studies have suggested that the regular use of β_2AR agonists is associated with an increased risk for asthma exacerbations and mortality (Crane, Pearce et al. 1989, Pearce, Beasley et al. 1995, Anderson, Ayres et al. 2005, Johnston and Edwards 2009). The initial concerns surrounded the excessive use of high intrinsic efficacy, short acting β-agonists isoproterenol and fenoterol, leading to their discontinuation in asthma therapy. Later, safety concerns arose regarding the use of long acting β-agonists (LABAs), such as salmeterol, when clinical trials studying the safety of salmeterol in asthmatic patients found a significant increase in asthma related mortality (Nelson, Weiss et al. 2006, Salpeter, Buckley et al. 2006). These safety concerns led the Food and Drug Administration (FDA) to require black-box warnings on the use of LABAs in asthma therapy (Walker, Penn et al. 2011).

More recently, *in vivo* studies from our laboratory have shown that mice deficient in epinephrine, the endogenous agonist for the β_2AR, do not develop the full asthma phenotype (Thanawala, Forkuo et al. 2013). Further, chronic treatment with β_2AR agonists, formoterol and salmeterol, restored the asthma phenotype in these mice (Thanawala, Forkuo et al. 2013, Forkuo, Kim et al. 2016). Another study showed that chronic salbutamol treatment in mice produced airway wall thickening by promoting the proliferation of airway epithelial cells (Tamaoki, Tagaya et al. 2004). Numerous in vitro studies have

also shown a pro-inflammatory effect of β_2AR agonists. For example, both short and long acting β_2AR agonists promoted the cytokine-stimulated accumulation of Th2 cells (Loza, Foster et al. 2008), and an up-regulation of the epithelial derived cytokine, thymic stromal lymphopoietin (TSLP) (Futamura, Orihara et al. 2010). Several molecular mechanisms contribute to these adverse effects of chronic β_2AR agonist use in asthma.

a) Repeated use of β_2AR agonists causes PKA and GRK-mediated desensitization of the receptors present on the airway smooth muscle cells, leading to a loss of the bronchial relaxant response to the agonists (Billington, Ojo et al. 2013).
b) Transgenic mice with β_2AR overexpression in ASM showed an increased activity of PLC-β1, and an increased G_q- mediated bronchial constrictor response to methacholine and histamine (McGraw, Almoosa et al. 2003). Similarly, an *in vivo* murine asthma model also showed an up-regulation of PLC-β1 (Lin, Peng et al. 2008). These studies suggested that chronic use of β_2AR agonists might increase the contractile response to airway constrictors, such as methacholine and histamine.
c) Continued exposure to β_2AR agonists may cause a switch in β_2AR coupling from Gs to Gi, which is mediated by PKA-phosphorylation of the receptor (Daaka, Luttrell et al. 1997). This switching in the β_2AR signaling decreases the bronchial relaxation response to agonists, and leads to

ERK1/2 signaling, which is associated with an increased mitogenic activity in the airway smooth muscle cells (Zou, Komuro et al. 1999, Shore and Drazen 2003).

d) The GRK-mediated β_2AR phosphorylation promotes recruitment of the adaptor protein β-arrestin-2, which in addition to causing receptor desensitization also stimulates further G-protein independent signaling leading to ERK/12 activation. Several *in vivo* studies using murine models of asthma have shown that β_2AR-stimulated β-arrestin-2 signaling regulates the development of asthma phenotype (Walker, Fong et al. 2003, Deshpande, Theriot et al. 2008, Chen, Hegde et al. 2015).

2.2.4 Optimizing the Use of β_2 Adrenoceptor Ligands in Asthma

Based on the evidence discussed in the previous sections, chronic use of β_2AR agonists appears to promote airway inflammation and mucus plugging, worsen airway hyper-responsiveness, and induce airway remodeling in asthma. Our studies on murine asthma models have shown that certain β-blockers, such as ICI 118,551 and nadolol attenuated the asthma phenotypes (Nguyen, Omoluabi et al. 2008, Thanawala, Valdez et al. 2015, Joshi, Valdez et al. 2017). These β-blockers could be used as add-on drugs to minimize receptor desensitization in airway smooth muscle cells, and to suppress the underlying airway inflammation in asthma. However, our studies showed that

certain other β-blockers, such as carvedilol and propranolol did not attenuate the asthma phenotypes in murine models (Nguyen, Omoluabi et al. 2008, Thanawala, Valdez et al. 2015, Joshi, Valdez et al. 2017). The differential effects of β-blockers were also seen in clinical trials where nadolol, but not propranolol, increased the amount of methacholine required to produce a 20% reduction in forced expiratory volume in 1 second (FEV1) in different subsets of asthmatics (Hanania, Singh et al. 2008, Hanania, Mannava et al. 2010, Short, Williamson et al. 2013).

The differences in the efficacies of the various β-blockers may be explained by their different signaling properties. Therefore, it is critical to determine the β_2AR-mediated 'beneficial' vs 'adverse' signaling pathways, in order to optimize the use of β_2AR ligands in asthma therapy. The ideal drugs would be those that preferentially activate the 'beneficial' signaling pathway over the 'adverse' pathway compared to a reference ligand, a phenomenon known as *biased agonism*. In case of the β_2AR, the Gs-protein dependent cAMP pathway appears to be beneficial in asthma, whereas the β-arrestin-dependent ERK1/2 signaling pathway may be responsible for the deleterious effects of chronic agonist treatment (Walker, Penn et al. 2011). β_2AR signaling exhibits cell-specific bias (system bias) in mediating the effects on the asthma phenotypes. For example, β_2AR signaling in the ASM is predominantly mediated by the G_s-cAMP pathway and causes the beneficial

effect of ASM relaxation, while in the airway epithelium, β_2AR signaling may be mediated by the β-arrestin dependent pathway causing deleterious effects of airway inflammation. An ideal β_2AR agonist for asthma therapy would therefore be a G_s-protein biased agonist with inverse or antagonist properties at the ERK1/2 signaling pathway. As no such G_s-biased β_2AR agonist has yet been identified, we indirectly studied the role of β_2AR-G_s-pathway using phosphodiesterase (PDE) 4 inhibitors roflumilast and rolipram with β_2AR agonists to increase cAMP accumulation and found a protective effect of β_2AR-Gs-cAMP pathway in murine asthma models (Forkuo, Kim et al. 2016). A recently identified bifunctional compound GS- 5759 exhibits dual synergistic effects of β_2AR agonism and PDE4 inhibition suggesting a therapeutic potential in obstructive airway diseases such as COPD and asthma (Joshi, Yan et al. 2017).

2.3 Receptor Theory

2.3.1 'Classical' Receptor Theory

A.J. Clark was the first to mathematically describe the effects of a drug at a receptor. He proposed the 'occupation theory' in 1937 which suggested that receptor occupation by agonists correlated positively with the magnitude of the response. According to this initial model, a drug's effect was a function of its affinity for the receptor, with an agonist producing an effect, and an

antagonist producing no effect. However, Ariens et al. in 1954 noted that different agonists could produce different maximal responses in a given system. They introduced the term ‘intrinsic activity’, and gave a value of 1 to full agonists (those that produced the largest maximal response). Partial agonists were assigned values between 0 and 1 reflecting their ratios relative to a full agonist (Ariens 1954).

Classical receptor theory was then further developed by Stephenson in 1956, who introduced the term ‘efficacy’ to describe the ability of a ligand to activate a receptor. According to Stephenson’s theory, agonist potency was a product of the ligand’s affinity and efficacy. An antagonist had no efficacy and therefore did not activate the system. Partial agonists had efficacies between that of agonists and antagonists and produced a lower response (Stephenson 1956). Classical receptor theory further evolved through the contributions of Colquhoun, Ehlert, Furchgott, Gaddum, Rang, Schild and others to further refine concepts. For example, Furchgott proposed dividing Stephensen’s efficacy (which was a function of both the ligand, and the system), by the total number of receptors in a system to introduce a system-independent property of a ligand termed as ‘intrinsic efficacy’ (Furchgott 1966). Schild’s contribution also allowed the determination of another system-independent constant, affinity. Schild reasoned that antagonist had zero efficacy and therefore only possessed affinity. He deduced that while antagonists did not

produce a directly measurable effect, one could measure the 'effect' of a competitive antagonist by quantifying the effect of the antagonist on the agonist response curves (Arunlakshana and Schild 1959). Schild plots became one of the most valuable tools in the discovery of antagonists and led to the discovery of drugs like propranolol and cimetidine.

2.3.2 'Two-State' Model of Receptor Theory

Earlier work on ligand-gated ion channel receptors proposed that ligand binding caused a conformational change in the ion channel resulting in channel opening. Experimental data suggested the existence of two states of the ion channel, a closed state, and an open state. However, receptor theory for G-protein coupled receptors (GPCRs) could be explained by a single quiescent state of the receptor that was only activated by ligands possessing both affinity and efficacy. Then in the 1980's and 90's, studies primarily dealing with δ-opioid receptors and β-adrenergic receptors suggested that the receptor existed in two conformational states. The first mathematical model proposing 2 states for GPCRs was the ternary complex model (De Lean, Stadel et al. 1980). The model was based on receptor interaction with a membrane component that was later identified as GTP-binding protein (or G-protein). According to the ternary complex model, the receptor existed in an equilibrium between a low affinity unbound state and a high affinity G-protein-

bound state. Agonists interacted with the high affinity conformation and produced a ternary complex consisting of agonist-receptor-G protein. The equilibrium constant for this reaction depended on the intrinsic activities of the agonists. Ligand affinity and efficacy were independent parameters according to this model. The ternary complex model was successfully applied to other GPCRs coupled to adenylate cyclase system.

In 1989, Costa and Herz described the phenomenon of constitutive activity (the ability of unliganded receptors to spontaneously activate G-proteins) at the δ-opioid receptor and its reduction by certain ligands that were a subset of antagonists. Costa and Herz proposed naming the subset of antagonists that could inactivate spontaneously active receptors as 'negative antagonists', but this term was subsequently replaced with the term, 'inverse agonists' (Fig. 6). Constitutive receptor signaling was later observed at other GPCRs including β_2-adrenoceptors (β_2ARs) in recombinant systems overexpressing the receptor (Chidiac, Hebert et al. 1994) or expressing a mutated receptor (Samama, Cotecchia et al. 1993), and *in vivo* in transgenic mice overexpressing the β_2AR in the myocardium (Bond, Leff et al. 1995). Certain antagonists of the β_2AR, such as timolol, propranolol and ICI-118, 551 were able to inhibit the constitutive activation of the receptors and were named inverse agonists (Chidiac, Hebert et al. 1994, Bond, Leff et al. 1995). The ternary complex model could not explain both constitutive receptor

activity and inverse agonism. Therefore, it became necessary to extend the ternary complex model by incorporating an additional receptor state that led to the 'two state' receptor theory.

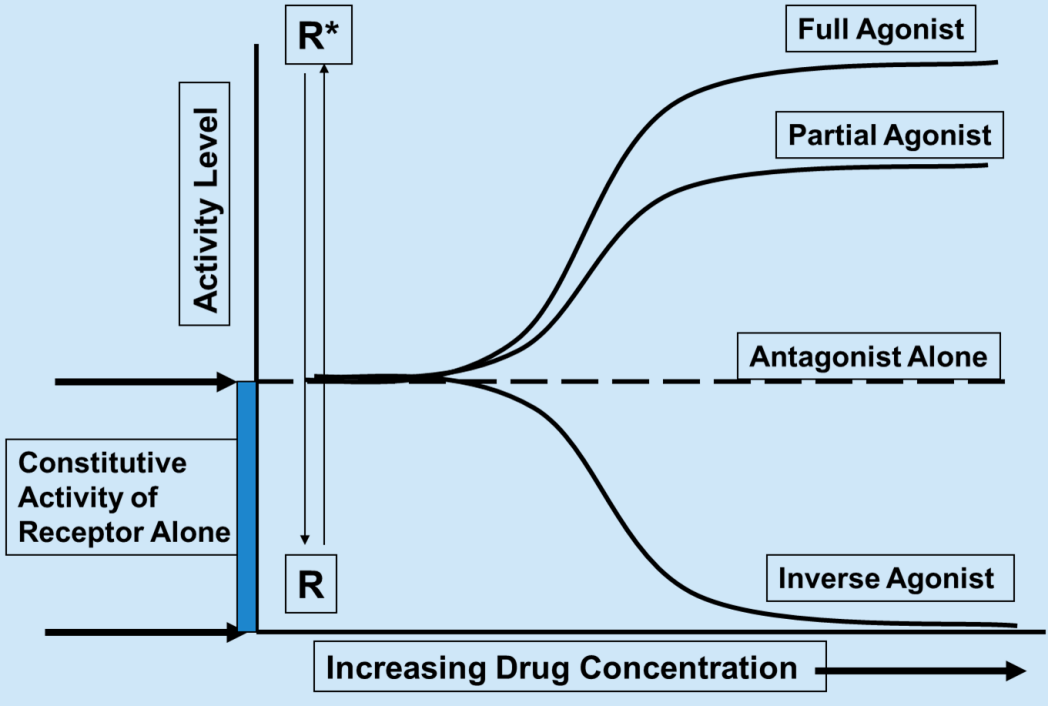

Figure 6. Constitutive Receptor Activity and Inverse Agonism The figure is a schematic representation of the dose response curves for an agonist, partial agonist, antagonist and an inverse agonist. The receptor exists in equilibrium between the inactive state R and the active state R*. Agonist and Partial agonist stabilize the R* conformation and cause receptor activation. Antagonist does not affect the equilibrium between R and R*, and does not alter the basal activity level. An inverse agonist stabilizes R conformation and reduces the constitutive activity of the receptor. Figure adapted from (Hanania, Dickey et al. 2010)

The two-state receptor theory model (Fig. 7) stated that a receptor exists in equilibrium between an inactive state R and an active state R*. In the absence of a ligand, the equilibrium constant L describes the distribution between the two receptor states, and therefore the basal activity at the receptor. Higher proportion of the active state R* gives constitutive activity to the receptor. Binding of a ligand can displace the equilibrium towards one of the two receptor states, depending on its relative affinity for the two states. An agonist has a relatively higher affinity for the active state R*, and its binding stabilizes R* at the expense of R leading to receptor activation. On the other hand, an inverse agonist has a higher affinity for the inactive state R shifting the equilibrium towards R, thereby inhibiting constitutive activation of the receptor. A competitive antagonist, also called a neutral antagonist, has equal affinity for both R and R* and does not affect the equilibrium between the two states. Therefore, unlike inverse agonists, antagonists cannot inhibit constitutive activation of the receptor, but can prevent the binding and activity of both agonists and inverse agonists. Partial agonists, like full agonists, still have a relatively higher affinity for R* than R, but the difference in their affinities for the two states is lower than that of full agonists. Similarly, partial inverse agonists have a higher affinity for R compared to R*, but the difference in the affinities for the two states is lower than that of full inverse agonists (Leff 1995, Thanawala, Forkuo et al. 2014).

Unlike traditional receptor theory models, the two-state theory suggested that ligand affinity and efficacy were dependent on the equilibrium dissociation constants for the two states, and therefore correlated with each other.

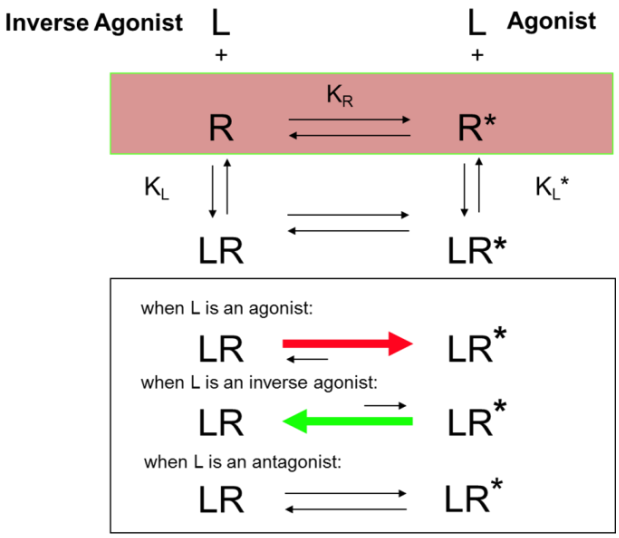

Figure 7. Two-State Receptor Theory The figure is a schematic representation of the two-state receptor theory model proposed by Paul Leff. R and R* represent the inactive and active receptor states, respectively. K_R is the equilibrium constant between the two states. The ligand L binds to the receptor with an overall affinity dictated by the equilibrium dissociation contants K_L and K_L* for the inactive and the active states, respectively. Figure adapted from (Bond, Leff et al. 1995)

2.3.2.1 Constitutive Receptor Signaling and Inverse Agonists in Asthma

Several *in vitro* studies have shown that the β_2-adrenoceptor (β_2AR) is capable of constitutively activating downstream signaling in the absence of a ligand (Samama, Cotecchia et al. 1993, Chidiac, Hebert et al. 1994); and the first *in vivo* evidence for the constitutive activity of the β_2AR was published in 1995 using transgenic mice with cardiac-specific overexpression of the human β_2AR (Bond, Leff et al. 1995).

In our previous studies using an ovalbumin driven murine model, we showed that the inverse agonist nadolol, but not the antagonist alprenolol, reduced the asthma phenotype (Callaerts-Vegh, Evans et al. 2004, Nguyen, Omoluabi et al. 2008). This suggested that constitutive activation of the β_2AR may be required for developing the asthma phenotype. However, our later studies using pharmacological and genetic means to deplete the endogenous agonist epinephrine from mice showed that agonist stimulation of the β_2AR was required for developing the asthma phenotype in a murine asthma model (Thanawala, Forkuo et al. 2013). These findings rejected our earlier hypothesis that constitutive β_2AR signaling was required for developing asthma. Furthermore, we found that only certain inverse agonists like nadolol and ICI-118, 55, but not others like carvedilol and propranolol, were able to attenuate the asthma phenotype in the ovalbumin

model of asthma (Thanawala, Valdez et al. 2015). Pilot clinical studies also showed that nadolol reduced AHR in mild asthmatics (Hanania, Singh et al. 2008), while another inverse agonist propranolol did not show similar beneficial effects in another subset of asthmatics maintained on inhaled corticosteroids (Short, Williamson et al. 2013). These results necessitated formulation of a new hypothesis that could explain the variable efficacies of β_2AR inverse agonists in asthma (vide infra).

2.3.3 'Three-State' Model of Receptor Theory

Experimental evidence for the coupling of a receptor to multiple G-proteins led Terry Kenakin to propose 'receptor promiscuity', the notion whereby a receptor could activate more signaling pathways than the canonical pathway (Kenakin 1995). However the observation that agonists could variably activate different signaling pathways did not necessitate more than 2 receptor states. To explain the differential agonist responses, Kenakin proposed a mechanism based on the differences in the 'strength of signal', which suggested that agonists with higher intrinsic efficacy could activate multiple signaling pathways, and agonists with lower efficacy could activate the most efficiently coupled signaling pathway (Kenakin 1995). The 'strength of signal' argument was also congruent with the accepted two-state receptor theory at the time.

However, the ‘strength of signal’ argument and the two-state receptor theory model could not explain the accumulating evidence showing the reversal of agonist potency or efficacy orders between different signaling pathways. This is because the two-state receptor theory described agonists as ligands that stabilized the active R* conformation over the inactive R conformation. If R* was coupled to different signaling pathways, then all agonists would show a similar pattern of signaling, and there would be no alteration of agonist potency orders between pathways (Leff, Scaramellini et al. 1997).

Evidence for different agonist potencies between pathways led to the concept of agonist-receptor trafficking, now commonly known as ‘biased agonism’ (Kenakin 1995, Kenakin 1996, Kenakin 2009). According to this concept, a receptor can activate several signaling pathways, each associated with a specific receptor conformation, and an agonist can preferentially stabilize one of these conformations relative to a reference ligand. In theory, this would suggest the existence of an unlimited number of receptor conformations. However, at the time, experimental evidence pointed to the existence of only two different signaling pathways at certain receptors. For example, agonists at the 5-hydroxytryptamine (5-HT_{2A} and 5-HT_{2C}) receptors showed differential efficacies relative to 5-HT at the phospholipase A2 (PLA_2) and phospholipase C (PLC) pathways measured in CHO cells stably

expressing 5-HT_{2A} or 5-HT_{2C} receptors. The authors based these findings on the concept of agonist-receptor trafficking (Berg, Maayani et al. 1998). For a simple prediction of altered agonist behavior at two different pathways, Paul Leff proposed the 'three-state' receptor theory model.

According to the three-state receptor theory (Fig. 8), a receptor exists in three states, an inactive state R, and two active states R* and R**. The two active states are coupled to different signaling pathways, and the ligand's activities at the two pathways are governed by their relative affinities for the three states. For agonists, their activity is dependent on their relative affinities for R* and R**. An agonist with higher affinity for R* would preferentially activate the R*-coupled signaling pathway, and an agonist with higher affinity for R** would activate the other pathway. Here, two different scenarios arise depending on the intactness of the assay systems used for measuring the signaling pathways. In the '*intact'* three-state model, all three states are in equilibrium, and activity at one pathway can affect activity at the other pathway. Therefore, agonists can show different efficacies at the two pathways but must have the same potency order. In an *isolated system*, the two pathways exist independent of each other and can be considered to operate as 2 two-state systems. Therefore, agonists may show different efficacy and potency orders between the two pathways (Leff, Scaramellini et al. 1997).

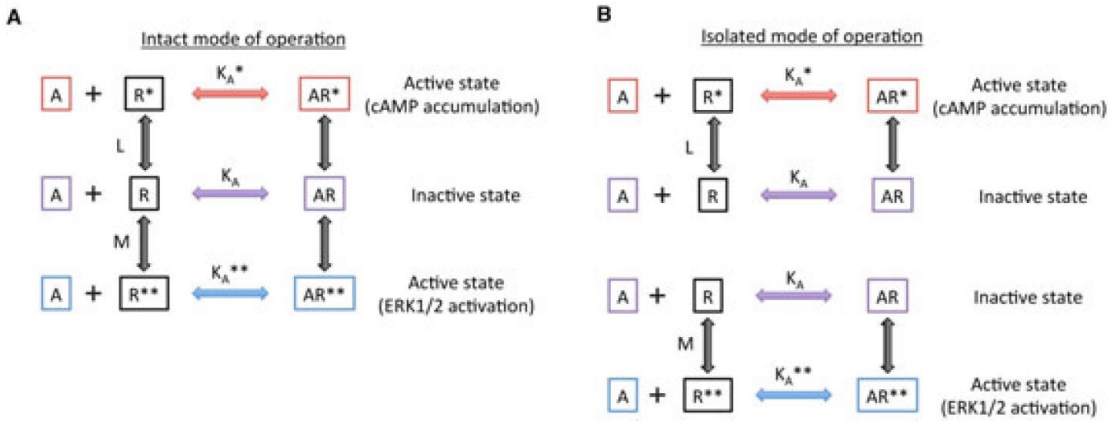

Figure 8. Three-State Receptor Theory The figure represents a schematic of the three-state receptor theory model proposed by Paul Leff et al. R is the inactive state, and R*, R** are the two active states that interact with the G proteins, G1 and G2, respectively. L is the equilibrium constant between R and R*; M is the equilibrium constant between R and R**. K_A, K_A* and K_A** represent the equilibrium dissociation constants of the agonist for the three states R, R* and R**, respectively. Panel A represents the intact-system, and panel B represents the isolated-system. Figure adapted from (Thanawala, Valdez et al. 2015).

2.3.4 Biased Signaling

Ligand bias refers to a ligand's ability to preferentially activate one signaling pathway over the other at the same receptor. The concept is based on the notion that a receptor exists in multiple conformations that interact with specific signaling pathways, and different ligands stabilize unique receptor

conformations to produce differential signaling profiles (Kenakin 2009, Kenakin 2011, Kenakin and Christopoulos 2013). The phenomenon has become an important aspect in the drug discovery process as biased ligands can have therapeutic advantages over unbiased ligands. For example, in the case of μ-opioid receptors, G-protein signaling is associated with analgesic effect and β-arrestin mediated signaling is associated with respiratory depression. Recent studies have identified biased μ-opioid receptor agonists that are potent analgesics but lack abuse potential and respiratory depression (Manglik, Lin et al. 2016). Several μ-opioid receptor biased ligands have now advanced to clinical trials. Oliceridine, also known as TRV130, is a μ-opioid receptor G protein pathway selective (μGPS) modulator that has recently been granted the 'breakthrough therapy' designation by FDA (Lewis 2017). Oliceridine is currently under phase III clinical studies for moderate to severe acute pain requiring parenteral opioid therapy (Clinical trials- NCT02656875).

2.3.4.1 Types of Bias

Biased agonism between two signaling pathways can be visualized in a bias plot, where the agonist response produced in one pathway is plotted as a function of the agonist response produced in the other pathway (Fig. 9).

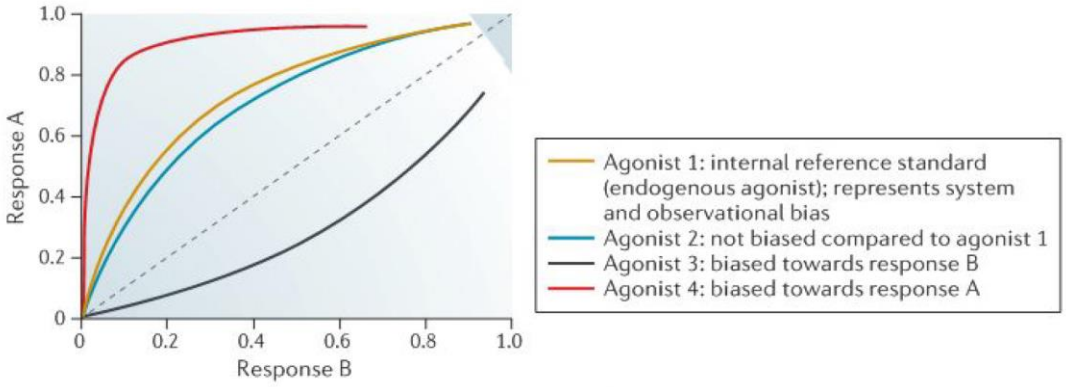

Figure 9. Bias Plot The figure is a graphical representation of ligand bias for four agonists between pathways A and B. The bias plot for the four agonists in the figure represents agonist response in pathway plotted as a function of the corresponding response in pathway B. The reference agonist (agonist 1) represents system and observational bias. Compared to agonist 1, agonist 2 is unbiased, while agonists 3 and 4 are biased towards pathway B and A, respectively. Figure adapted from (Kenakin and Christopoulos 2013).

However, biased signaling observed in *in vitro* assays can be attributed to three kinds of bias- *system bias*, *observational bias* and *ligand bias*. *System bias* stems from the differences in the efficiencies of coupling of signaling pathways in a cell. For example, in the rat atrium, inotropy (force of contraction) and lusitropy (rate of relaxation) are differentially coupled and therefore require different concentrations of cAMP. *Observational bias* arises due to differences in the sensitivities of the assays used to measure ligand response in different pathways. Therefore, the pathways cannot be directly compared to measure ligand bias. As both

system and observational biases equally affect all ligands, they do not hold any significant therapeutic advantage. System and observational biases can be removed by comparing the activities of agonists to a reference ligand at each pathway, and then comparing the relative agonist activities between different pathways. Therefore, *ligand bias* is always measured and reported relative to a reference ligand (Kenakin and Christopoulos 2013).

2.3.4.2 Quantification of Ligand Bias

Ligand bias is essentially quantified by comparing the efficacy of a ligand for stimulation of different signaling pathways relative to the same reference ligand in each pathway. However, such comparisons between agonist efficacies have to be made using system independent parameters. The Black/Leff operational model relates the agonist response to its functional affinity and efficacy according to the equation below (Kenakin 2014).

$$Response = \frac{[A]\tau Em}{[A](1+\tau) + \mathrm{K_A}}$$

Here, τ incorporates the intrinsic efficacy of the agonist as well as the system sensitivity parameters including receptor density and the coupling efficiency to the signaling pathway. The dissociation constant K_A denotes the reciprocal of the functional affinity of the agonist. Based on the model,

the ratio of τ/K_A, known as the *transduction coefficient*, can be derived as a system independent measure of agonist activity (Kenakin 2014). The parameter K_A is a measure of the functional affinity of the agonist and differs from the value obtained in simple binding assays. K_A can be calculated by the application of the Black/Leff operational model to the data or from the EC_{50} of a partial agonist (Kenakin 2014).

$$EC_{50} = \frac{K_A}{1 + \tau}$$

As receptor density is reduced, $\tau \longrightarrow 0$, then EC_{50} approximates the K_A value.

Thus, the transduction coefficient, τ/K_A, can be used to quantify ligand bias by first expressing the log (τ/K_A) for each agonist relative to a defined reference ligand to obtain $\Delta\log(\tau/K_A)$ that would control for system and observational bias. Ligand bias of an agonist between two pathways is then calculated as $\Delta\Delta\log(\tau/K_A)$ from the difference between $\Delta\log(\tau/K_A)$ values for the agonist between the pathways. Thus, log bias for an agonist for pathway p_1 over pathway p_2 is calculated from the equation below (Kenakin, Watson et al. 2012).

$$\log bias = \Delta\Delta\log\left(\frac{\tau}{K_A}\right)p_1 - p_2 = \Delta\log\left(\frac{\tau}{K_A}\right)p_1 - \Delta log\left(\frac{\tau}{K_A}\right)p_2$$

2.3.4.3 Applications of Biased Signaling and the 'Three-State' Receptor Theory in Asthma

The β_2AR is coupled to at least two different signaling pathways- the canonical Gs-cAMP pathway, and the βarrestin-2-ERK1/2 pathway (Galandrin and Bouvier 2006, Shenoy, Drake et al. 2006). Comprehensive studies of the activities of a wide range of β_2AR ligands at these two pathways showed that many of them exhibit biased signaling properties (Wisler, DeWire et al. 2007, van der Westhuizen, Breton et al. 2014). Our previous studies using the ovalbumin-driven murine asthma models and some pilot clinical trials have shown that certain β-blockers such as nadolol and ICI-118,551, but not others such as cavedilol and propranolol, are able to attenuate the asthma phenotype (Hanania, Singh et al. 2008, Short, Williamson et al. 2013, Thanawala, Valdez et al. 2015). Based on *in vitro* studies, these β-blockers are similar in inhibiting the cAMP pathway but differ in their effects at the ERK1/2 pathway. Nadolol and ICI-118,551 inhibit the ERK1/2 pathway, while carvedilol and propranolol activate ERK1/2 in cell-based assays (Fig. 10) (Galandrin and Bouvier 2006, Wisler, DeWire et al. 2007, van der Westhuizen, Breton et al. 2014). Furthermore, β-agonists like formoterol and epinephrine that were able to restore the asthma

phenotype in epinephrine deficient mice activated the ERK1/2 pathway *in vitro (*Thanawala, Valdez et al. 2015). These *in vitro* and *in vivo* studies suggest a correlation between the biased signaling properties of the β_2AR ligands (at the cAMP and ERK1/2 pathways) and their differential effects in asthma.

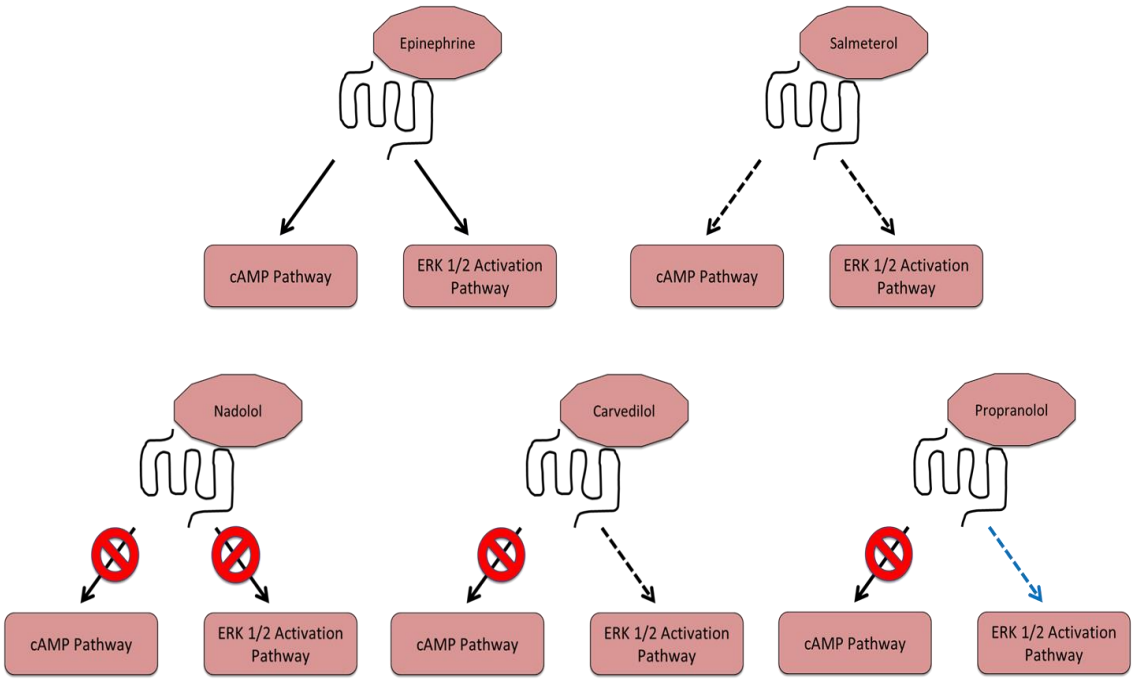

Figure. 10 Biased Signaling Profiles of β2AR Ligands The figure represents biased signaling profiles of β_2AR ligands at the cAMP and ERK1/2 activation pathways, in reference to the endogenous agonist epinephrine (depicted in the first panel). Relative to epinephrine, formoterol is a partial agonist at the cAMP and ERK1/2 pathways; nadolol inhibits both pathways; carvedilol and propranolol inhibit the cAMP pathway, but are partial agonists at the ERK1/2 pathway. The differently colored arrows for the activation of the ERK1/2 pathway by carvedilol and propranolol signify different mechanisms for the activation of ERK1/2 by the two ligands. Figure modified from (Thanawala, Valdez et al. 2015).

In collaboration with Paul Leff, our laboratory previously performed mathematical modeling using the three-state receptor theory to characterize the signaling properties of the β_2AR ligands. We compared the experimental data on cAMP and ERK1/2 activation of 12 β_2AR ligands (obtained from Van der Westhiuzen et al. 2014) with the simulated data for the intact and isolated three-state receptor theory models. The results showed that the experimental data fit the isolated three-state model better; since only the isolated model can accommodate for changes in both the potency and efficacy orders of β_2AR ligands at the two pathways (Thanawala, Valdez et al. 2015). These results of the mathematical modeling suggested that the cAMP and ERK1/2 pathways downstream of the β_2AR were independent of each other and therefore ligands could modulate one pathway without affecting the other. This could lead to high clinical relevance in designing new drugs for asthma while minimizing their adverse effects.

An important caveat of our studies so far is that the *in vitro* measurements of the signaling profiles of β_2AR ligands are made within minutes of their treatment, and therefore cannot accurately predict the behavior of these ligands after their chronic exposure *in vivo*. Furthermore, dynamic changes to a system resulting from ageing, altered pathophysiology in disease, and chronic exposure to drugs may affect ligand bias making it necessary to study bias in more dynamic systems than

in vitro cell-based models (Michel, Seifert et al. 2014). Dynamic bias as proposed by Michel et al. may also be able to explain why only a subset of drugs within a therapeutic class are beneficial in a disease (for example, only certain β-blockers have therapeutic potential in asthma). However, correlation to in vitro results may not explain these differential drug effects as chronic treatment with drugs may have a different effect on the system than their acute treatment studied in cell-based assays. Therefore, in the present studies, we have attempted to measure ERK1/2 activation *in vivo* after chronic treatment with certain β-blockers using murine asthma models to test our 'correlation' hypothesis.

2.4 Murine models of asthma

Animal models constitute an invaluable tool to study human diseases. They are being increasingly used in translational research to thoroughly investigate *in vitro* findings in intact animal systems before progressing to clinical studies. Besides humans, the only animal species that are known to develop asthma-like disease are cats and horses. However, they do not make good asthma models due to ethical and technical limitations. Mice are the most widely used animal models for asthma research as they are easy to breed, require low housing costs, and are available in a multitude of genetic variants. Anatomically, their airways are considerably different from that of humans (Fig. 11). Mice exhibit fewer numbers and orders of branching in their airways. Unlike humans,

they have much larger airways in relation to their body size, and also have fewer numbers of smaller airways (Kumar, Herbert et al. 2008). The types of cells and their location in the airways also vary between mice and humans. For example, mice have a large number of club cells, a type of secretory cells in the bronchial epithelium, but less smooth muscle cells in the airways, and their basal cell population is also restricted to tracheas. They also have no bronchial circulation, which could affect leukocyte recruitment (Mullane and Williams 2014). These anatomical differences may explain why mice, like most other animals, do not spontaneously develop features of asthma (Kumar, Herbert et al. 2008). Instead, they are sensitized to allergens to induce an immune reaction that resembles some of the features of human asthma, such as allergen specific IgE production, a Th2 biased inflammation leading to airway eosinophilia, mucus hypersecretion, airway hyper-responsiveness, and some features of airway remodeling. Nevertheless, mouse models have invariably become the species of choice to study asthma pathogenesis given the extensive availability of transgenic animals, and other features discussed above.

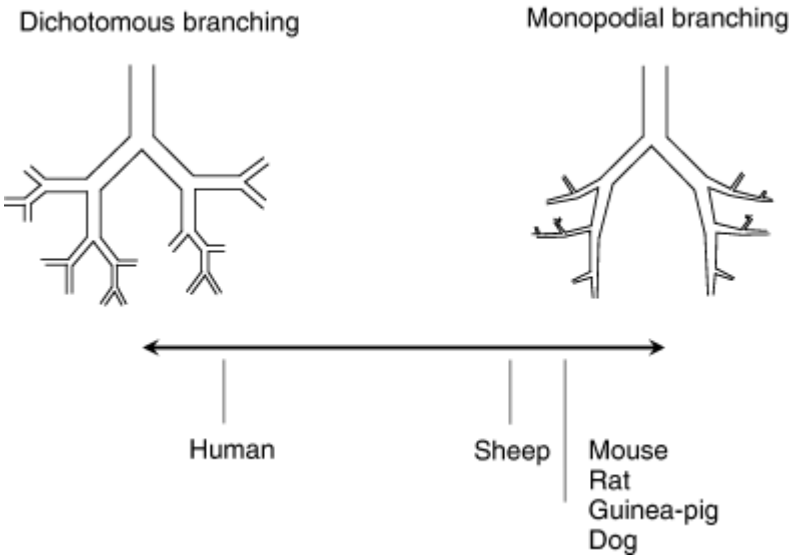

Figure 11. Lung Branching Patterns Varying Across Animal Species The figure represents the varying branching pattern of the airways between humans and other animal species that have been used as models of asthma. The airway branching pattern in humans is nearly symmetrical and dichotomous, where the daughter branches have similar diameters, while most other animals have a monopodial branching system, with one daughter branch larger than the other. Humans also have a vast network of smaller airways, unlike other species. Figure adapted from (Zosky and Sly 2007).

The heterogeneity of asthma makes it impossible for a single mouse model to mimic all phenotypes of the disease. Numerous models are available to study specific features of asthma, but their limitations must be carefully considered for the correct interpretation of results and their extrapolation to clinical relevance. The most commonly used models are allergen-driven and involve sensitization of the animals to air-borne allergens including house dust

mites, aspergillus, ragweed, cockroach extracts and even atypical allergens like chicken egg albumin (ovalbumin). The following sections discuss the two commonly used murine allergen-driven models of asthma- the 'classical' ovalbumin model, and the more recently developed house dust mite (HDM) drive model.

2.4.1 Ovalbumin-driven murine asthma model

The ovalbumin sensitization/challenge (Ova S/C) model of asthma has been the most commonly used model in asthma research. It involves peripheral sensitization of mice to ovalbumin by intra-peritoneal, subcutaneous or dermal routes in the presence of an adjuvant to boost the Th2 immune response (Mullane and Williams 2014, Sagar, Akbarshahi et al. 2015). Aluminium hydroxide (Alum) is routinely employed as an adjuvant to increase the immunogenic response to ovalbumin. However, its use is highly debated, and adjuvant-free protocols have been demonstrated to generate a comparable asthma phenotype (Conrad, Yildirim et al. 2009). Sensitization is usually carried out once a week over a period of 2-3 weeks, and induces ovalbumin specific IgE production in mice. Sensitization phase is followed by challenge with ovalbumin by intranasal or aerosol-inhalation routes, which develops a robust inflammatory response in mice mediated by the release of Th2 cytokines and chemokines (Fig. 12). The phenotype that develops is characterized by eosinophilic infiltration in broncho-alvoelar lavage fluid

(BALF), goblet cell metaplasia and mucus hypersecretion in the airways, and an increase in airway hyper-responsiveness (Mullane and Williams 2014, Sagar, Akbarshahi et al. 2015).

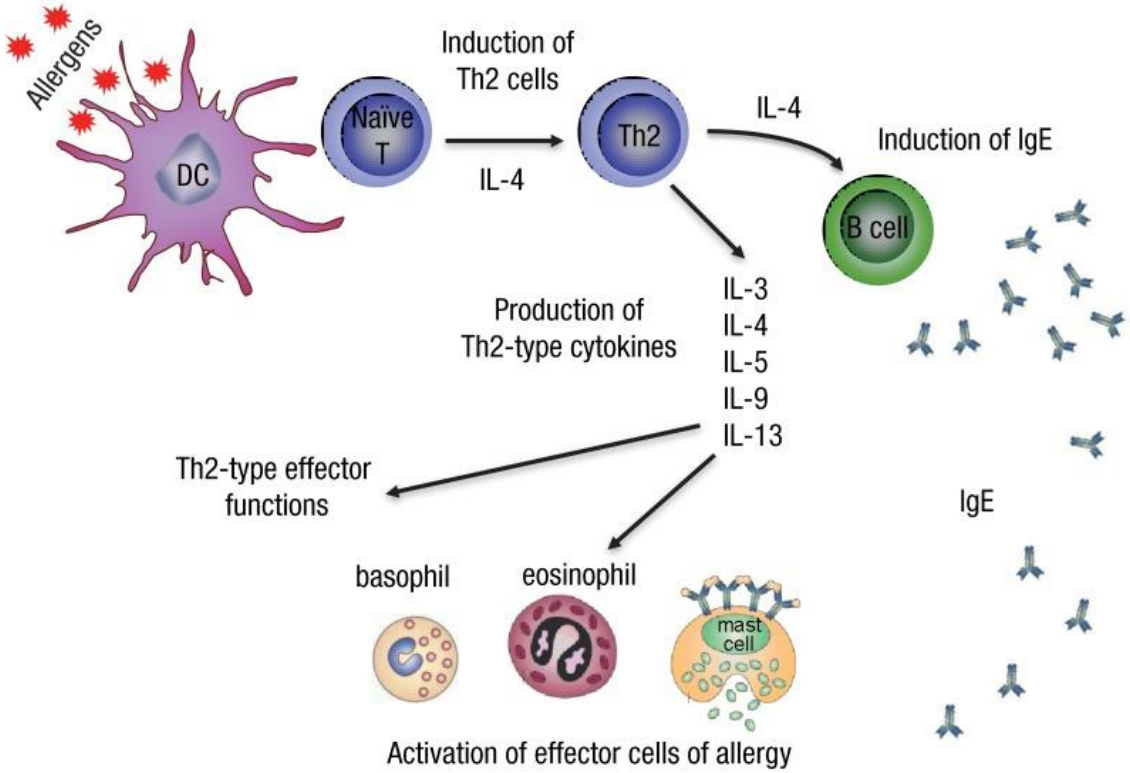

Figure 12. Inflammatory Response in Asthma Mediated by Th2 Cells Upon exposure to allergens, dendritic cells present the allergens to naïve CD4+ T cells causing their differentiation into a Th2 phenotype. The Th2 cytokine IL-4 induces the production of allergen specific immunoglobulin IgE from B cells. IgE binds to specific receptors on mast cells and basophils causing their degranulation, and releasing inflammatory mediators. Th2 cells produce inflammatory cytokines, such as IL-5, IL-9, and IL-13, which promote eosinophil recruitment, mucous metaplasia and airway hyper-responsiveness (AHR). Figure adapted from (Kucuksezer, Ozdemir et al. 2013).

The ovalbumin model has been increasingly criticized over the last decade, mainly for the lack of clinical relevance of the allergen, as ovalbumin is not implicated in human asthma. Moreover, the systemic delivery of the antigen for sensitization is not clinically relevant as it is not how humans are exposed or sensitized to allergens (Cates, Fattouh et al. 2004). Most of the sensitization and challenge protocols with ovalbumin produce an acute asthma phenotype, such as eosinophilic inflammation, mucous metaplasia, Th2 cytokines in BALF, and AHR. But, these features resolve within a few days of the last allergen challenge (Nials and Uddin 2008). In order to generate a more chronic inflammatory response with persistent AHR, the numbers of allergen challenges have to be increased. However, continuous respiratory exposure to ovalbumin could lead to immune tolerance with loss of inflammatory response (Swirski, Sajic et al. 2002, Van Hove, Maes et al. 2007, Kumar, Herbert et al. 2008).

Despite these limitations, ovalbumin models have been extensively and successfully used to understand the pathogenesis of asthma, particularly the role of Th2 immunity in the development of the disease. In fact, ovalbumin-driven asthma models led to the identification of several of the key Th2 cytokines, such as IL-5 and IL-13 as therapeutic targets. These were later translated to clinical trials and demonstrated promising results in certain asthma phenotypes (Sagar, Akbarshahi et al. 2015).

2.4.2 House Dust Mite (HDM)-driven murine asthma model

House dust mites are implicated in causing a range of respiratory symptoms in 50-85 % of asthmatics worldwide. Out of the nearly 50,000 identified species of dust mites, *Dermatophagoides pteronyssinus (Der p)* and *Dermatophagoides farinae (Der f)* are most commonly associated with allergic reactions in humans. The extract from Der p is frequently employed to induce an asthma phenotype in murine models. The phenotype development involves a complex interplay of the innate and adaptive immunity, and mediated by $CD4^+$ Th2 immune cells (Gregory and Lloyd 2011, Buday 2014).

The major contributors to the allergenicity of HDM are discussed in table 2. The serine and cysteine proteases contained in the allergens disrupt the airway epithelial barrier by causing cleavage of junctional proteins, such as zonula occluden 1 (ZO-1), occludin and claudin-1. This allows for the delivery of antigens to the antigen presenting cells in the sub-mucosal region. Furthermore, these proteases activate the protease activated receptors (PRRs) on the epithelium, which promotes leukocyte infiltration, and stimulates the release of epithelial derived cytokines, chemokines and growth factors (Gregory and Lloyd 2011). The innate, epithelial derived cytokines, such as IL-25, IL-33 and TSLP promote the recruitment of eosinophils, allergen-specific IgE production, and trafficking of activated dendritic cells to

the mediastinal lymph nodes that initiates a Th2 immune response (Gregory and Lloyd 2011).

Table 2. Allergic constituents in house dust mites (Buday and Plevkova, 2014)

Constituent	Function
Cysteine and Serine Proteases- Der p1, 3, 6 and 9	• **Disruption of epithelial barrier by proteolytic cleavage of ZO-1, occludin and E-cadherin** • **PAR2 activation leading to inflammatory cell infiltration**
β-glucan from fungal spores	**Activation of chemokine CCL20-dendritic maturation**
Chitin in HDM exoskeleton	**Potent adjuvant regulating innate and adaptive immune response**
Lipopolysaccharides endotoxin	**TLR2 and TLR4 mediated immune response**

Over the past decade, asthma researchers have switched to using HDM-driven murine models from using the “classical” ovalbumin-driven murine models of asthma. Besides using clinically relevant allergens, the HDM models offer several advantages over the ovalbumin models. Unlike

ovalbumin, the HDM models require topical (intranasal or intra-tracheal) delivery of the allergens for sensitization, thus simulating allergic sensitization in humans (Cates, Fattouh et al. 2004, Gregory and Lloyd 2011). Also, HDM models do not need an adjuvant for boosting the immune response, which avoids the potentially confounding allergenicity of the adjuvants. Unlike ovalbumin, long-term respiratory exposure to HDM can be used to generate chronic inflammation with airway remodeling in mice (Johnson, Wiley et al. 2004). However, another study showed that chronic (11 week) exposure to HDM led to diminished airway eosinophilia and AHR in mice (Bracken, Adami et al. 2015).

Despite the growing popularity of HDM-driven murine models of asthma, there is concern regarding the “allergic” component of the inflammation developed in response to repeated nasal insults with HDM (Birrell, Van Oosterhout et al. 2010). Unlike the ovalbumin models, there is no clear distinction between the sensitization and challenge phases in the HDM models. There is also limited evidence to show increased levels of total and HDM specific IgE in the murine models, while these levels are high in HDM allergic individuals (Birrell, Van Oosterhout et al. 2010). Therefore, more research is required before making an absolute switch from ovalbumin to HDM-driven murine models of asthma.

2.5 Background

2.5.1 'Paradigm Shift' in the Treatment of Congestive Heart Failure

Congestive heart failure (CHF) is associated with a decrease in cardiac contractility, blood pressure and cardiac output. Cardiac contractility (inotropy) is regulated by catecholamine stimulation of the β-adrenoceptors in the heart producing cAMP and causing muscle contraction. The reduced cardiac contractility and the decreased output from the failing heart produce a compensatory increase in the levels of epinephrine and nor-epinephrine, which initially improve cardiac function. However, the constant stimulation of the adrenergic system decreases β-adrenoceptor density in the heart and desensitizes the cAMP response, thereby reducing cardiac contractility over time (Bristow, Ginsburg et al. 1986).

Initially, treatment for CHF aimed to improve myocardial contractility by using positive inotropic drugs that stimulated the β-adrenoceptors to increase cAMP production. The β-adrenoceptor agonists, such as dobutamine improved cardiac performance when used for a short term. However, their chronic use resulted in a loss of the initial beneficial effects and an increase in mortality (Bond 2003). Therefore, the β-adrenoceptor agonists were beneficial when used acutely, but proved to be detrimental when used chronically.On the other hand, β-adrenoceptor blockers (β-blockers) were considered to be

negative inotropic drugs as they produced an acute decrease in myocardial contractility. Therefore, these drugs were contraindicated in CHF patients for many years. However, several studies in the 1990s showed that chronic treatment with certain β-blockers improved cardiac function and decreased mortality. The treatment caused an initial decrease in hemodynamic effects, but upon well-controlled dose-escalation, there was a gradual improvement in the overall cardiac performance over several months of continued therapy. Several clinical trials found promising results with chronic use of certain β-blockers such as carvedilol and metoprolol, which led to their FDA approval for the treatment of CHF (Bond et al. 2003). Therefore, there was a paradoxical therapeutic benefit of using β-blockers for treating heart failure, which later caused a ‘paradigm shift’ in the treatment of heart failure changing the once contraindicated drugs into first line therapy (Fig. 13).

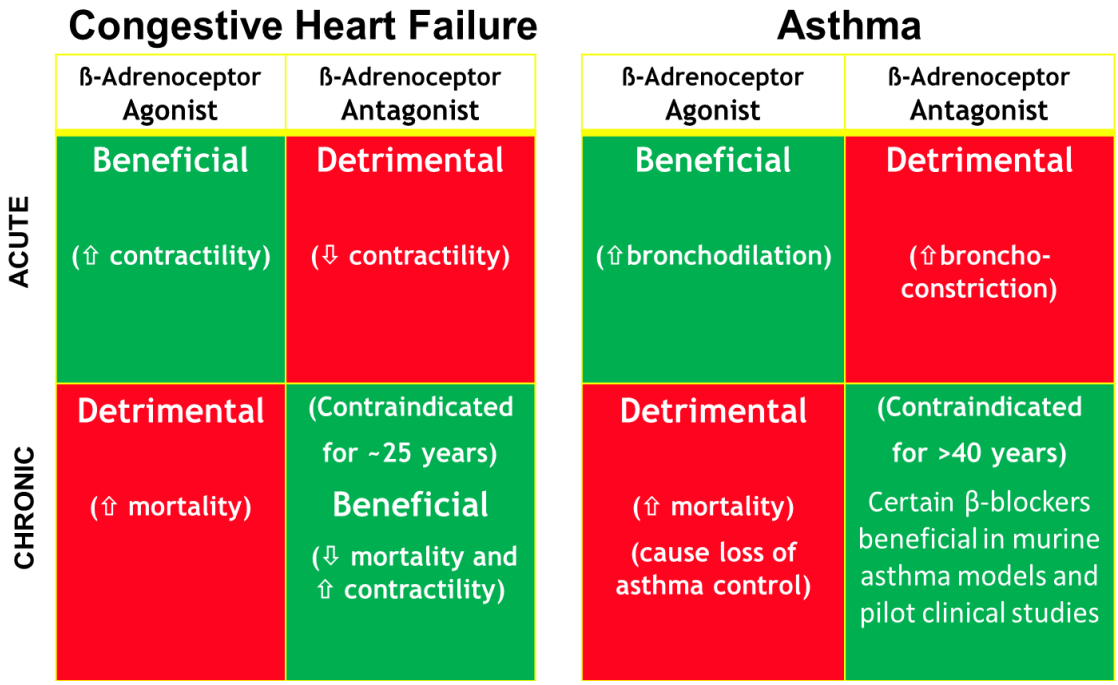

Figure 13. Pharmacological Analogy between Congestive Heart Failure and Asthma The figure represents a summary of the acute and chronic effects of β-adrenoceptor ligands in the treatment of CHF and asthma. Figure adapted and modified from (Bond 2001).

2.5.2 β-blockers in Asthma

Based on the paradoxical effects of β-blockers in the treatment of heart failure, and the analogies that existed between heart failure and asthma, our laboratory hypothesized that just as in heart failure, chronic use of certain β-blockers could be beneficial in the treatment of asthma (Fig. 13). To test the

hypothesis, we previously studied the effects of acute vs chronic administration of β-blockers including carvedilol and nadolol in an ovalbumin-driven murine asthma model. We found that chronic administration of certain β-blockers, as opposed to their acute administration, decreased the peak airway constrictor response to methacholine (Callaerts-Vegh, Evans et al. 2004). However, carvedilol caused a leftward shift in the AHR curve suggesting worsening of the airway sensitivity, whereas nadolol showed beneficial effects. In the same study, acute administration of partial β_2AR agonist salbutamol (albuterol) and another β-blocker alprenolol with partial agonist properties lowered the peak airway constriction to methacholine, but chronically did not affect the airway response. In subsequent studies with a range of β-blockers, our laboratory found that chronic treatment with β-blockers showing inverse agonist properties at the β_2 adrenoceptor (β_2AR), such as ICI-118,551 and nadolol, were able to attenuate the asthma phenotype in the ovalbumin model (Nguyen, Omoluabi et al. 2008). To further test the role of β_2AR signaling in asthma, we subjected β_2AR-null mice to the ovalbumin-driven model of asthma, and found that the β_2AR-null mice did not develop the phenotype (Nguyen, Lin et al. 2009).

The permissive role of the β_2AR signaling in the development of asthma phenotype and the attenuation of the phenotype by certain β_2AR inverse agonists led to the hypothesis that inverse agonism might be a

desirable property of β-blockers in the treatment of asthma. To test whether constitutive signaling at the β_2AR was required to develop the asthma phenotype in mice, we removed epinephrine, the endogenous agonist for the β_2AR. We used two different approaches for depleting epinephrine from mice-

a) Genetic approach using mice that lacked phenylethanolamine N-Methyltransferase (PNMT) enzyme required for the biosynthesis of epinephrine from nor-epinephrine
b) Pharmacological approach using reserpine to deplete all catecholamines from mice

Both these approaches resulted in the complete depletion of epinephrine levels in the adrenal glands and plasma. When subjected to the asthma model, these epi-null mice did not develop the asthma phenotype suggesting that agonist stimulation and not constitutive signaling of the β_2AR was required to develop the asthma phenotype (Thanawala, Forkuo et al. 2013). It also showed that β_2AR inverse agonism was not the desired property of drugs in the treatment of asthma.

Further studies on the effects of β-blockers in murine asthma models showed that only certain β-blockers like nadolol and ICI-118,551 and not others like carvedilol and propranolol were able to attenuate the asthma phenotype in murine models (Thanawala, Valdez et al. 2015). Pilot clinical

trials also showed the differential effects of β-blockers in asthma, where nadolol but not propranolol increased the PC_{20} value in different subsets of asthmatics (Hanania, Singh et al. 2008, Hanania, Mannava et al. 2010, Short, Williamson et al. 2013). The next obvious question was why only certain β-blockers showed beneficial effects on the asthma phenotype. To address this question, we looked at the biased signaling properties of the β-blockers at the Gs-cAMP and ERK1/2 activation pathways downstream of the β_2AR. These drugs were similar in inhibiting the Gs-cAMP pathway but differed in their effects on the ERK1/2 pathway. Based on the results from our *in vivo* studies and the *in vitro* signaling profiles of the various β-blockers reported previously (Galandrin and Bouvier 2006, Wisler, DeWire et al. 2007, van der Westhuizen, Breton et al. 2014), we suggested β_2AR mediated ERK1/2 activation correlated with the development of asthma phenotype. We tested this correlation hypothesis in the present studies by measuring the effect of β-blockers on ERK1/2 phosphorylation in murine asthma models.

3. Methods

3.1 Animals

For the house dust mite (HDM) driven murine models of asthma, male Balb/c mice, aged 4-8 weeks, were purchased from Jackson laboratories ((Bar Harbor, ME, USA). The β-arrestin-2 knockout (βarr2-KO) mice for use in the ovalbumin-driven asthma model studies were purchased as homozygotes from Jackson laboratories (Strain $Arrb2^{tm1Rjl}$) and subsequently bred in-house. This strain was originally created by targeted mutation of the Arrb2 (βarr2) gene in the laboratory of Robert Lefkowitz at Duke University Medical Center and later donated to Jackson laboratories. These mice lack the expression of βarr2 gene which regulates the development of asthma phenotype (Walker, Fong et al. 2003). Their wild type (WT) controls, C57BL6/J mice, were purchased from Jackson laboratories. To maintain the genotype, heterozygotes were generated by crossing the homozygous βarr2-KO mice and the WT C57BL6/J mice and subsequently bred. The offspring resulting from breeding the heterozygotes consisted of 50 % homozygous mice ($\beta arr2^{+/+}$ WT and $\beta arr2^{-/-}$ KO) which were used in experiments and 50 % heterozygous mice ($\beta arr2^{+/-}$) which were used for further breeding.

All mice were housed in the animal facility at the University of Houston under specific pathogen free conditions. They were kept on ALPHA-dri®

bedding and maintained in ambient conditions of 22-24 °C temperature and 45-48 % humidity. All mice were subjected to 12h light/dark cycle, and provided access to food and water *ad libitum*. All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Houston (protocol # 13-021, 16-022) and followed the NIH guidelines.

3.2 Genotyping

All mice produced by the heterozygous βarr2-KO breeders were genotyped to identify the βarr2-KO and WT mice. In addition, a batch of βarr2-KO and WT mice were tested for the presence of the βarr2 protein in whole lung homogenate by immunoblotting.

For genotyping, a portion of the tail (~3 mm) was clipped and digested in tail lysis buffer (50 mM KCl, 10 nM Tris-HCl, 0.1 % Triton X-100 in distilled water) mixed with proteinase K (Promega®, Madison, WI, USA) in a ratio of 60:1. Approximately, 305 µl of the mixture was added per tail sample and shaken overnight at 55-60 °C. A concentrated solution of ammonium chloride (100 µl) was added to the digested tail samples to separate the protein and 300 µl isopropanol was added to the remaining solution to precipitate the DNA. The isolated DNA was then washed with 70 % ethanol and dissolved in 25 µl PCR-grade water.

Approximately 100 ng of DNA was run in a PCR using primers for the sequences: βARR2CIN: 5’ – GAT CAA AGC CCT CGA TGA TC – 3’; βARR2FLAG22: 5’ – ACA GGG TCC ACT TTG TCC A – 3’; βARR2FLAG23: 5’ – GCT AAA GCG CAT GCT CCA GA – 3’ according to the protocol listed in table 3.

Table 3. PCR conditions for genotyping

Step	Temperature	Time (min:sec)
1	94 °C	1:30
2	94 °C	0:30
3	57 °C	0:30
4	65 °C	3:00
5	4 °C	∞

X 35 cycles

cDNA was run on a 1-2 % agarose gel containing ethidium bromide with positive and negative control samples and visualized under UV illumination. A 1 Kb ladder (Promega®, Madison, WI, USA) was used to determine the positions of

the bands on the DNA gel. The KO and WT bands appeared at 300 and 600 bp, respectively.

3.3 Murine models of asthma

Two murine models of asthma were used in these studies.

1. Ovalbumin-driven asthma model

In this model, mice were immunized with ovalbumin, the chicken egg albumin (Sigma Aldrich, St. Louis, MO, USA). After careful optimization of the dose, mice were sensitized with 0.4 mg/kg/day ovalbumin adsorbed to 2 mg alum intraperitoneally (i.p.) on days: 0, 7 and 14. Following sensitization, mice were challenged on days: 43-45 by inhalation of 1 % w/v ovalbumin solution nebulized in a chamber using the Pulmo-Aide® Compressor Nebulizer System (Devilbiss, Fulton, MD, USA) (Fig. 12). In another protocol, mice were challenged on days: 24-28 by intranasal administration of 1 mg/kg/day ovalbumin (Fig. 14). Twenty-four hours post-challenge, mice were evaluated for the various parameters of asthma.

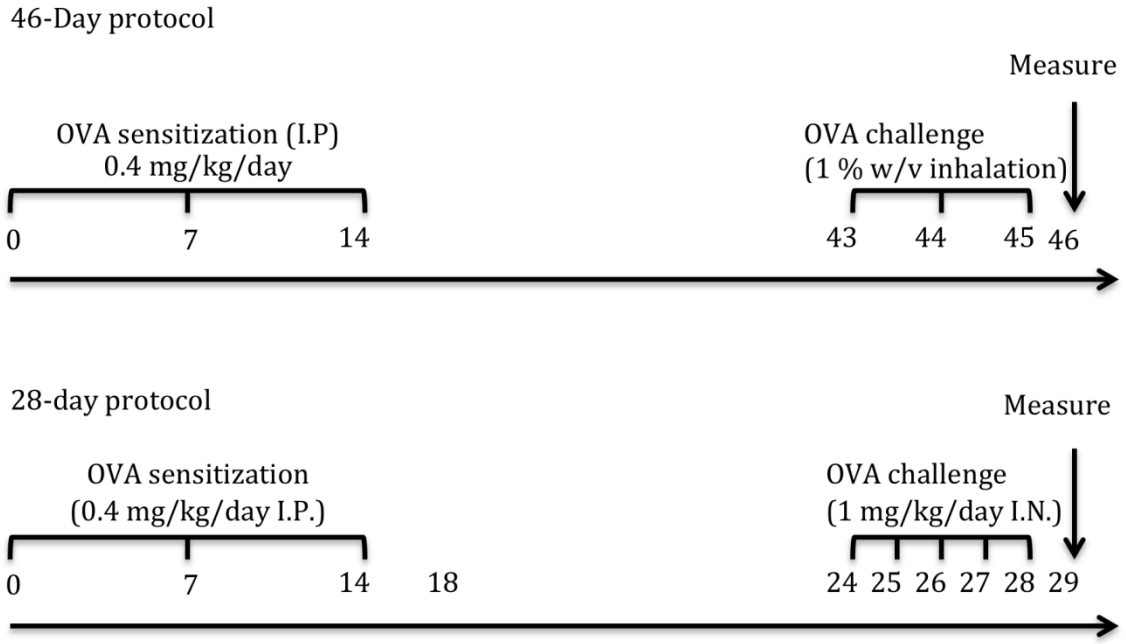

Figure 14. Ovalbumin-Driven Murine Asthma Models The figure represents ovalbumin sensitization and challenge (Ova S/C) protocols where mice were sensitized with 0.4 mg/kg/day ovalbumin mixed with 2 mg of alum and subsequently challenged with (A) intranasal delivery of ovalbumin on days 43-45; and (B) intranasal delivery of 1 mg/kg/day ovalbumin on days 24-28. Twenty-four hours later, mice were evaluated for the asthma phenotypes.

2. House Dust Mite (HDM)-driven asthma model

To overcome the limited clinical relevance of the ovalbumin allergen in causing asthma, we used the house dust mite-driven murine asthma models. Nearly 85 % of asthmatic patients are allergic to house dust mites (Gregory and Lloyd 2011). As a result, their whole body extract is commonly employed as an allergen driving the asthma phenotype in mice.

In the present studies, mice were challenged by once daily intranasal administration of 25 µg of HDM protein dissolved in 10 µl sterile saline according to the timelines for the 'prophylactic' and 'therapeutic' drug treatment protocols (Fig. 15).

a. 'Prophylactic' model

In this model, mice were challenged by intranasal delivery of 25 µg of HDM protein for 5 days per week over a period of 4 weeks and were simultaneously treated with drugs. On day 27, forty-eight hours after the final challenge with HDM, mice were evaluated for the asthma phenotypes (Fig. 15A).

b. 'Therapeutic' model

In this model, mice were initially challenged by daily intranasal administration of 25 µg of HDM protein for 10 consecutive days. A control and a vehicle group were evaluated seventy-two hours post-final HDM-challenge (day 12 of the protocol, Fig. 15B1). After the phenotype had been established, the remaining mice were started on the respective drug treatments for 4 weeks. Another vehicle group was evaluated four weeks post-final HDM challenge (protocol day 37, Fig. 15B2), while the remaining vehicle and drug treatment groups were re-challenged with HDM (25 µg) for 3 days (protocol days 35, 36, 37) and evaluated 5 days later (protocol day 42, Fig. 15B3).

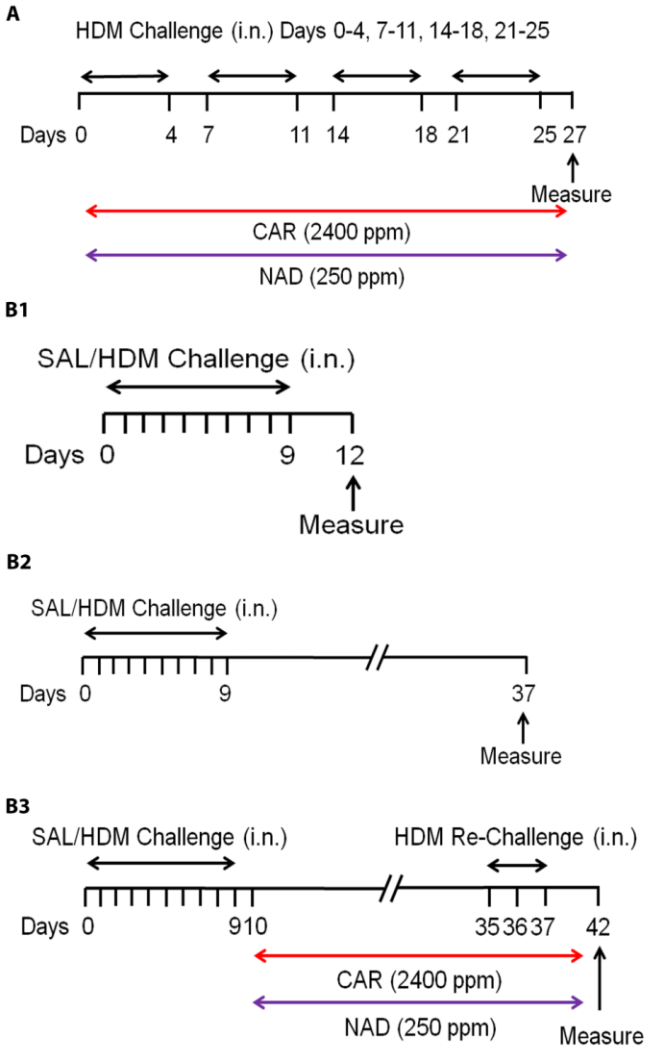

Figure 15. HDM-driven Murine Asthma Models The figure represents HDM challenge protocols for the (A) prophylactic, and (B1-B3) therapeutic models. (A) Mice were intranasally challenged with saline or 25 µg HDM protein for 4 weeks, 5 days per week. 48 hours post final challenge, mice were evaluated for asthma parameters. (B1-B3) Mice were challenged with saline or HDM for 10 days (0-9) and a subset evaluated on day 12 (B1). After the establishment of the phenotype, the remaining mice were treated with vehicle, 2400 ppm carvedilol or 250 ppm nadolol for 4 weeks and subsequently re-challenged on days 35-37. 5 days following re-challenge, mice were evaluated for the asthma parameters (B3). A vehicle treated group was evaluated 4 weeks post-final challenge on day 37 (B2) (Joshi, Valdez et al. 2017).

3.4 Drug Treatment

The non-selective β-adrenoceptor (βAR) blockers nadolol, carvedilol and propranolol were administered for at least 28 days in mice to achieve chronic treatment. Nadolol (250 ppm) and carvedilol (2400 ppm) were triturated with powdered rodent chow and provided to mice *ad libitum*. Mice were housed in groups of 3 to 4 and provided access to powdered food containing sufficient quantities of drugs in J-feeders. The feeders were filled with sufficient quantity of food to last for one day, such that each mouse ate ~ 5g of food per day. The feeders were replenished with fresh food every day. Propranolol was dissolved in drinking water at a dose of 140 mg/L, and provided to mice *ad libitum* for 28 days. The doses of nadolol, carvedilol and propranolol were selected based on our previous studies (Nguyen, Lin et al. 2009, Thanawala, Valdez et al. 2015).

3.5 Broncho-alevolar Lavage (BAL) and differential cell counts

Mouse tracheas were euthanized with 100 mg/kg i.p. pentobarbital sodium (Patterson Veterinary, Blythewood, SC, USA) and cannulated using a 20 G luer stub adapter. After isolating the left lung lobe with a hemostat, the right lobes were lavaged with 500 µl saline to obtain the broncho-alveolar lavage fluid (BALF). In another method, mouse tracheas were cannulated using PE90 (# 427421) tubing connected to silastic tubing (Dow Corning, #508-005). The other end of the silastic tubing was attached to a perfusion set containing sterile

saline solution. BALF was collected by passing through ~1 ml of saline twice into both lung lobes using a pressure of 25 mmH_20.

The collected BALF was centrifuged at 300 G rcf at 4°C, and the supernatant was flash frozen for subsequent analysis. The pelleted cells were re-suspended in 500 µl phospho-buffered saline (PBS) and counted using a hemocytometer (Hausser Scientific, Horsham, PA). Cells contained in 10 µl of the suspension were counted in the four chambers of the hemocytometer and the number multiplied by 0.5 X 10^4 to obtain the total cell count in each sample. The remaining BALF was spun onto charged slides for 5 minutes using a Cytospin® (Thermo Scientific, Waltham, MA, USA) and stained with modified Wright-Geimsa stain (Sigma Aldrich, St. Louis, MO, USA) for visualizing granulocytes. The percentage of eosinophils and neutrophils were determined in 5 random fields on each slide using 40X magnification on a light microscope. The absolute numbers of eosinophils and neutrophils were calculated by multiplying their percentages by the total cell number obtained from the hemocytometer, and expressed as eosinophils/ ml and neutrophils/ ml of BALF, respectively.

3.6 Mucous metaplasia in airways

1. **Tissue Fixation:** Following BALF collection, the hemostat isolating the left lung lobe was released. The right lung lobes were removed and flash frozen

for further analysis, while the left lobe was perfused with cold 10 % buffered formalin using a pressure of 25 mmH_20. The tracheal cannula was removed, and the open end of the trachea was tied off with a suture. The perfused lung was removed from the thoracic cavity and placed in cold 10 % formalin for overnight fixation at 4 ºC.

In another method where BALF was collected from all lung lobes, lungs were removed from the chest cavity while still connected to the tracheal cannula. The right lobes were isolated and flash frozen. The left lobe was perfused with cold 10 % buffered formalin using a pressure of 25 mmH_2O, and further fixed overnight at 4 °C.

2. Sectioning and Staining: Twenty-four hours post-fixation, the top and bottom ends of the left lung lobe were removed and the remaining part was sectioned transversely into two halves. The sectioned lobe was placed into tissue cassettes and dehydrated and fixed in paraffin using the paraffin robot. The two halves were then embedded into paraffin blocks for further sectioning.

The paraffin blocks were mounted on the sample holder of a microtome to obtain 5 µm thick transverse sections. At least 2-3 sections from each sample were collected onto positively charged slides and stained with periodic acid fluorescent Schiff's stain for mucin quantification. Prior to staining, sections

were de-paraffinized with Histo-Clear® or xylene substitute and rehydrated by incubating in gradients of ethanol solutions (100 %, 95 %, 80 %, 70 %) and PBS. The rehydrated sections were then incubated with 1 % periodic acid to carefully oxidize alcohols in the glycoproteins to aldehydes. Following oxidation, sections were incubated with fluorescent Schiff's reagent (0.5 % acriflavine, 1 % HCl, 1 % sodium metabisulfite in distilled water) in dark for 20 minutes and rinsed in distilled water and acid alcohol. The stained sections were air dried in the dark and mounted with #1.5 coverslip using Fluoro-Gel® mounting medium. The slides were allowed to set overnight on bench top and subjected to fluorescence microscopy the following day.

3. Image Acquisition: Images of bronchial airway sections were captured at 40X magnification on a fluorescence microscope for morphometric analysis of airway mucin content. At least 6 images of focused areas on bronchial airways were captured for each sample. The fluorescent acriflavine Schiff's reagent reacts with the aldehydes in mucin glycoproteins (obtained after periodic acid oxidization during staining) and fluoresces red when excited over a broad range of wavelength (380-580 nm). The parenchymal cells in the epithelium fluoresce green when excited at the same range (Piccotti, Dickey et al. 2012). For analysis, the airway sections were excited at the peak excitation spectrum of 500/573 nm and images were acquired using dual band pass filters for GFP (green)/TRITC (red) with peaks at 530/628 nm. The

acquired images were stored in tiff format for morphometric quantification (Piccotti, Dickey et al. 2012).

4. Morphometric Analysis: The airway mucin content was quantified by morphometric analysis of the airway images using ImageJ® software. The scale was set for each image based on the magnification used during image acquisition. The length of the basement membrane and the area of mucin globules were measured for each image and the mucin volume density with the units of nl/mm^2 calculated by dividing the mucin area by the product of basement membrane length (BML) and $4/\pi$ (Piccotti, Dickey et al. 2012).

$$\text{Mucin Volume Density} = \text{Mucin Area} / (\text{BML} * 4/\pi)$$

3.7 Airway Hyper-responsiveness (AHR)

Airway hyper-responsiveness (AHR) is a cardinal feature of asthma characterized by an increased sensitivity of airways to constrictors such as methacholine. In the present studies, AHR was measured in response to dose increments of nebulized methacholine (0-50 mg/ml) by the forced oscillation technique (FOT) using the Flexivent® (Scireq, Montreal, Canada) equipped with an in-line nebulizer. At the beginning of the experiment each day, airway calibration was performed at 0 and 300 mmHg using a sphygmomanometer attached to the Y-tube of the Flexivent. Tube calibration was performed for each subject prior to the experiment by attaching the cannula to be used for the

subject to the Y-tube. Upon calibration, mice were anesthetized and paralyzed using 240 mg/kg ketamine and 48 mg/kg xylazine with booster doses as required during the experiment. After the loss of corneal and nociceptive reflexes was extablished, mice were tracheostomized and cannulated with a 20 G luer stub adapter. The anesthetized mice were connected to the Y-tube of the instrument through the cannula and ventilated at 150 breaths per minute using a small animal ventilator within the Flexivent. A positive end-expiratory pressure (PEEP) of 3 cmH_2O was maintained within the lungs during the experiment. Heart rate was monitored throughout the experiment using EKG leads attached to the limbs and if it fell below 40 bpm, the mouse was declared dead for the experiment and euthanized immediately.

After about 4 minutes of ventilation, the perturbations were started and the baseline resistance measurement was acquired. Saline followed by increasing doses of methacholine (1, 2.5, 10, 25 and 50 mg/ml) were nebulized using the in-line ultrasonic nebulizer (Aeroneb®), and the total respiratory system resistance (Rrs) measurements were recorded. Each dose was nebulized for 15 seconds and the airway resistance was measured every 10 sec over a duration of 3 min. Each dose administration was followed by two deep inflations to bring the airway resistance back to baseline.

The total airway resistance (Rrs) was calculated by averaging the peak and one value on either side of the peak. Airway sensitivity (PC100), was

determined by measuring the concentration of methacholine causing doubling of the baseline airway resistance; and airway reactivity (K) was determined from the slope of the Rrs-methacholine dose-response curve. These parameters were calculated using non-linear regression analysis on the dose-response curve fitted to an exponential growth function (Joshi, Valdez et al. 2017).

3.8 Cytokine and Chemokine Analysis in BALF

Airway inflammation in asthma is majorly mediated by Th2 type lymphocytes that produce pro-inflammatory Th2 cytokines IL-4, IL-5 and IL-13 and chemokines CCL11/EOTAXIN and RANTES. In the present studies, we measured the concentration of Th1- and Th2- cytokines and chemokines in the broncheo-alveolar lavage fluid (BALF) in the house dust mite (HDM)-driven murine asthma models.

BALF samples were collected from mice subjected to the 'prophylactic' HDM model 4 hours post-final saline/HDM challenge as described earlier in section 3.3. The levels of the pro-inflammatory cytokines IL-13 (Th2), IFN-γ (Th1) and IL-17 (Th17), and chemokines EOTAXIN (Th2) and CXCL1/KC (Th1) were measured by sandwich ELISAs according to the manufacturer's instructions (R&D Systems,, Inc., Minneapolis, MN, USA). The catalogue numbers and the sensitivities of the ELISA kits are mentioned in table 4. Briefly,

50 µl of the samples, standards or controls were added to each well of a microplate pre-coated with the polyclonal antibody specific for each analyte. After washing the wells to remove the unbound antigens, a specific enzyme-linked polyclonal antibody was added to form a sandwich of antibody-antigen-antibody, which when incubated with a substrate yielded a blue product. Upon addition of the stop solution containing HCl, the blue product turned yellow. The intensity of the color corresponded with the amount of the analyte present in the samples.

The optical density was measured for each well and a standard curve was generated using log-log curve fitting or 4-point logistic curve fitting, as recommended by the kit. The sample values were calculated by interpolation on the standard curve.

Table 4. ELISA kits for cytokines and chemokines

ELISA Kit	Catalogue Number (R&D Systems)	Sensitivity (pg/ml)
IL-13	M1300	1.5 pg/ml
IFN-γ	MIF00	2 pg/ml
IL-17	M1700	5 pg/ml
CCL11/EOTAXIN	MME00	3 pg/ml

CXCL1/KC	MKC00B	2 pg/ml

3.9 Ovalbumin-specific IgE

Ovalbumin allergen-specific immunoglobulin IgE was measured in serum samples collected from mice post-mortem. Prior to performing lung lavage in mice, blood was collected by cardiac puncture and allowed to clot for ~30 min at room temperature. Serum was separated by centrifugation at 2500 rpm for 10 min, flash frozen in liquid nitrogen, and stored at -80 °C until further analysis. The concentration of ovalbumin-specific IgE in mouse serum was measured by a sandwich ELISA using the manufacturer's instructions (Cat # 439807, Biolegend®, San Diego, CA, USA) as described briefly in the earlier section.

3.10 Immunoblotting

Extracellular-signal regulated kinases (ERK) 1/2 phosphorylation and Exchange Protein Directly Activated by cAMP (EPAC) 1/2 expression were measured in the whole lung homogenates by immunoblotting. After collecting BALF, the right lung lobes were isolated and flash frozen in liquid nitrogen. The frozen lungs were homogenized in lysis buffer (50 mM Tris-base, 1.25 mM EDTA, 1.2 mM PMSF in distilled water) containing protease and phosphatase inhibitor cocktail (Halt™, Thermo Scientific, Waltham, MA, USA) and briefly sonicated. The homogenized lung samples were centrifuged at 5000 rpm for 8

minutes and the supernatants were collected and stored at -80 °C until further use. The total protein concentration in the samples was determined by Pierce bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). The protein samples were mixed with laemmli buffer containing 2-mercaptoethanol to get a final protein concentration of 2 µg/µl. Samples were heated at 95 °C for 6 minutes and stored at -20 °C.

Twenty-five µg of protein from each sample was loaded on 4-20 % pre-cast polyacrylamide gels and subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) at 90V for 90 minutes. The separated proteins on the gels were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with milk powder (5%) in 50 mM Tris, 0.15 M NaCl and 0.1% Tween 20 (TBS-T) for 2 hours. The membranes were immunoblotted with the mouse monoclonal anti-P-p44/42 MAPK (pERK1/2), anti-p44/42 MAPK (ERK1/2), anti-EPAC1 and 2 antibodies (Cell Signaling, Danvers, MA, USA) using the recommended antibody dilutions. The catalogue information and the dilutions used for the various antibodies are mentioned in table 5. The proteins were detected by chemiluminescence and subsequently imaged and analyzed using the Fluor-Chem 8800® imaging system (Alpha Innotech, CA, USA).

Table 5. Primary antibodies used for immune-blotting

Antibody	Manufacturer	Catalogue No.	Dilution
Phospho-p44/42 MAPK mouse mAb	Cell Signaling	9106	1:2000
p44/42 MAPK mouse mAb	Cell Signaling	9107	1:2000
Epac1 mouse mAb	Cell Signaling	4155	1:1000
Epac2 mouse mAb	Cell Signaling	4156	1:1000
GAPDH Rabbit mAb	Cell Signaling	2118	1:1000

3.11 Statistical Analysis

Statistical analysis was performed using Graph-Pad Prism® 7 software (San Diego, CA, USA). Data are expressed as mean ± SEM (standard error of means). Assuming normal distribution of the data, statistical analysis was performed using student's t-test for comparing two groups and one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple

comparison tests for more than 2 groups. Airway sensitivity and reactivity were measured using non-linear regression analysis of the dose-response curve data fitted to an exponential growth function. Standard curves for ELISAs were generated using log-log curve fitting or 4-point logistic curve fitting and the unknown values calculated by interpolation.

4. Chapter 1.

The 'prophylactic' and 'therapeutic' effects of β-blockers in a house dust mite (HDM) driven murine asthma model*

*The data discussed in this chapter has been published in the manuscript titled "Effects of β-blockers on house dust mite-driven murine models pre- and post-development of an asthma phenotype" in *Pulmonary Pharmacology and Therapeutics,* 2017

4.1 Rationale

We have previously shown the differential effects of β-blockers on the asthma phenotype using ovalbumin-driven murine asthma models. However, the ovalbumin models suffer from several limitations and have been increasingly criticized for the lack of clinical relevance of the allergens involved in producing the phenotype. To address these limitations, we switched to using the well-recognized clinically relevant allergens in house dust mites to develop an asthma phenotype in murine models.

4.2 Objective and Hypothesis

The present studies aimed to investigate the effects of the β-blockers carvedilol and nadolol on the asthma phenotype, when the drugs were administered pre- and post- the development of the phenotype, using a more clinically relevant HDM-driven murine model of asthma.

Based on our previous studies, we hypothesized that similar to their effects in the ovalbumin models, nadolol, but not carvedilol, would attenuate the asthma phenotype in the HDM-driven murine models of asthma.

4.3 Approach

To test our hypothesis, we employed protocols to study the effects of 'prophylactic' (pre-development of phenotype) and 'therapeutic' (post-development of phenotype) treatment with β-blockers using HDM-driven murine models of asthma. The models have been described in detail in 'Methods' section and are only briefly discussed here. In the *prophylactic* model, Balb/c mice were exposed to daily intranasal administration of 25 µg of HDM protein 5 days a week for 4 weeks. Drug treatment with carvedilol (2400 ppm) and nadolol (250 ppm) began simultaneously with the HDM challenges and continued throughout the duration of HDM exposure. Calculated amounts of the drugs were mixed in powdered chow and fed to mice in J-feeders *ad libitum*. At the end of 4 weeks, we evaluated the mice 48 hours post-final HDM challenge for the presence of the three cardinal features of asthma-

a) Inflammatory cellular infiltration in broncheo-alveolar lavage fluid (BALF)
b) Airway mucous metaplasia
c) Airway hyper-responsiveness (AHR)

In the *therapeutic* model, we initially challenged mice with HDM protein for 10 days, and evaluated a subset of mice 72 hours later for the presence of the asthma phenotype. After establishing the asthma phenotype, we treated the remaining mice with vehicle, carvedilol (2400 ppm) or nadolol (250 ppm) for four weeks. At the end of four weeks, we evaluated another vehicle-treated subset of mice, and then re-challenged the other vehicle and drug treatment groups with HDM for 3 more days. Five days following re-exposure to HDM, we evaluated all the remaining groups of mice.

In addition to measuring the asthma phenotype, we also characterized the HDM model for the inflammatory cytokine/chemokine profile driving the phenotype. We measured the levels of cytokines- IL-13 (Th2), IFN-γ (Th1), and IL-17 (Th17); and chemokines- EOTAXIN (Th2) and CXCL1/KC (Th1) in BALF. However, the cytokine and chemokine levels are sensitive to the time of measurement after the last HDM exposure. Based on literature, we selected the time point of 4 hours after the final HDM challenge for collection of BALF for the analysis of inflammatory mediators.

4.4 Results

4.4.1 'Prophylactic' effect of β-blockers on inflammatory cellular infiltration in BALF

Mouse lungs were lavaged with saline through an intra-tracheally implanted 20-G leur stub adaptor and the total cellular counts and eosinophil counts were determined as described in 'Methods'. In the 'prophylactic' model, 4 weeks of HDM exposure in mice significantly increased both total cells and eosinophils in BALF. Concomitant treatment with carvedilol did not affect this increase in the total cells and eosinophils, whereas nadolol treatment significantly attenuated the increase in the inflammatory cells in response to HDM (Fig. 16). The HDM-induced inflammatory cellular infiltration in nadolol treated mice was also significantly lower than in carvedilol treated mice; however it was still higher than the inflammatory cells in saline control mice (Fig. 16).

4.4.2 'Therapeutic' effect of β-blockers on inflammatory cellular infiltration in BALF

In the 'therapeutic' model, the initial HDM exposure significantly increased the numbers of total cells (Fig. 17A, C) and eosinophils (Fig. 17B, D) in BALF over saline control mice. The increase in the inflammatory cells was completely resolved at 4 weeks after the final HDM challenge in these

mice (as evaluated on protocol-day 37, Fig. 17A-D). Re-exposure to HDM restored the increase in both the total cells and eosinophils in the vehicle and carvedilol treated mice (Fig. 17A, B). However, nadolol treatment significantly lowered the increased numbers of total cells and eosinophils in response to HDM re-exposure in mice (Fig. 17C, D).

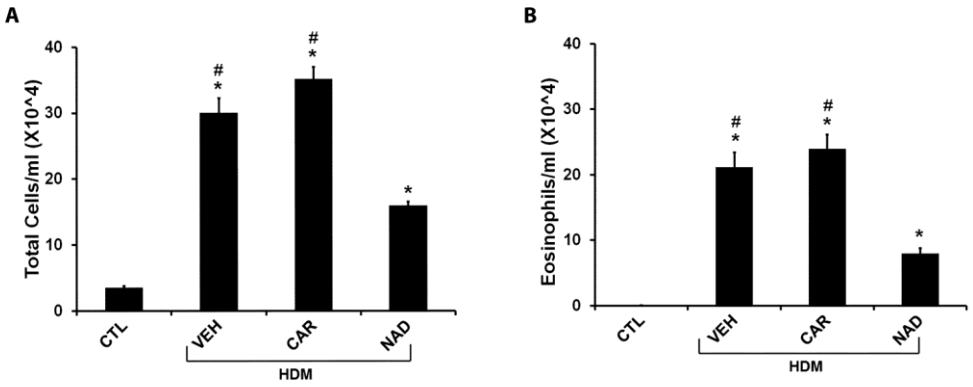

Figure. 16 'Prophylactic' Effect of β-blockers on Inflammatory Cellular Infiltration in BALF The figure is a graphical representation of the total cells (A) and eosinophils (B) in broncheo-alveolar lavage fluid (BALF) collected from mice in response to HDM exposure. Mice were concomitantly treated with vehicle (VEH), 2400 ppm carvedilol (CAR) or 250 ppm nadolol (NAD). A. Total cells in HDM exposed mice treated with VEH, CAR or NAD compared to the saline exposed CTL group. B. Eosinophil cells in HDM exposed mice treated with VEH, CAR or NAD compared to the saline exposed CTL group. Data are plotted as mean ± SEM from 5-6 mice per group. * represents statistical significance at $p < 0.05$ in reference to the CTL group. # represents statistical significance at $p < 0.05$ in reference to the NAD group. Figure adapted from (Joshi, Valdez et al. 2017).

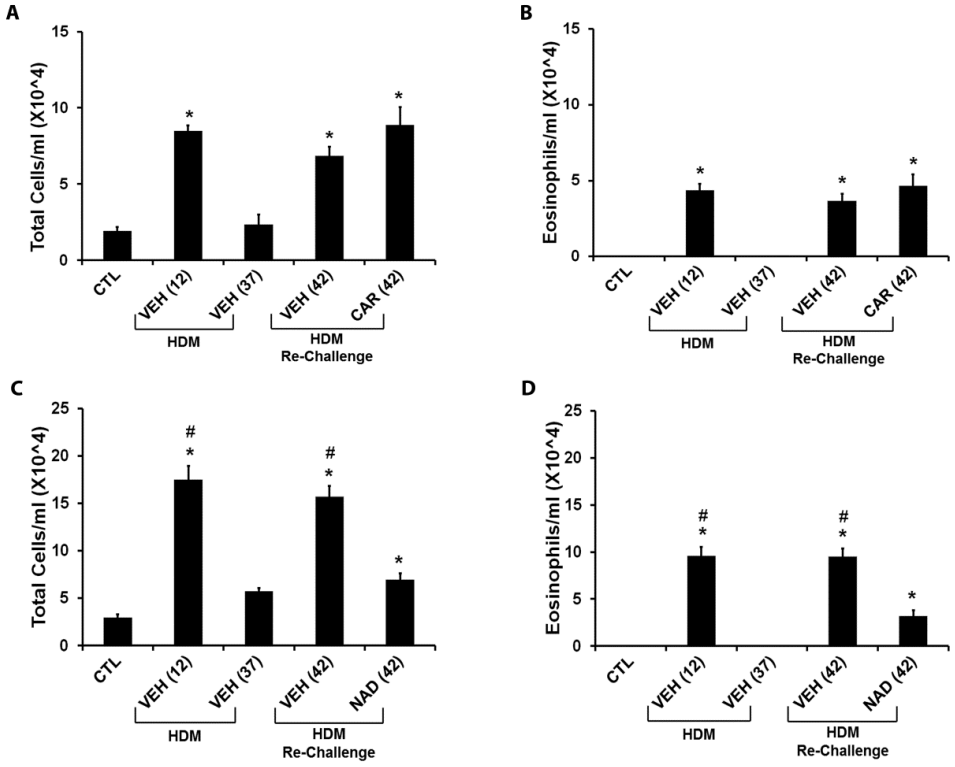

Figure. 17 'Therapeutic' Effect of β-blockers on Inflammatory Cellular Infiltration in BALF
The figure is a graphical representation of the total cells (A, C) and eosinophils (B, D) in broncheo-alveolar lavage fluid (BALF) collected from mice in response to HDM exposure. A. Total cells and B. eosinophils in mice evaluated on days 12, and 37 after initial HDM exposure; and mice treated with VEH or CAR and evaluated on day 42 after HDM re-exposure, compared to saline control mice. C. Total cells and D. eosinophils in mice evaluated on days 12, and 37 after initial HDM exposure; and mice treated with VEH or NAD and evaluated on day 42 after HDM re-exposure, compared to saline exposed CTL mice. Data are plotted as mean ± SEM from 5-8 mice per group. * represents statistical significance at $p < 0.05$ in reference to the CTL group. # represents statistical significance at $p < 0.05$ in reference to the NAD (42) group. Figure adapted from (Joshi, Valdez et al. 2017).

4.4.3 'Prophylactic' effect of β-blockers on mucous metaplasia in the airways

Airway sections from the left lung lobes were stained with periodic acid fluorescent Schiff's (PAFS) stain to measure mucin content in goblet cells in the airway epithelium. As represented in the Fig. 18, panel A, mucin appears as red globules (fluorescing in TRITC emission spectrum) over green epithelium (fluorescing in GFP emission spectrum). In the prophylactic model, HDM exposure significantly increased the mucin volume density contained in the airway epithelium. Carvedilol treatment did not affect, while nadolol treatment significantly attenuated the increase in the mucin volume density in response to HDM (Fig. 18). Similar to inflammatory cellular infiltration, nadolol treated mice showed significantly lowered mucin density compared to carvedilol treated mice, but it was still higher than the mucin density in saline control mice (Fig. 18).

4.4.4 'Therapeutic' effect of β-blockers on mucous metaplasia in the airways

In the therapeutic model, the initial 10 days of HDM exposure significantly increased mucin volume density in the airways which was completely resolved after four weeks as evaluated on day 37 of the protocol (Fig. 19). As with the inflammatory cells, HDM re-exposure restored the

increase in mucin volume density in the vehicle and carvedilol treated mice (Fig. 19). Nadolol treated mice showed a significant decrease in the mucin content compared to the vehicle and carvedilol treated mice after HDM exposure, but the mucin content was still higher than in saline control mice (Fig. 19).

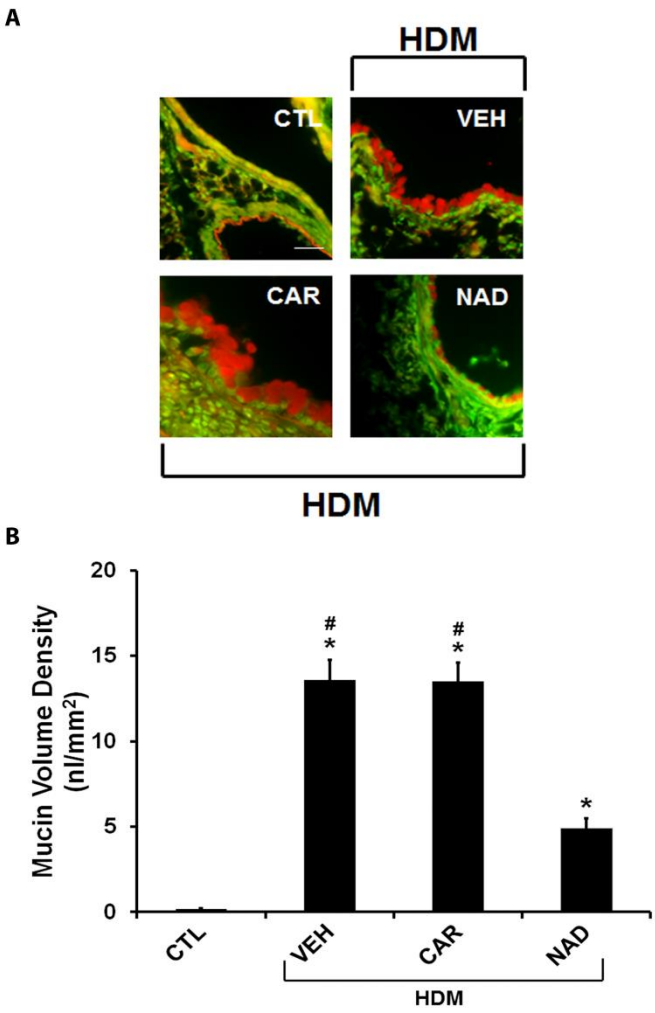

Figure. 18 'Prophylactic' Effect of β-blockers on Mucous Metaplasia in BALF The figure represents (A) PAFS stained images showing mucin content (red) in airway epithelial cells (green) and (B) morphometric quantification of mucin volume density from mice in response to HDM exposure in the prophylactic model. Mice were concomitantly treated with vehicle (VEH), 2400 ppm carvedilol (CAR) or 250 ppm nadolol (NAD). B. Graphical representation of mucin volume density in HDM exposed mice treated with VEH, CAR or NAD compared to the saline exposed CTL group. Data are plotted as mean ± SEM from 5-6 mice per group. * represents statistical significance at $p< 0.05$ in reference to the CTL group. # represents statistical significance at $p < 0.05$ in reference to the NAD group. Figure adapted from (Joshi, Valdez et al. 2017).

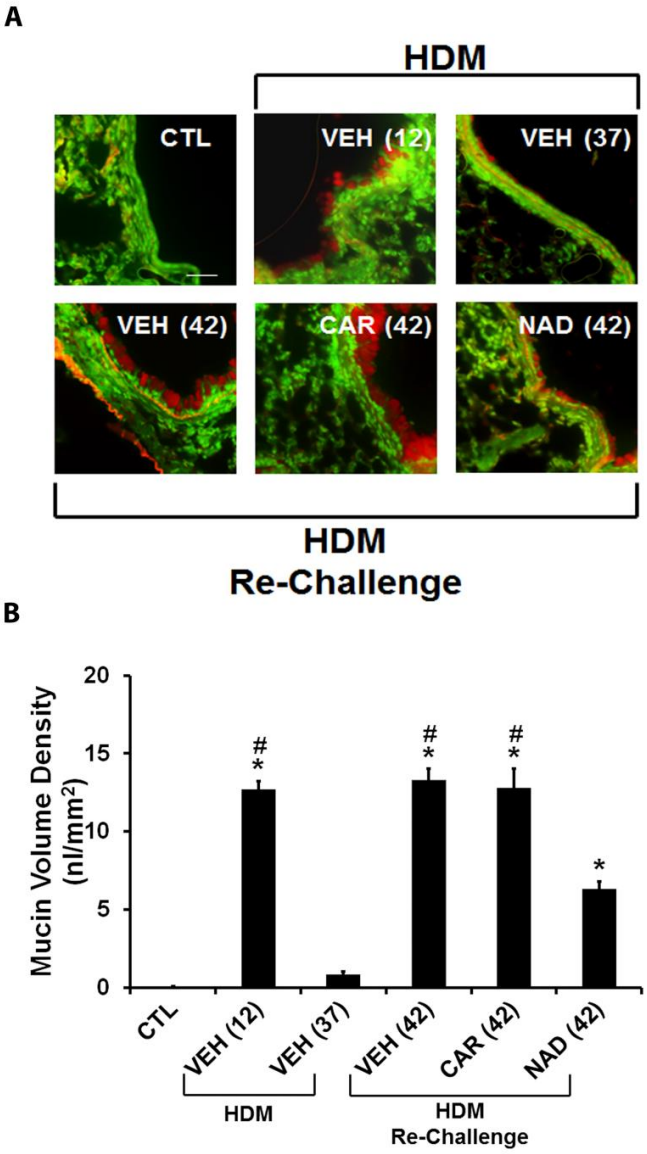

Figure. 19 'Therapeutic' Effect of β-blockers on Mucous Metaplasia in BALF The figure represents (A) PAFS stained images showing mucin content (red) in airway epithelial cells (green) and (B) morphometric quantification of mucin volume density from mice in response to HDM exposure in the therapeutic model. The graph represents mucin volume density in mice evaluated on days 12, and 37 after initial HDM exposure; and mice treated with VEH, CAR or NAD and evaluated on day 42 after HDM re-exposure, compared to saline control mice. Data are plotted as mean ± SEM from 5-8 mice per group. * represents statistical significance at $p < 0.05$ in reference to the CTL group. # represents statistical significance at $p < 0.05$ in reference to the NAD group. Figure adapted from (Joshi, Valdez et al. 2017).

4.4.5 'Prophylactic' effect of β-blockers on airway hyper-responsiveness (AHR)

We measured the total respiratory resistance (Rrs) to dose increments of the airway constrictor methacholine (0-50 mg/ml) using the Flexivent® as detailed in 'Methods'. As additional parameters of AHR, we measured airway sensitivity, which is calculated from the dose of methacholine causing doubling of the baseline airway resistance (PC_{100}); and airway reactivity calculated from the slope (K) of the methacholine-Rrs dose-response curve.

In the 'prophylactic' model, HDM exposure in mice significantly increased Rrs over saline exposure in the control mice. The increase in Rrs reached significance at the 25 mg/ml and 50 mg/ml doses (Fig. 20A). Carvedilol treated group of mice exposed to HDM showed a similar increase in Rrs in response to methacholine (Fig. 20A). Nadolol treated mice showed a significant decrease in Rrs compared to the vehicle and carvedilol treated mice exposed to HDM, but showed a higher airway resistance than saline control mice (Fig. 20A). We also plotted peak airway resistance to methacholine and found that vehicle and carvedilol treatment with HDM exposure in mice significantly increased the peak Rrs, while nadolol treatment with HDM exposure did not increase the peak Rrs (Fig. 20B). HDM exposure in both vehicle and carvedilol treated mice, but not in nadolol treated mice,

significantly increased airway sensitivity (depicted by a decrease in PC_{100} value) and airway reactivity (Fig. 20C, D).

4.4.6 'Therapeutic' effect of β-blockers on airway hyper-responsiveness (AHR)

In the therapeutic model, the initial HDM exposure in mice increased the total airway resistance (Rrs) to methacholine, which remained elevated even 4 weeks after the last HDM challenge (Fig. 21A, B). Re-challenge with HDM 4 weeks later maintained the increase in Rrs in mice, which was not affected by carvedilol treatment, but was attenuated by nadolol treatment (Fig. 21A). However, both carvedilol and nadolol treatment lowered the peak airway resistance compared to the vehicle treatment in mice re-exposed to HDM (Fig. 21B). In terms of airway sensitivity and reactivity, neither carvedilol nor nadolol caused a change in these parameters. However, HDM exposure did not significantly change airway sensitivity and reactivity over saline exposure making it difficult to draw conclusions (Fig. 21C, D).

4.4.7 Inflammatory cytokines and chemokines in BALF

We characterized the HDM model for the Th1/Th2/Th17 phenotype by measuring the levels of inflammatory cytokines and chemokines in BALF. In this model, HDM exposure increased the level of Th2 cytokine IL-13 in BALF,

which was attenuated by nadolol treatment, but not by carvedilol treatment in mice (Fig. 22A). However, HDM exposure with or without any drug treatment, did not affect the levels of IFN-γ and IL-17 in BALF (Fig. 22B, C).

HDM exposure significantly increased the level of the eosinophilic chemoattractant EOTAXIN in BALF, which was further increased by concomitant carvedilol treatment (Fig. 23A). Nadolol treatment did not increase EOTAXIN level in response to HDM exposure (Fig. 23A). HDM exposure with vehicle or nadolol treatment did not significantly increase the neutrophilic chemoattractant KC in BALF, but carvedilol treatment showed a significant increase in KC over the groups (Fig. 23B). The levels of the chemokines EOTAXIN and KC correlated well with the levels of eosinophils (Fig. 23C) and neutrophils (Fig. 23D), respectively according to the correlation plots depicted in Fig. 23E, F.

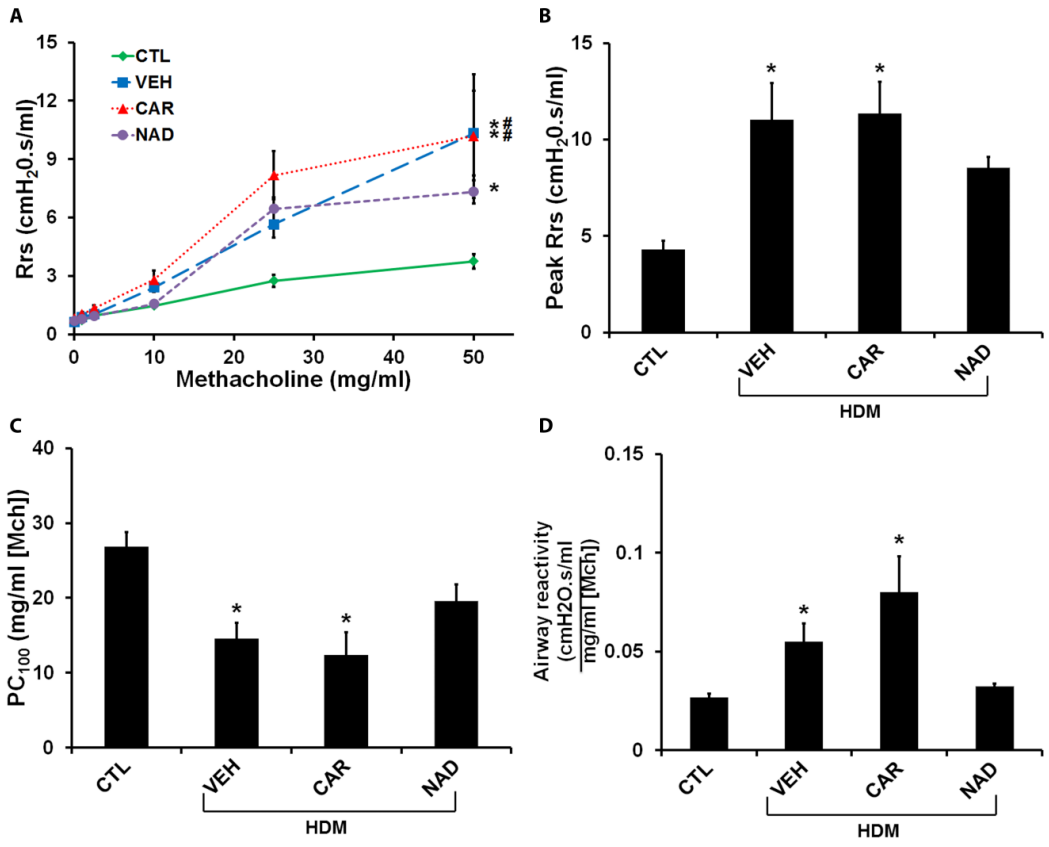

Figure. 20 'Prophylactic' Effects of β-blockers on Airway Hyper-responsiveness (AHR) in BALF AHR was measured in response to increasing doses of methacholine (0-50 mg/ml) using the forced oscillation technique (FOT) with a Flexivent®. The figure represents (A) total respiratory resistance (Rrs)- methacholine dose-response curve, (B) peak airway resistance, (C) PC_{100} as a measure of airway sensitivity, and (D) airway reactivity in mice exposed to HDM and concomitantly treated with vehicle (VEH), 2400 ppm carvedilol (CAR) or 250 ppm nadolol (NAD) in the prophylactic model. The figures compare Rrs, peak Rrs, PC_{100} and airway reactivity in HDM exposed mice treated with VEH, CAR or NAD to the saline exposed CTL mice. Data are plotted as mean ± SEM from 5-6 mice per group. * represents statistical significance at $p < 0.05$ in reference to the CTL group. # represents statistical significance at $p < 0.05$ in reference to the NAD group. Figure adapted from (Joshi, Valdez et al. 2017).

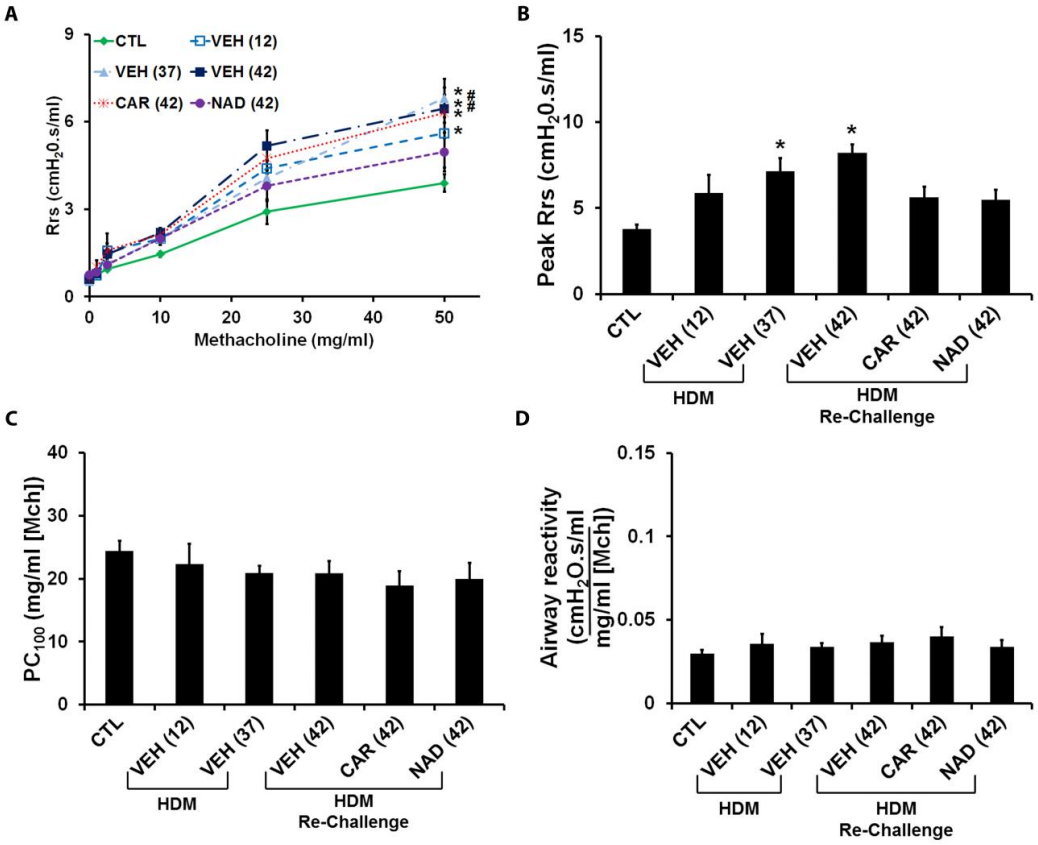

Figure. 21 'Therapeutic' Effect of β-blockers on Airway Hyper-responsiveness (AHR) in BALF AHR was measured in response to increasing doses of methacholine (0-50 mg/ml) using the forced oscillation technique (FOT) with a Flexivent®. The figure represents (A) total respiratory resistance (Rrs)- methacholine dose-response curve, (B) peak airway resistance, (C) PC_{100} as a measure of airway sensitivity, and (D) airway reactivity in mice exposed to HDM in the therapeutic model. The figures compare Rrs, peak Rrs, PC_{100} and airway reactivity in mice evaluated on days 12, and 37 after initial HDM exposure; and mice treated with VEH, CAR or NAD and evaluated on day 42 after HDM re-exposure with saline control mice. Data are plotted as mean ± SEM from 7-13 mice per group. * represents statistical significance at $p< 0.05$ in reference to the CTL group. # represents statistical significance at $p < 0.05$ in reference to the NAD group. Figure adapted from (Joshi, Valdez et al. 2017).

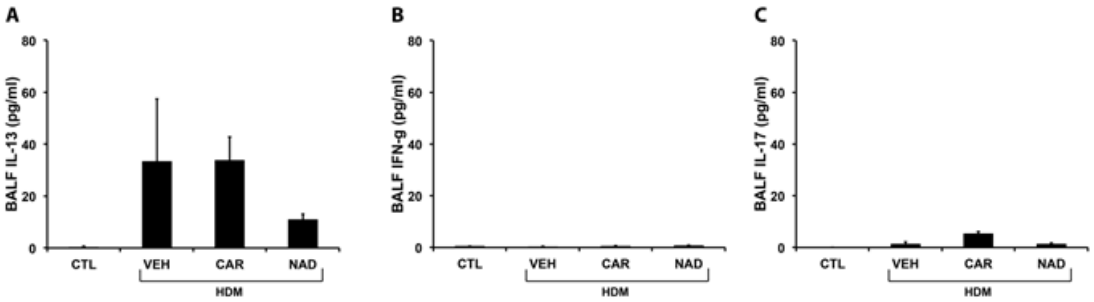

Figure. 22 'Prophylactic' effects of β-blockers on Inflammatory Cytokines in BALF The figure represents (A) concentration of IL-13 (B) concentration of IFN-γ, and (C) concentration of IL-17 in the BALF of mice exposed to HDM and concomitantly treated with vehicle (VEH), 2400 ppm carvedilol (CAR) or 250 ppm nadolol (NAD) compared to the respective CTL mice in the prophylactic model. Data are plotted as mean ± SEM from 5-6 mice per group. Figure adapted from (Joshi, Valdez et al. 2017).

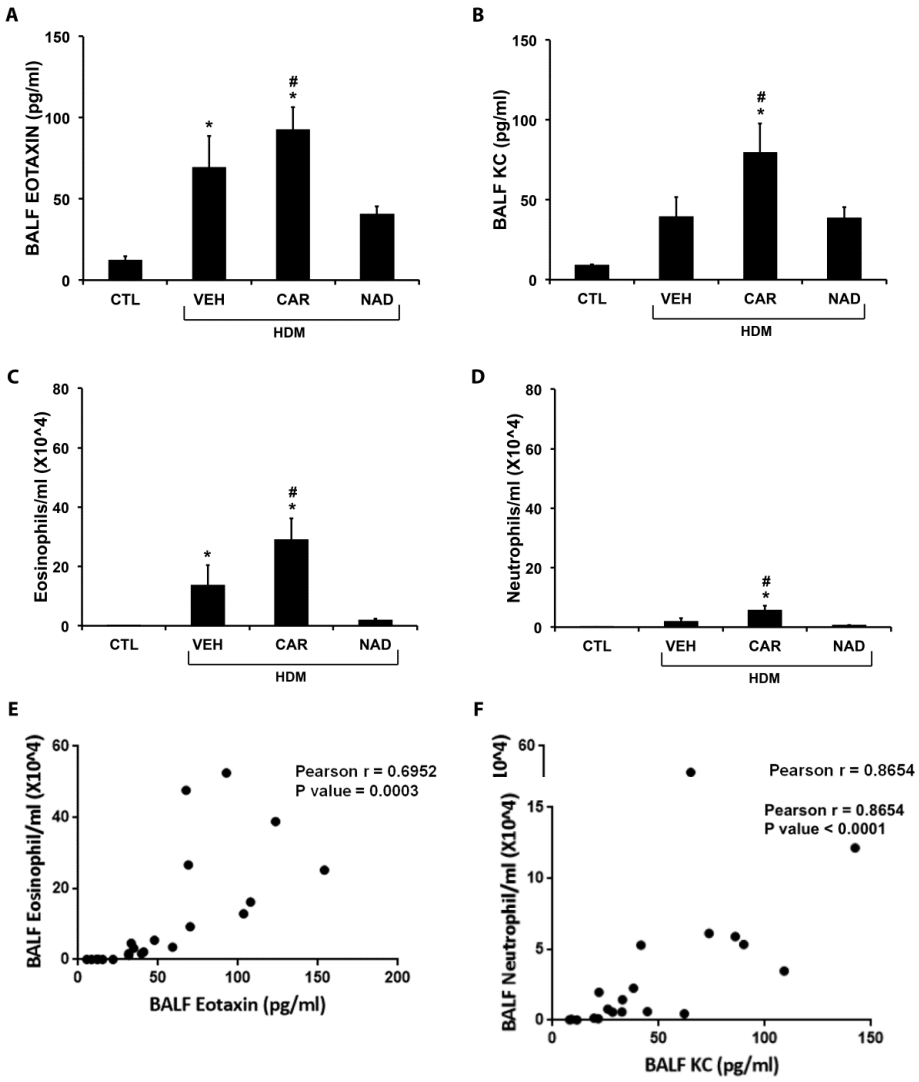

Figure. 23 'Prophylactic' effects of β-blockers on inflammatory chemokines in BALF and their correlation with inflammatory cells The figure represents (A) concentration of EOTAXIN (B) concentration of CXCL1/KC, (C) cellular counts of eosinophils, and (D) cellular counts of neutrophils in the BALF of mice exposed to HDM and concomitantly treated with vehicle (VEH), 2400 ppm carvedilol (CAR) or 250 ppm nadolol (NAD) compared to the respective CTL mice in the prophylactic model. Figures (E) and (F) represent correlation plots between eosinophil and Eotaxin levels in BALF, and neutrophil and KC levels, respectively. Data are plotted as mean ± SEM from 5-6 mice per group. * represents statistical significance at $p < 0.05$ in reference to the CTL group. # represents statistical significance at $p < 0.05$ in reference to the NAD group. Figure adapted from (Joshi, Valdez et al. 2017).

4.5 Discussion

We have previously shown that only certain β-blockers like nadolol and ICI-118,551, but not others like carvedilol, propranolol and alprenolol, prevent the development of asthma phenotype in ovalbumin-driven murine models of asthma (Nguyen, Omoluabi et al. 2008, Thanawala, Valdez et al. 2015). All of these previous studies had used ovalbumin asthma models to study the development of asthma phenotype. However, the ovalbumin model uses chicken egg albumin as a source of allergens, which has been criticized for lack of clinical relevance (Cates, Fattouh et al. 2004). Furthermore, the model itself suffers from limitations of requiring systemic delivery of the allergen, which again is not a clinically relevant route of exposure to asthma-causing allergens in humans. On the other hand, chronic exposure to ovalbumin through the respiratory route leads to immune tolerance in mice making the model unsuitable for use in a chronic setting (Swirski, Sajic et al. 2002, Van Hove, Maes et al. 2007, Kumar, Herbert et al. 2008). The ovalbumin model also requires co-administration of an adjuvant such as aluminium hydroxide (alum) to boost the immune response (Mullane and Williams 2014, Sagar, Akbarshahi et al. 2015); however, the need for adjuvants has been debated for many years (Conrad, Yildirim et al. 2009).

In light of these limitations, we switched our asthma models to using house dust mite (HDM) as a source of allergens to produce an asthma

phenotype in mice. These models utilize clinically relevant allergens from the HDM species *Dermatophagoides pteronyssinus* (Derp), which are implicated in causing respiratory symptoms in 50-85 % of asthmatic patients worldwide. Besides being clinically relevant, the model offers several advantages, such as intranasal delivery of the allergens which mimics human exposure to allergens during asthma, and at least 6 weeks of continued respiratory exposure to HDM that does not cause immune tolerance in mice (Cates, Fattouh et al. 2004, Johnson, Wiley et al. 2004). The model also does not require the use of adjuvants to boost the immune response.

In the present studies, we tested the β-blockers nadolol and carvedilol in HDM-driven murine models of asthma. As observed, these models developed a robust asthma phenotype in mice that was qualitatively similar to the phenotype produced in the ovalbumin models, characterized by an increase in eosinophilic inflammation, mucus hypersecretion and airway hyper-responsiveness. The characteristic features of allergic asthma are mediated by the release of Th2 specific cytokines IL-4, IL-5 and IL-13 from Th2 cells and the recruitment of the eosinophil chemoattractant protein eotaxin (Barnes, Chung et al. 1998). In order to confirm that the ovalbumin and HDM models followed a similar immune pathway, we characterized the HDM model for the presence of Th1/Th2/Th17 inflammatory mediators. We found that the HDM model, similar to our previous

ovalbumin model, exhibited a Th2 biased immune response characterized by an increase in IL-13 and eotaxin levels in BALF (discussed in detail below).

Our previous studies had only investigated the prophylactic effects of β-blockers on the asthma phenotype, where drug treatment was initiated before mice developed the phenotype. To increase the clinical relevance of our studies, the current set of experiments also investigated the therapeutic effects of β-blockers in mice that had already developed an asthma phenotype to HDM allergic insult. We selected the drugs carvedilol and nadolol as prototype β-blockers that had shown differential effects on the asthma phenotype in the ovalbumin models (Thanawala, Valdez et al. 2015). Similar to our previous results, the current experiments showed that nadolol, but not carvedilol, decreased the asthma phenotype in both the prophylactic and therapeutic models.

Allergic asthma is an eosinophilic disease, and our model produced a significant increase in the number of eosinophils in BALF after exposure to HDM. Concomitant treatment with nadolol in the prophylactic model reduced the eosinophilic inflammation, whereas treatment with carvedilol did not affect the increase in eosinophil numbers in response to HDM (Fig. 16). In the therapeutic model as well, mice developed a robust increase in the number of eosinophils in BALF after an initial exposure to HDM, which returned to baseline values after four weeks. Re-exposure to HDM again increased

eosinophil numbers in these mice. Nadolol treatment significantly attenuated, but carvedilol treatment was ineffective at lowering the eosinophil numbers (Fig. 17). Therefore, the results suggest that nadolol and carvedilol varied in their effects on the HDM-induced inflammatory cellular infiltration in BALF. Other studies in the literature have shown an increase in both eosinophils and neutrophils after intranasal HDM exposure in mice, but the response depended on the protocol for allergen exposure and the time-points of measuring the phenotype (Piyadasa, Altieri et al. 2016). In order to observe a peak increase in inflammation and AHR, previous studies by others suggested evaluating mice 48 hours post-final HDM challenge in the prophylactic model (Piyadasa, Altieri et al. 2016), and 72 hours to 5 days after the final HDM challenge in the therapeutic model (Cates, Fattouh et al. 2004). As neutrophils peak at 6-12 hours following the final HDM exposure, we did not see an increase in neutrophil numbers in our HDM models of asthma. However, even at the 4 hour time-point of BALF collection for cytokine/chemokine analysis, neutrophil expression was negligible other than in the carvedilol treated mice (Fig. 23D). These results are consistent with the HDM model being an eosinophilic model.

Mucous metaplasia is another feature of asthma and is characterized by an increased intracellular accumulation of mucin glycoproteins in the airway epithelium. HDM exposure in the current models significantly increased the mucin volume density in the airway epithelium. Prophylactic treatment with

nadolol prevented the increase in airway mucin content, whereas carvedilol treatment was ineffective at reducing the mucin content (Fig. 18). In the therapeutic model as well, nadolol, but not carvedilol treatment attenuated the increased mucous metaplasia in response to HDM exposure (Fig. 19). These results suggest that nadolol is effective at reducing mucus hypersecretion in asthma irrespective of whether treatment is begun before or after the phenotype develops in mice. Despite nadolol's ability to reduce the asthma phenotypes of airway inflammation and mucous metaplasia compared to vehicle or carvedilol treatment, it should be noted that these phenotypes were still higher in the HDM challenged nadolol group over saline controls, suggesting that nadolol treatment did not retrurn the elevated phenotypes to baseline values (Fig. 16-19). This is in contrast to our previous results in the ovalbumin models where nadolol had completely attenuated the asthma phenotypes. This slight variation may indicate some differences between the ovalbumin and HDM models and may suggest a harsher phenotype developed in the HDM model, as also observed for airway hyper-responsiveness (AHR) discussed below.

The intranasal HDM challenges produced an increased airway resistance to dose increments of methacholine when measured by the forced oscillation technique (Flexivent®). Nadolol treatment attenuated the increased airway resistance in the 'prophylactic' model, while carvedilol was ineffective.

As additional parameters of AHR, we also measured airway sensitivity and reactivity to methacholine. An increase in airway sensitivity is indicated by lowering of the dose of methacholine that produced a doubling of the baseline airway resistance (PC_{100}). Airway reactivity is measured from the slope (K) of the methacholine-Rrs dose response curve. Our results show that prophylactic treatment with nadolol, but not with carvedilol decreased airway sensitivity and reactivity in response to HDM (Fig. 20). On the other hand, nadolol only caused a moderate decrease in airway resistance in the 'therapeutic' model, and had no effect on airway sensitivity and reactivity in this model (Fig. 21). However, an interesting observation is that the increase in airway resistance was persistent even four weeks after the final HDM challenge in the therapeutic model, while inflammation and mucous metaplasia had completely resolved at this time-point (Fig. 21). This is consistent with a previous report showing only a partial resolution of AHR and a complete reduction of inflammation in the HDM model after 9 weeks (Johnson 2001). The limited effect of nadolol in reducing AHR in the therapeutic model suggests a very strong AHR phenotype in this model. This is also evidenced by another study showing that the standard combination therapy with glucocorticoids and β_2AR agonist had completely resolved inflammation but not AHR when given concomitantly with HDM exposure in mice (Johnson, Pacitto et al. 2008). Nonetheless, nadolol treatment sufficiently

reduced the asthma phenotypes when used prophylactically or therapeutically in the HDM models of asthma to support its therapeutic potential in asthma.

With respect to the immune response, there have been conflicting reports in the literature suggesting the involvement of Th1 and Th2 mediators driving the HDM-induced immune response (Gregory, Causton et al. 2009, Piyadasa, Altieri et al. 2016). Our results suggest that the HDM models in the present studies followed a Th2 phenotype. Based on a previous report that had performed a time-course of the expression of inflammatory mediators in the HDM model (Gregory, Causton et al. 2009), we evaluated the mice in the current study at 4 hours after the final HDM challenge. At this time point, our HDM model showed an increase in the concentration of the Th2 cytokine IL-13 and chemokine eotaxin in BALF, while the Th1 and Th17 cytokines IFN-γ and IL-17 and the neutrophilic chemoattractant CXCL1/KC were almost undetected. Consistent with our previous results in the ovalbumin model, prophylactic treatment with nadolol, but not carvedilol, prevented the increase in the levels of IL-13 and eotaxin in BALF (Fig. 22, 23). These results suggest that nadolol's beneficial effect in reducing the asthma phenotype involves attenuation of the Th2 immune response.

The present experiments tested only two β-blockers nadolol and carvedilol in the HDM models of asthma. As the ovalbumin and HDM models

differ in some features, particularly in the intensity of the phenotype developed in these models, future experiments should be designed to test additional β-blockers in the HDM models to determine their differential effects produced on the asthma phenotypes. Also, additional experiments are required to study the AHR phenotype in the HDM models, as the current results saw no significant increase in airway sensitivity and reactivity in the therapeutic model compared to the prophylactic model. These anomalous results from the AHR experiments made it difficult to draw definitive conclusions about the therapeutic effects of β-blockers on the AHR phenotype. As a result, a complete switch from ovalbumin to HDM models must be carefully considered as both models present advantages and limitations and therefore should be used in combination.

4.6 Conclusions

Results from the present studies suggest that nadolol reduces the asthma phenotype in the HDM-driven murine models of asthma both as prophylactic and therapeutic treatment, while carvedilol is ineffective. These results are consistent with our previous results in the ovalbumin model and provide further strength to the therapeutic potential of nadolol in the treatment of asthma.

5. Chapter 2

The role of extracellular-signal regulated kinases (ERK1/2) and β-arrestin-2 signaling in mediating the effects of β-blockers in murine asthma models

*Parts of the data discussed in this chapter have been published in the manuscript titled “Effects of β-blockers on house dust mite-driven murine models pre- and post-development of an asthma phenotype” in *Pulmonary Pharmacology and Therapeutics,* 2017

5.1 Rationale

Our previous studies have suggested the differential effects of β-blockers on the asthma phenotype correlates with their biased signaling profiles at the Gs-cAMP and ERK1/2 pathways downstream of the β_2-adrenoceptor (β_2AR) (Thanawala, Valdez et al. 2015). Our results indicated that β-blockers like nadolol that inhibited ERK1/2 phosphorylation reduced the asthma phenotype, while β-blockers like carvedilol and propranolol that activated ERK1/2 did not affect the asthma phenotype produced with the ovalbumin driven murine models (Thanawala, Valdez et al. 2015). However, a study using HEK-293 cells overexpressing the β_2AR showed that carvedilol stimulates ERK1/2 phosphorylation via β-arrestin-2 mediated pathway while, propranolol does so independent of β-arrestin-2. Studies have implicated the role of β-

arrestin-2 in the pathogenesis of asthma as the β-arrestin-2 knock-out (β-arr2 KO) mice have an attenuated asthma phenotype (Walker, Fong et al. 2003, Chen, Hegde et al. 2015). The rationale for the present studies was to identify the signaling mechanism leading to the development of asthma downstream of the β_2AR activation.

5.2 Objective and Hypothesis

The objectives of our present studies were

1. To investigate the effect of β-blockers on ERK1/2 activation in murine asthma models as a potential mechanism of their differential effects on the asthma phenotype
2. To test whether in β-arrestin-2 null mice, β-blockers carvedilol and propranolol restore the asthma phenotype in a murine model of asthma

Based on our previous studies, we hypothesized that

1. Nadolol would decrease ERK1/2 phosphorylation in murine lungs in the HDM models of asthma correlating with attenuating the asthma phenotype, while carvedilol being ineffective at reducing the asthma phenotype would not lower ERK1/2 phosphorylation in the murine lungs.
2. Propranolol, but not carvedilol, would restore the asthma phenotype in β-arrestin-2 null mice.

5.3 Approach

For ***objective 1***, we measured ERK1/2 phosphorylation by immunoblotting using whole lung homogenates of mice from HDM-driven asthma models. We used both prophylactic and therapeutic models of HDM-driven asthma to study the effects of β-blockers nadolol and carvedilol on ERK1/2 phosphorylation. To further test the role of ERK1/2 signaling in the development of asthma phenotype, we measured ERK1/2 phosphorylation in an ovalbumin model of asthma as it allows studying the development of the phenotype in two stages: the sensitization phase where the phenotype is absent, and the challenge phase that develops the phenotype. We then compared ERK1/2 phosphorylation in naïve mice with mice that were sensitized but not challenged with ovalbumin (Ova S/N), and mice that were sensitized and challenged with ovalbumin (Ova S/C). Our studies were however limited as they lacked the cell-specific measurement of ERK1/2 phosphorylation in the lung. As ERK1/2 activation in different lung cells may be associated with different cellular functions and each cell type may contribute differently towards the total ERK1/2 phosphorylation in the lung homogenates. Future studies should be aimed to measure cell-specific modulation of ERK1/2 phosphorylation by β-blockers.

For ***objective 2***, we studied the effects of β-blockers carvedilol and propranolol on the development of asthma phenotype in β-arr2 KO mice using

the ovalbumin-driven murine asthma model. Mice were sensitized to 0.4 mg/kg/day of ovalbumin by intraperitoneal delivery on days 0, 7 and 14, and subsequently treated with β-blockers for 28 days. Following treatment, mice were challenged with 1% w/v ovalbumin solution by inhalation on the last three days of the protocol (days 43-45) and evaluated 24 hours later for mucous metaplasia in the airways. The control mice were sensitized to ovalbumin but challenged with saline.

5.4 Results

5.4.1 'Prophylactic' effect of β-blockers carvedilol and nadolol on ERK1/2 phosphorylation in mouse lungs in the HDM-driven asthma model

In the 'prophylactic' model, phospho-ERK1/2 expression increased in the lungs of mice after four weeks of HDM exposure (Fig. 24). Concomitant drug therapy with either nadolol or carvedilol did not affect the increased phospho-ERK1/2 expression in these mice (Fig. 24). These results suggested that despite the reduction in the asthma phenotype in nadolol treated mice, phospho-ERK1/2 expression remained elevated.

5.4.2 'Therapeutic' effect of nadolol on ERK1/2 phosphorylation in the HDM-driven asthma model

In the therapeutic model, the initial HDM exposure for 10 days increased the phospho-ERK1/2 expression in the lung (Fig. 25A, B). However, phospho-ERK1/2 expression remained elevated even at 4 weeks following the final HDM exposure in mice, while the phenotypes of airway inflammation and mucous metaplasia had completely resolved at this time-point (Fig. 25C, D). Subsequent re-exposure to HDM further increased phospho-ERK1/2 expression in the vehicle treated mice (Fig. 25C, D). Nadolol treatment initiated after the initial HDM exposure when mice had developed the asthma phenotype, attenuated the phenotype upon re-exposure to HDM (discussed in the previous chapter), but failed to reduce phospho-ERK1/2 expression in the lungs (Fig. 25C, D). These results also suggested that ERK1/2 phosphorylation remained upregulated in mouse lungs after exposure to HDM, even when the asthma phenotype had been resolved.

5.4.3 ERK1/2 phosphorylation in the ovalbumin-driven murine asthma model

In the ovalbumin model of asthma, ovalbumin sensitization in the absence of challenge did not produce an asthma phenotype in mice, while subsequent intranasal challenge with ovalbumin produced a significant

increase in inflammatory cellular infiltration and mucous metaplasia (Fig. 26). In this model, ovalbumin sensitization increased the phospho-ERK1/2 expression even in the absence of respiratory challenge with the allergen, when the asthma phenotype was not present (Fig. 27). Subsequent ovalbumin challenge further increased ERK1/2 phosphorylation in these mice (Fig. 27). These results confirm our findings in the HDM model by showing that ERK1/2 phosphorylation increases in response to allergen exposure in mice even when the asthma phenotype is absent.

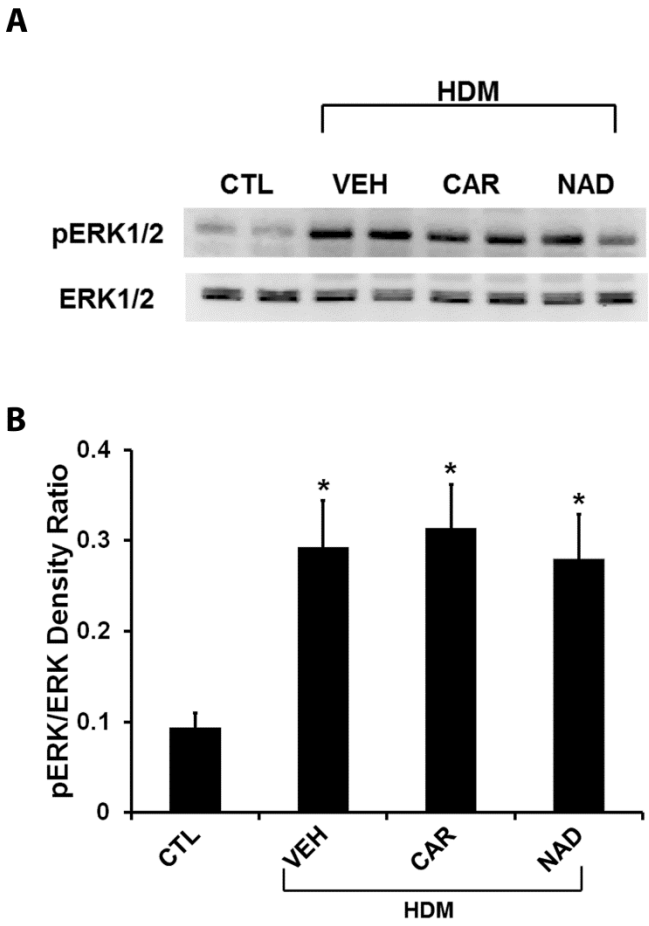

Figure. 24 'Prophylactic' Effect of β-blockers on Extracellular-signal Regulated Kinase (ERK1/2) Phosphorylation in the HDM Model of Asthma The figure represents (A) western blot, and (B) quantification of the relative phospho-ERK1/2 protein expression in lung homogenates of mice subjected to the prophylactic HDM model of asthma. The figures compare phosphoERK/ERK density ratio in whole lungs of mice exposed to HDM and concomitantly treated with vehicle (VEH), 2400 ppm carvedilol (CAR) or 250 ppm nadolol (NAD) compared to the respective CTL mice. Data are plotted as mean ± SEM from 6 mice per group. * represents statistical significance at $p < 0.05$ in reference to the CTL group. Figure adapted from (Joshi, Valdez et al. 2017).

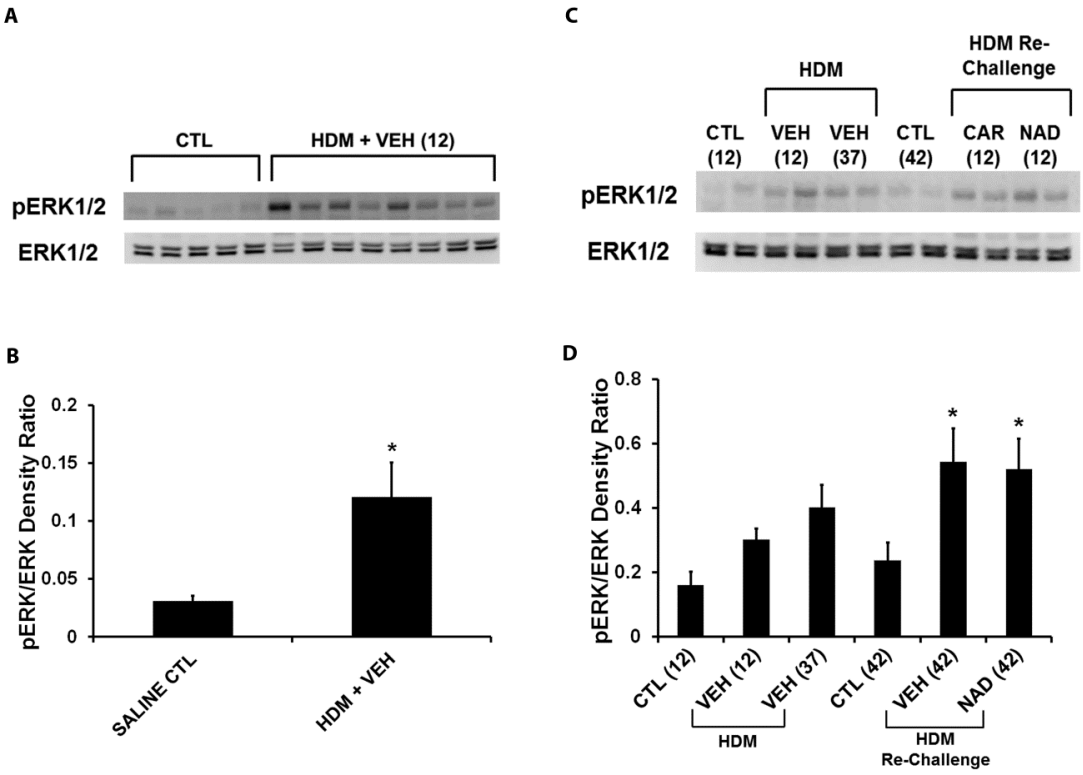

Figure. 25 'Therapeutic' Effect of Nadolol on Extracellular-signal Regulated Kinase (ERK1/2) Phosphorylation in the HDM Model of Asthma The figure panels A and B represent (A) western blot, and (B) quantification of the relative phospho-ERK1/2 protein expression in lung homogenates of mice exposed to HDM for 10 days compared to the saline control mice. The figure panels C and D represent (C) western blot, and (D) quantification of the relative expression of phospho-ERK to total ERK in mice evaluated on days 12, and 37 after the initial HDM exposure; and mice treated with VEH or NAD and evaluated on day 42 after HDM re-exposure compared to the respective saline control mice. Data are plotted as mean ± SEM from 5-8 mice per group. * represents statistical significance at $p < 0.05$ in reference to the CTL group. Figure adapted from (Joshi, Valdez et al. 2017).

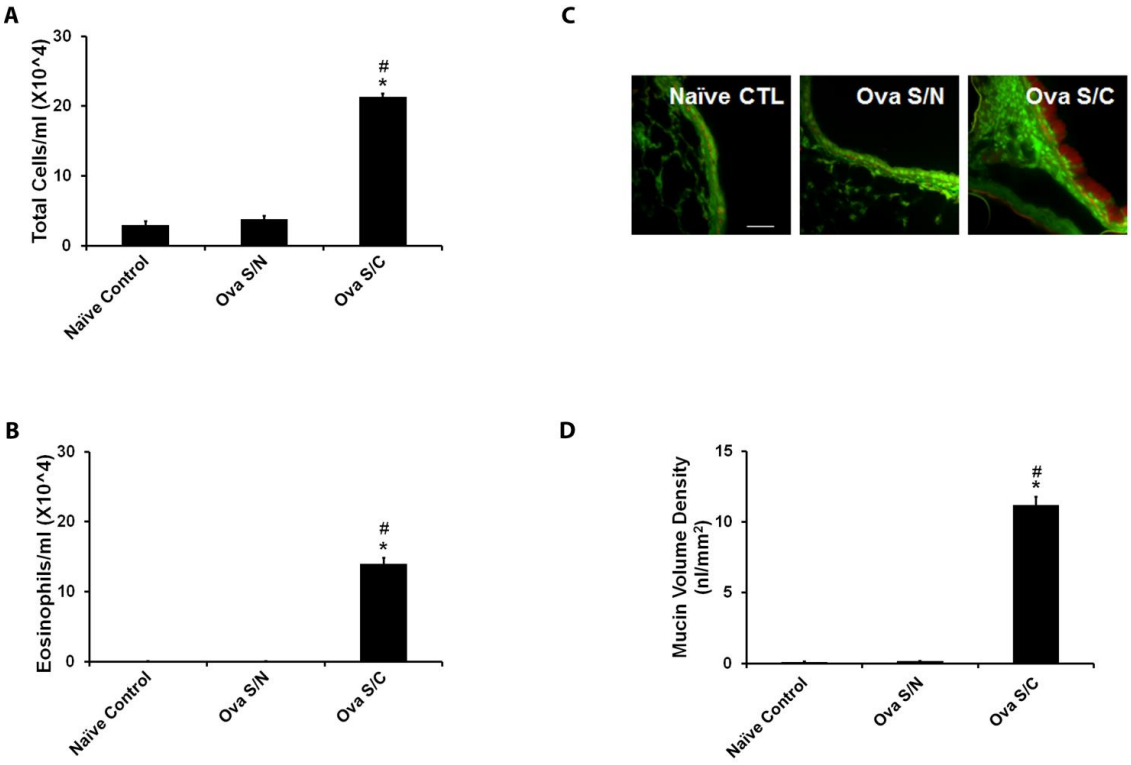

Figure. 26 Inflammatory Cellular Infiltration in BALF and Airway Mucous Metaplasia in the Ovalbumin Model of Asthma The figure represents BALF inflammatory cells (A, B), and mucous metaplasia (C, D) in mice subjected to ovalbumin sensitization and challenge. A. Total cells and B. eosinophil numbers in BALF of mice sensitized to ovalbumin with (Ova S/C) or without (Ova S/N) subsequent challenge compared to naïve control mice. C. PAFS stained images showing mucin content (red) in airway epithelial cells (green), and D. morphometric quantification of mucin volume density in mice sensitized to ovalbumin with or without subsequent challenge compared to naïve control mice. Data are plotted as mean ± SEM from 4-5 mice per group. * represents statistical significance at $p< 0.05$ in reference to the naïve control group. # represents statistical significance at $p < 0.05$ in reference to the Ova S/N group. Figure adapted from Suppl. Material of (Joshi, Valdez et al. 2017).

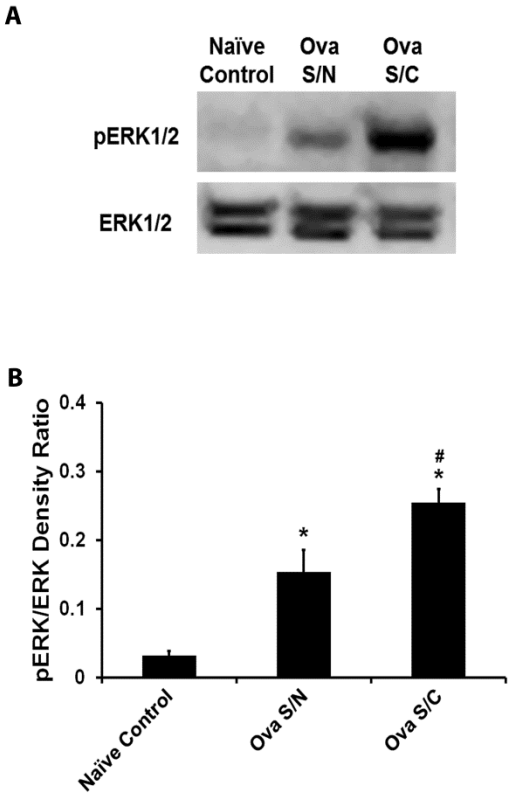

Figure. 27 Extracellular-signal Regulated Kinase (ERK1/2) Phosphorylation in the Ovalbumin Model of Asthma The figure represents (A) western blot, and (B) quantification of the phospho-ERK1/2 protein expression in lung homogenates of mice sensitized and challenged with ovalbumin (Ova S/C) compared to mice sensitized to ovalbumin without challenge (Ova S/N), and naïve control mice. Data are plotted as mean ± SEM from 4-5 mice per group * represents statistical significance at $p< 0.05$ in reference to the naïve control group. # represents statistical significance at $p < 0.05$ in reference to the Ova S/N group. Figure adapted from (Joshi, Valdez et al. 2017).

5.4.4 β-arrestin-2 gene and protein expression in the β-arrestin-2 KO and WT mice

We performed PCR-amplification of DNA from the β-arr2 KO and WT mice for the Arrb2 (β-arr2) gene and visualized the gene on a 1-2% agarose gel under UV fluorescence to confirm their genotype. In addition to genotyping, we measured the expression of β-arr2 protein in these mice by western blotting.

The β-arr2 KO and WT mice showed bands at 300 and 600 bp, respectively on the UV-illuminated agarose gel (Fig. 28A). The β-arr2 KO and WT mice were used in experiments only after their genotype had been identified. In the western blot experiments, the β-arr2 protein expression in the whole lung homogenate was significantly reduced in the β-arr2 KO mice compared to the WT mice (Fig. 28B, C). However, the protein was not completely undetected in the β-arr2 KO mice (Fig. 28B, C).

5.4.5 Effect of β-blockers carvedilol and propranolol on airway mucous metaplasia in β-arrestin-2 KO mice in the ovalbumin model of asthma

We measured the mucin volume density in the periodic acid fluorescent Schiff (PAFS) stained airway sections from the β-arr2 KO mice and their WT littermates. Ovalbumin sensitization and challenge significantly increased the mucin volume density in both the β-arr2 KO and WT mice over

the ovalbumin sensitized control mice (Fig. 29). The data from these experiments conflicted with the previously published reports of an attenuated eosinophilic inflammation in the β-arr2 KO mice. Also, the β-blockers carvedilol and propranolol did not affect the increase in the mucin volume density in either β-arr2 KO or WT mice.

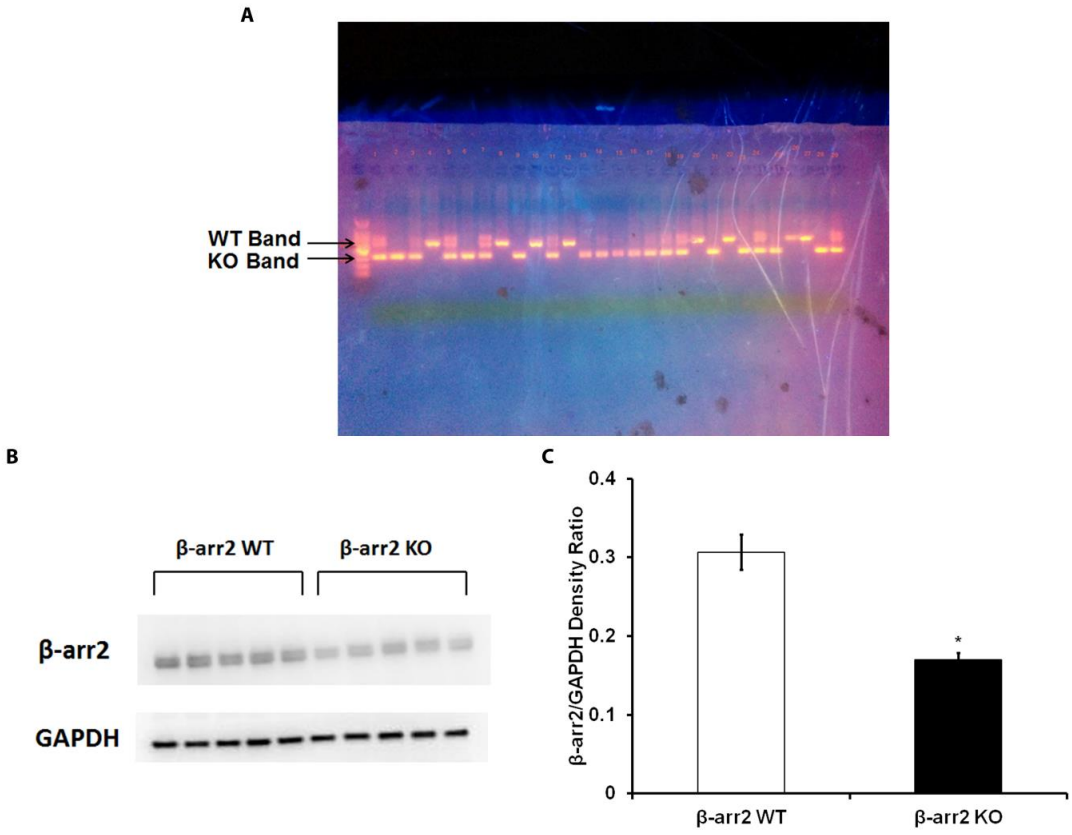

Figure. 28 β-arrestin-2 Gene and Protein Expression in the β-arrestin-2 KO and WT Mice
The figure represents (A) PCR gel for β-arr2 gene visualization, (B) western blot for β-arr2 protein, and (C) quantification of the β-arr2/GAPDH density ratio in lung homogenates of β-arr2 KO and WT mice. (A). Representative image of the DNA gel shows the presence of the WT band at ~600 bp and the KO band at ~300 bp. Data are plotted as mean ± SEM from 5 mice per group and analyzed by student t-test. * represents statistical significance at $p < 0.05$ in reference to the β-arr2 WT group.

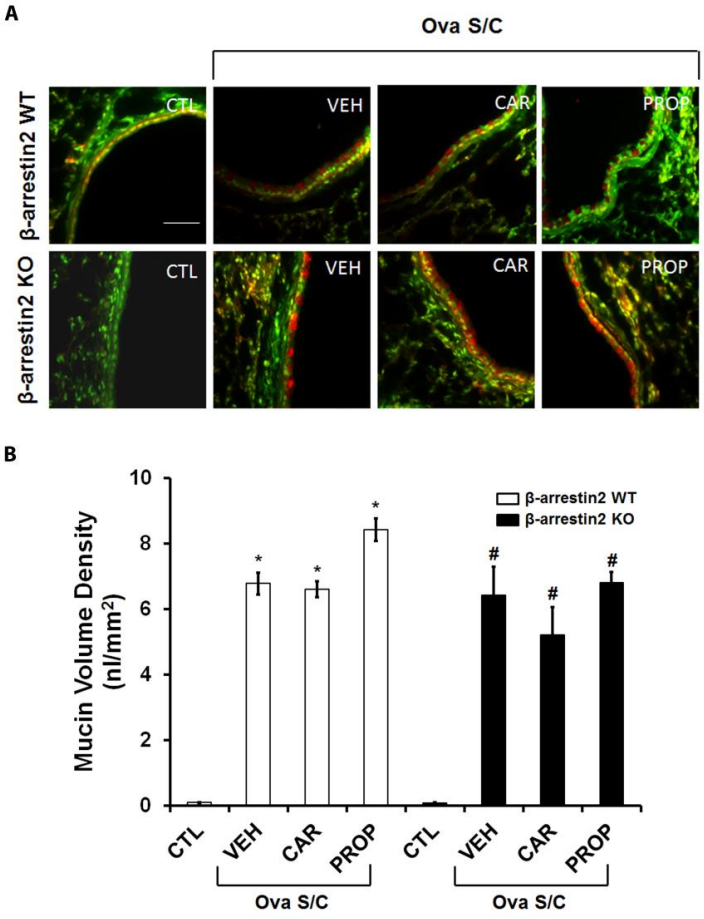

Figure. 29 Effect of β-blockers on Airway Mucous Metaplasia in β-arrestin-2 Null Mice in the Ovalbumin Model The figure represents (A) PAFS stained images showing mucin content (red) in airway epithelial cells (green), and (B) morphometric quantification of mucin volume density in β-arr2 WT and KO mice sensitized/challenged with ovalbumin and treated with vehicle, carvedilol (CAR) or propranolol (PROP) compared to their respective control mice. Data are plotted as mean ± SEM from 5-8 mice per group and analyzed by one-way ANOVA with Tukey's post-hoc test. * represents statistical significance at $p < 0.05$ in reference to the β-arr2 WT control group. # represents statistical significance at $p < 0.05$ in reference to the β-arr2 KO control group.

5.5 Discussion

The experiments discussed in this chapter aimed to investigate the role of extracellular-signal regulated kinases 1/2 (ERK1/2) inhibition in mediating the attenuation of the asthma phenotype by nadolol in the HDM-driven asthma models. We found that nadolol did not reduce the ERK1/2 phosphorylation induced by HDM exposure in mice, despite preventing the development of the asthma phenotypes of inflammation and mucous metaplasia in the prophylactic model and attenuating these established phenotypes in the therapeutic model. However, nadolol's effect on attenuating AHR was only moderate indicating that ERK1/2 prosphorylation may play a role in developing and maintaining AHR in mice. With the caveats outlined above about the limits of interpretation of data obtained form whole lung homogenates, our results suggest nadolol's attenuation of the asthma phenotypes may not be associated with the long-term inhibition of ERK1/2 in the lungs. Upon further investigation using the ovalbumin-driven asthma model, we found that allergen sensitization alone upregulated ERK1/2 phosphorylation even prior to the development of airway inflammation and mucous metaplasia, suggesting ERK1/2 activation may not be required for perpetuation of these phenotypes.

As previously discussed, activation of the β_2AR is required for developing the asthma phenotype in mice since mice that lack the β_2AR, or the endogenous β_2AR agonist epinephrine, show an attenuated asthma phenotype

in the ovalbumin model of asthma (Nguyen, Lin et al. 2009, Thanawala, Forkuo et al. 2013). However, blocking the β_2AR with antagonists did not equally reduce the asthma phenotype as only a subset of β-blockers was found to be beneficial in murine asthma models and some pilot clinical studies (Hanania, Singh et al. 2008, Nguyen, Omoluabi et al. 2008, Hanania, Mannava et al. 2010, Thanawala, Valdez et al. 2015). To understand the differential effects of β-blockers in asthma, we looked at their effects on the signaling pathways downstream of the β_2AR. The two commonly studied pathways associated with the β_2AR are the G_s-cyclic AMP pathway, and the β-arrestin-2 signaling pathway leading to ERK1/2 activation (Galandrin and Bouvier 2006, Wisler, DeWire et al. 2007). Majority of the known β-blockers inhibit the canonical G_s-cAMP pathway, but some are biased towards activating the β-arrestin-2-ERK1/2 pathway. This serves as an example of biased signaling at GPCRs, where ligands preferentially activate one of the several signaling pathways at a receptor compared to a reference standard ligand (Kenakin 2009). With respect to the β_2AR, the endogenous agonist epinephrine is by convention considered to have natural bias at both cAMP and ERK1/2 activation pathways. Compared to epinephrine, the β-blockers nadolol, ICI-118, 551, and metoprolol are inverse agonists (inhibiting both constitutive and agonist stimulated activity) at the cAMP pathway and also inhibit ERK1/2 activation, whereas the β-blockers carvedilol and propranolol are inverse agonists at the cAMP pathway but are

partial agonists relative to epinephrine at ERK1/2 activation (Data from Wisler et al. 2007 re-plotted in Fig. 30). Alprenolol is almost a neutral antagonist at the cAMP pathway but activates ERK1/2 relative to epinephrine. Our studies in the ovalbumin and HDM-driven asthma models have shown differential effects of the chronic treatment with these β-blockers on the asthma phenotype, with nadolol, ICI-118, 551 and metoprolol attenuating the phenotype and carvedilol, propranolol and alprenolol not affecting the phenotype in murine asthma models (Lin, Peng et al. 2008, Nguyen, Omoluabi et al. 2008, Thanawala, Valdez et al. 2015, Joshi, Valdez et al. 2017). As such, these studies suggest a correlation between modulation of ERK1/2 activity by β-blockers and their effects on the asthma phenotype in murine models.

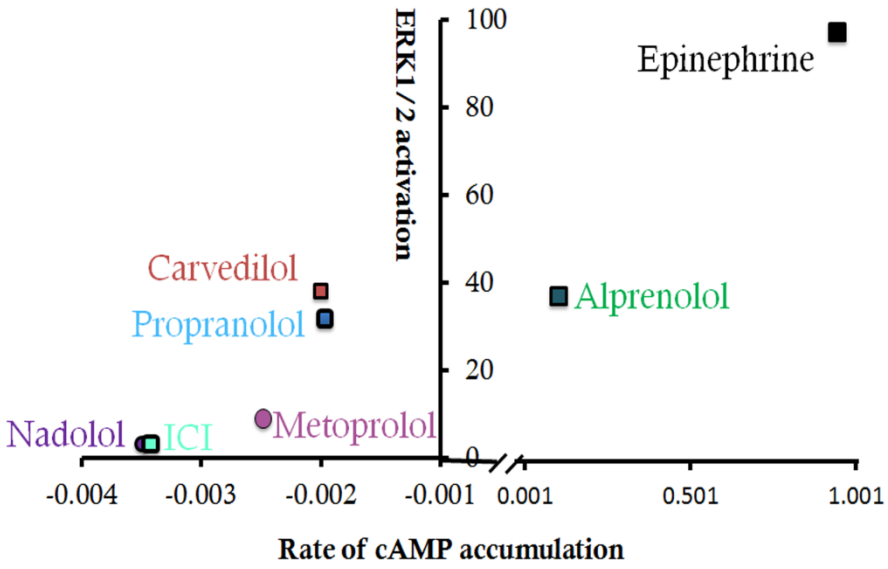

Figure. 30 Biased Signaling by β-blockers at the β2AR-Associated cAMP and ERK1/2 Pathways The figure represents a XY scatter plot of the relative activation of β-blockers at cAMP and ERK1/2 activation. Data for the plot was obtained from Wisler et al. 2007. Relative to the reference ligand epinephrine, nadolol, ICI 118,551, and metoprolol inhibit both cAMP and ERK1/2 pathways, while carvedilol and propranolol inhibit the cAMP pathway but activate ERK1/2. Alprenolol is a very weak partial agonist at the cAMP pathway activates ERK1/2.

Several studies in the literature have implicated ERK1/2 activation in the development of asthma. ERK1/2 phosphorylation is increased in the airway epithelial and smooth muscle cells from the biopsy tissues from asthmatic patients (Liu, Liang et al. 2008) and in a mouse model of asthma (Liu, Tundwal et al. 2010). Furthermore, a study showed that the mitogen activated protein kinase kinase 1/2 (MEK1/2) inhibitor U0126 that inhibits ERK1/2 activation reduced the asthma phenotype in the ovalbumin model of asthma (Duan, Chan et al. 2004). Based on these previous reports and the evidence of a correlation between ERK1/2 activation and development of an asthma phenotype in mice, we hypothesized that HDM exposure in mice would increase the expression of phospho-ERK1/2 in the lungs, which would be decreased by chronic treatment with nadolol, but not carvedilol. In the current experiments, we found that HDM exposure in mice indeed increased phospho-ERK1/2 expression in the lungs, but chronic prophylactic treatment with nadolol did not change the phospho-ERK1/2 expression despite preventing the development of airway inflammation and mucous metaplasia in response to HDM. Carvedilol also had no effect on phospho-ERK1/2 expression in this model (Fig. 24). Similarly in the therapeutic model, chronic nadolol treatment did not reduce phospho-ERK1/2 expression in lungs despite attenuating the asthma phenotypes of inflammation and mucous metaplasia in response to HDM (Fig. 25). However, nadolol produced only a moderate decrease in AHR in the therapeutic model. Also, 4 weeks of rest after

the initial HDM exposure in the therapeutic model did not lower phospho-ERK1/2 expression (Fig. 25B), while it reduced airway inflammation and mucous metaplasia, but not AHR. These results show that the initial development of asthma phenotypes in mice is associated with an increase in phospho-ERK1/2 expression in the lungs, but later the phenotypes of airway inflammation and mucous metaplasia, not AHR, may dissociate from phospho-ERK1/2 expression. The results also imply that the overall ERK1/2 phosphorylation in the lung may not be involved in mediating the opposite effects of β-blockers on the asthma phenotype.

Regardless of the apparent limitations of the ovalbumin model of asthma, the model offers an advantage in studying the development of the asthma phenotype in two stages- a sensitization stage where mice are sensitized to the allergen but the phenotype is not present, and a challenge stage that develops the phenotype. After careful optimization of the sensitization protocol (discussed in supplementary section below), we studied the role of ERK1/2 phosphorylation in the development of asthma phenotype in the ovalbumin model. We measured the expression of phospho-ERK1/2 in lungs after the allergen sensitization and challenge stages of the ovalbumin model. In this model, ovalbumin sensitization in mice without challenge was sufficient to increase phospho-ERK1/2 expression in lungs even though the asthma phenotype was absent (Fig. 26, 27). Results from the ovalbumin model

further support the notion that ERK1/2 phosphorylation may not be solely associated with the development of the asthma phenotype in the murine models, and may be associated only with the initial stages of the disease development.

As discussed earlier, a major limitation of our studies is the lack of cell-specific measurement of ERK1/2 phosphorylation in the lung. Therefore, further *in vivo* studies are required for making definitive conclusions regarding the role of ERK1/2 signaling in mediating the effects of β-blockers in asthma. Our studies suffer from another limitation in that measurement of *in vivo* ERK1/2 phosphorylation may not be ideal to study the in vivo implications of biased signaling by β-blockers in asthma. As ERK1/2 is activated by both G-protein and β-arrestin dependent pathways, and plays a role in multiple cellular functions such as proliferation, survival, growth, differentiation, apoptosis, it may be difficult to shut down ERK1/2 signaling once activated. Furthermore, ERK1/2 activation by G-protein and β-arrestin pathways differs in its spacio-temporal regulation (Ahn, Shenoy et al. 2004). The G-protein dependent ERK1/2 signaling is rapid, transient and results in nuclear translocation of phospho-ERK1/2, while the β-arrestin mediated ERK1/2 signaling is slower, more persistent and confined to the cytoplasm (Ahn, Shenoy et al. 2004, Shenoy, Drake et al. 2006). Therefore, in addition to measuring cell-specific

ERK1/2 phosphorylation *in vivo*, it is important to determine the sub-cellular localization of phospho-ERK1/2 in future studies.

The role of β-arrestin-2 signaling is also implicated in development of the asthma phenotype. Studies have shown that the β-arrestin-2 knock-out (β-arr2 KO) mice have an attenuated asthma phenotype in the ovalbumin model of asthma (Walker, Fong et al. 2003). These studies showed that β-arrestin-2 positively regulates migration of the inflammatory cells to the lung, and the contractile function of the airway smooth muscle cells (Walker, Fong et al. 2003, Deshpande, Theriot et al. 2008, Hollingsworth, Free et al. 2010). Furthermore, a recent study showed that the conditional genetic deletion of β-arrestin-2 in mice mitigated an already established asthma phenotype (Chen, Hegde et al. 2015). In our previous studies, we found that both carvedilol and propranolol promoted the development of asthma phenotype in mice lacking epinephrine (that do not develop the asthma phenotype by themselves) in the ovalbumin model of asthma (Thanawala, Valdez et al. 2015). According to in vitro studies in HEK cells stably expressing the β_2AR, both carvedilol and propranolol activate the ERK1/2 pathway at the β_2AR (Fig. 5.7) (Wisler, DeWire et al. 2007). However, in the same studies, carvedilol activated ERK1/2 via a β-arrestin-2 dependent pathway, while propranolol activated ERK1/2 independent of the β-arrestin-2 pathway. These reports led to our current hypothesis that it

is the activation of ERK1/2 signaling and not β-arrestin-2, that is critical for developing asthma.

To test the hypothesis, we measured the effects of carvedilol and propranolol on the asthma phenotype in the β-arr2 KO mice and their wild-type (β-arr2 WT) littermates. We hypothesized that propranolol and not carvedilol would restore the asthma phenotype in the β-arr2 KO mice since propranolol activates ERK1/2 independent of the β-arrestin-2 pathway. However, we did not see an attenuated asthma phenotype in the β-arr2 KO mice, contradictory to the previously published reports. Our results showed that ovalbumin sensitization and challenge produced a significant increase in mucous metaplasia in the airways of the β-arr2 KO mice, which was not affected by carvedilol or propranolol treatment (Fig. 29). To confirm these results, we performed several pilot experiments in the β-arr2 KO mice and measured airway eosinophilic inflammation and mucous metaplasia in response to ovalbumin sensitization and challenge (discussed in supplementary section below). The anomalous results in our experiments with the β-arr2 KO mice may suggest an alteration in their phenotype brought about by breeding over several generations or due to unknown mutations in our mouse colony. However, all mice were regularly genotyped by PCR based technique for the Arrb2 (β-arr2) gene and were used in experiments only after the identification of their genotype. In addition to identifying their genotype by PCR, we measured the

expression of β-arr2 protein in the lungs of some representative β-arr2 KO and WT mice from our colony. We found that the β-arr2 KO mice showed a significantly reduced expression of the β-arr2 protein in their lungs compared to their WT littermates (Fig. 28). However, the β-arr2 protein was not completely undetected in the KO mice probably due to the identification of some part of the non-functional β-arr2 epitope by the antibody. However, the protein expression studies did not confirm whether the β-arr2 protein remaining in the KO mice was functional. Further investigation into the anomalous phenotype of the β-arr2 KO in our colony was beyond the scope of this dissertation and was not pursued any further.

5.6 Conclusions

Results discussed in the chapter show that allergen exposure in mice promotes ERK1/2 phosphorylation in the lungs, but β-blocker treatment dissociates the ERK1/2 phosphorylation from the asthma phenotypes of inflammation and mucous metaplasia suggesting that ERK1/2 may play a limited role in maintaining these phenotypes.

6. Chapter 3 (Supplementary Results)

6.1 Optimization of the ovalbumin sensitization and challenge protocol for the development of asthma phenotype in mice

6.1.1 Rationale

The protocol for ovalbumin sensitization and challenge (Ova S/C) used in our previous studies in the ovalbumin model of asthma had developed a robust asthma phenotype in the PNMT-/- mice (that lack the endogenous agonist epinephrine) and the Svj/129 mice as their wild type controls. The Ova S/C protocol used in the previous experiments however did not produce an asthma phenotype in the C57BL/6 mouse strain in the current studies, therefore requiring optimization of the protocol.

6.1.2 Objective and Hypothesis

The objective of the current set of experiments was to optimize the dose of ovalbumin for sensitization in mice, and the method of challenge to produce a significant increase in airway inflammation and mucous metaplasia in the ovalbumin-driven murine asthma model.

Based on a previously published report on the optimization of ovalbumin dose for sensitization, we hypothesized that reducing the dose from 2 mg/kg/day to 0.4 mg/kg/day would produce an optimum phenotype in mice.

6.1.3 Approach

We modified the protocol for Ova S/C from our previous studies in the SvJ/129 mouse strain. The previous protocol involved sensitization of mice with three intraperitoneal injections with 2 mg/kg/day of ovalbumin adsorbed to 2 mg of alum on days 0, 7 and 14 of the protocol (Fig. 31A). Ovalbumin sensitization was followed by intranasal challenge with 1 mg/kg/day of ovalbumin on 5 consecutive days (Fig. 31A). In the present studies, we reduced the dose of ovalbumin for sensitization to 0.4 mg/kg/day of ovalbumin adsorbed to 2 mg of alum administered by intraperitoneal injections on days 0, 7 and 14 (Fig. 31B). In addition to the intranasal method of ovalbumin challenge, we used inhalation method where mice were exposed to 1 % w/v of ovalbumin solution nebulized in a chamber for 30 min on three consecutive days (Fig. 31B)

Using the two protocols for sensitization in C57BL/6 mice, we measured the concentration of immunoglobulin E (IgE) in serum in a subset of mice as readout to test the sensitization protocol. We then measured inflammatory cellular infiltration in the remaining mice after challenging them by intranasal administration of ovalbumin (as done previously in protocol A, Fig. 31)

In another set of experiments, we measured inflammatory cellular infiltration in mice after sensitization with 0.4 mg/kg/day ovalbumin and challenge by inhalation (Fig. 31B).

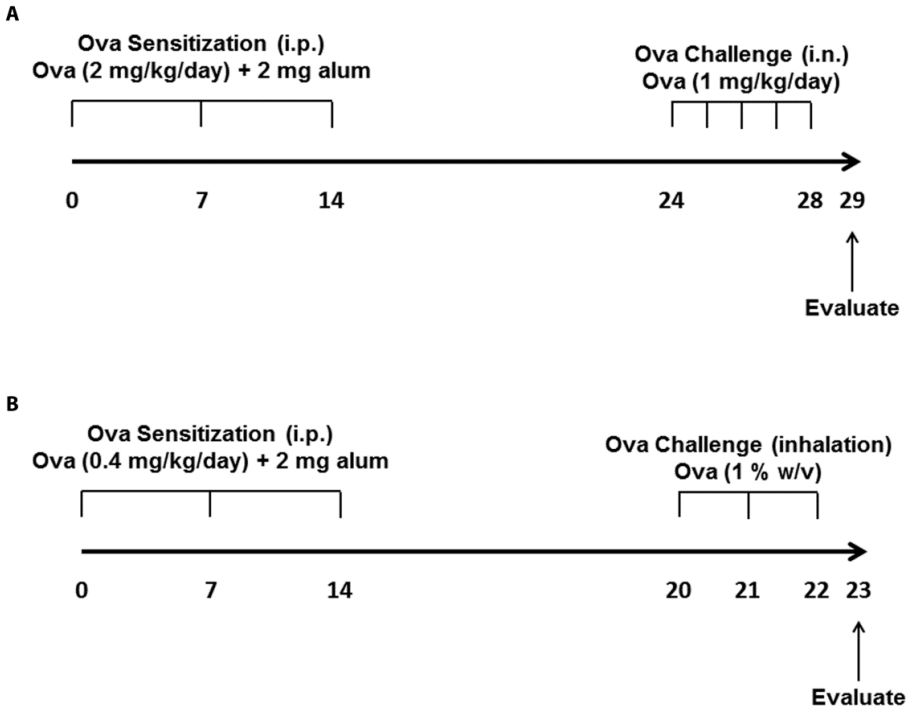

Figure. 31 Ovalbumin Sensitization and Challenge (Ova S/C) Protocols The figure represents (A) the previous protocol for Ova S/C in mice using a higher dose of ovalbumin (2 mg/kg/day) for sensitization and intranasal delivery of ovalbumin for challenge, and (B) the new protocol for Ova S/C in mice using a lower dose for sensitization (0.4 mg/kg/day) and inhalation route for challenge. The time point for challenge can vary from 1 week to several weeks after sensitization depending on the study requirements.

6.1.4 Results

6.1.4.1 Serum IgE levels in mice using different protocols of ovalbumin sensitization

Allergic asthma is mediated by the release of allergen specific immunoglobulin IgE in response to allergen sensitization. We sensitized C57BL/6 mice with the high and low doses of ovalbumin and measured the concentration of Ova-specific serum IgE. The higher dose of ovalbumin (2 mg/kg/day) did not increase the serum IgE concentration, while the lower dose (0.4 mg/kg/day) produced a significant increase in serum IgE concentration (Fig. 32).

6.1.4.2 Inflammatory cellular infiltration in BALF after different protocols of ovalbumin sensitization

We measured the levels of total cells and eosinophils in BALF of C57BL/6 mice sensitized with different doses of ovalbumin and challenged by intranasal administration of ovalbumin. The higher dose of ovalbumin (2 mg/kg/day) for sensitization only moderately increased the total cells but did not increase eosinophil numbers in BALF (Fig. 33A, B), while the lower dose of ovalbumin (0.4 mg/kg/day) produced a significant increase in both total

cells and eosinophils in BALF upon ovalbumin challenge by intranasal delivery (Fig. 33C, D).

6.1.4.3 Inflammatory cellular infiltration in BALF after ovalbumin challenge by inhalation

We tested inhalation delivery of ovalbumin challenge in C57BL/6 mice after sensitizing them with 0.4 mg/kg/day ovalbumin. In this experiment, ovalbumin challenge by inhalation again significantly increased the numbers of both total cells and eosinophils in BALF over control mice challenged with saline (Fig. 34).

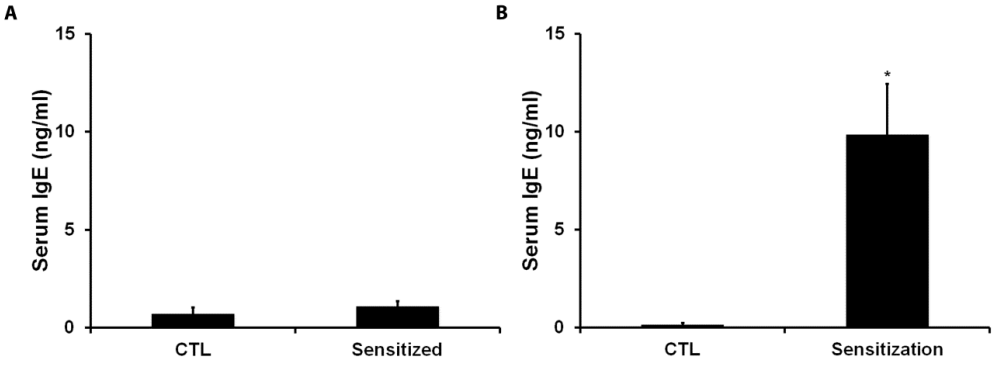

Figure. 32 Serum IgE Levels in C57BL/6 Mice Using Different Ovalbumin Sensitization Protocols The figure represents the serum levels of immunoglobulin IgE in (A) mice sensitized to 2 mg/kg/day of ovalbumin, and (B) mice sensitized to 0.4 mg/kg/day of ovalbumin compared to control mice sensitized to saline. Data are plotted as mean ± SEM from 3-6 mice per group and analyzed by student's t-test. * represents statistical significance at $p < 0.05$ in reference to the CTL group.

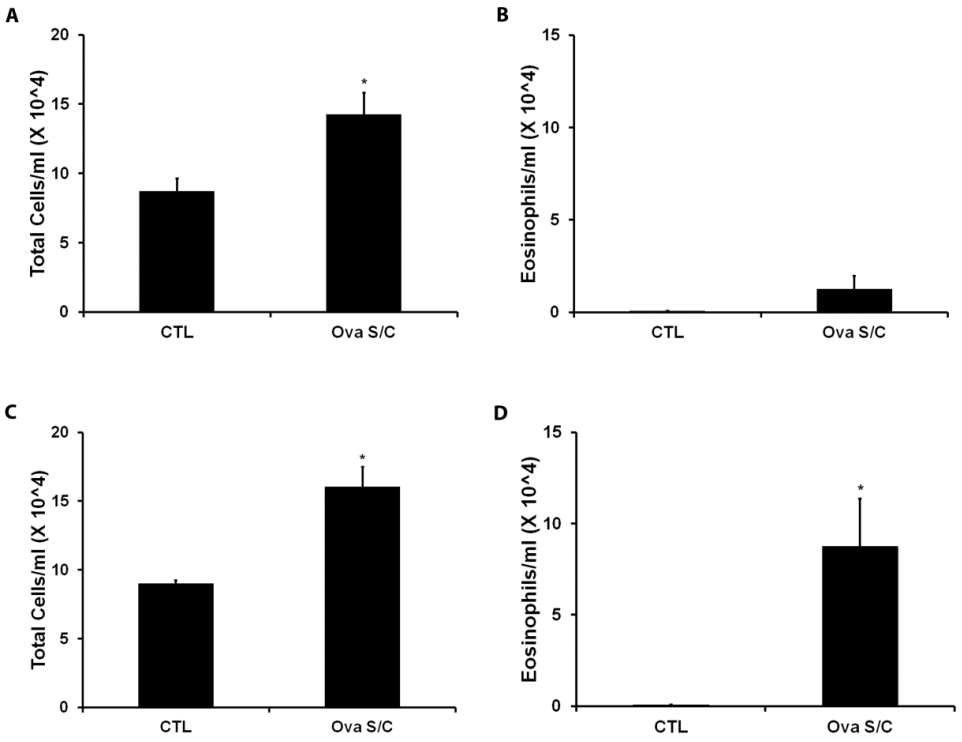

Figure. 33 Inflammatory Cellular Infiltration in BALF From Different Ovalbumin Sensitization Protocols The figure represents (A) total cells, and (B) eosinophils in BALF of mice sensitized to 2 mg/kg/day of ovalbumin; (C) total cells, and (D) eosinophils in mice sensitized to 0.4 mg/kg/day of ovalbumin and challenged with ovalbumin by intranasal delivery, compared to the respective control mice challenged with saline. Data are plotted as mean ± SEM from 3-5 mice per group and analyzed by student's t-test. * represents statistical significance at $p < 0.05$ in reference to the respective CTL groups.

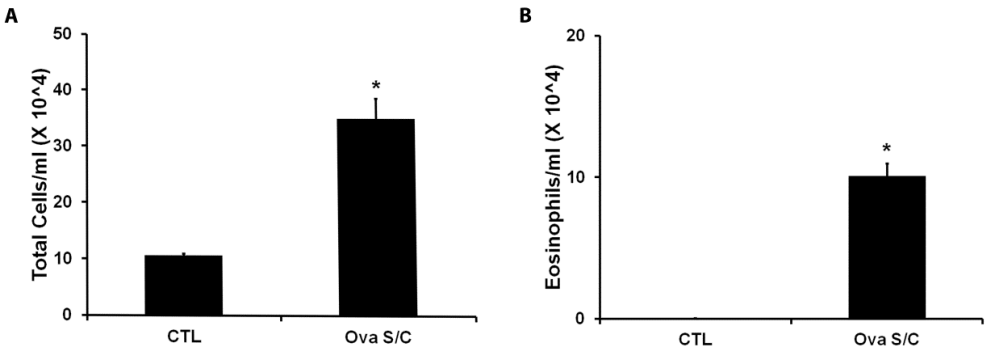

Figure. 34 Inflammatory Cellular Infiltration in BALF From Ovalbumin Challenge by Inhalation The figure represents (A) total cells, and (B) eosinophils in BALF of mice sensitized to 0.4 mg/kg/day of ovalbumin and challenged by inhalation of 1 % w/v ovalbumin compared to the control mice sensitized to ovalbumin but challenged with saline. Data are plotted as mean ± SEM from 5 mice per group and analyzed by student's t-test. * represents statistical significance at $p < 0.05$ in reference to the CTL group.

6.2 Testing different methods of ovalbumin challenge in the Ova S/C model of asthma in β-arrestin-2 KO mice

6.2.1 Rationale

As discussed in the previous chapter, ovalbumin sensitization and challenge resulted in development of the asthma phenotype in our β-arrestin-2 (β-arr2) KO mice contradictory to the previously published reports. We therefore tested different methods of ovalbumin challenge in our β-arr2 KO mice to check whether our mice produce consistent results across different protocols.

6.2.2 Approach

In one set of experiments, we sensitized the β-arr2 KO mice with 0.4 mg/kg/day of ovalbumin on days 0, 7 and 14, and challenged them by intranasal administration of 1 mg/kg/day ovalbumin on days 18-22 (Fig. 35A) or on days 41-45 (Fig. 35B). Twenty-four hours later, we measured the numbers of total cells and eosinophils in BALF.

In another set of experiments, we sensitized the β-arr2 KO mice with 0.4 mg/kg/day of ovalbumin on days 0, 7 and 14, and challenged them by inhalational exposure to a nebulized solution of 1 %w/v ovalbumin in a chamber on days 43-45 (Fig. 35C). We measured both eosinophilic inflammation and mucous metaplasia in the airways 24 hours later.

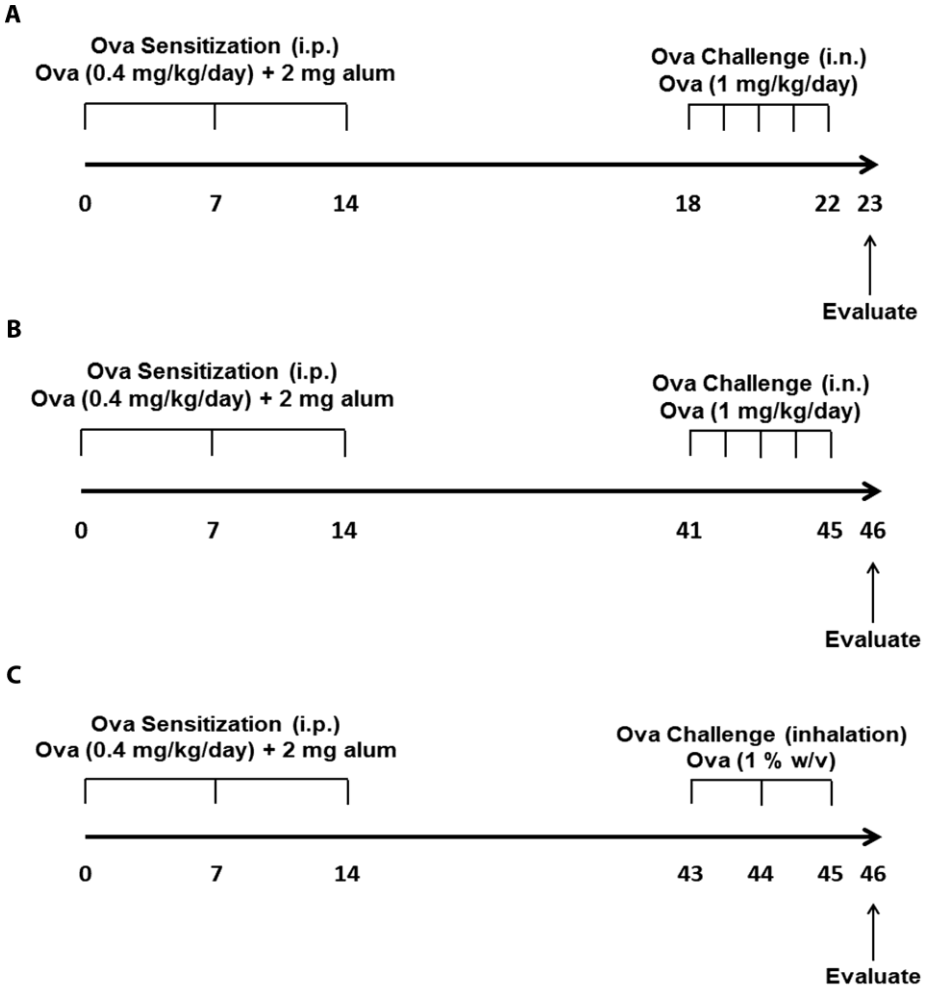

Figure. 35 Ovalbumin Sensitization and Challenge (Ova S/C) Protocols for Use in β-arr2 KO Mice The figure represents Ova S/C protocols where mice were sensitized with 0.4 mg/kg/day ovalbumin mixed with 2 mg of alum and subsequently challenged with (A) intranasal delivery of ovalbumin on days 18-22; (B) intranasal delivery of ovalbumin on days 41-45; and (C) inhalation delivery of ovalbumin on days 43-45. Twenty-four hours later, mice were evaluated for the asthma phenotype.

6.2.3 Results

6.2.3.1 Inflammatory cellular infiltration in BALF after intranasal challenge with ovalbumin using acute and chronic protocols

Ovalbumin sensitization followed by challenge via the intranasal route produced a significant increase in the total cell and eosinophil counts in BALF in β-arr2 KO mice over the saline challenged controls in both the acute 24 day (Fig. 36A, B) and the chronic 46 day (Fig. 36C, D) protocols.

6.2.3.2 Inflammatory cellular infiltration in BALF and mucous metaplasia after inhalation challenge with ovalbumin

In the protocol using inhalation method of ovalbumin challenge, β-arr2 KO mice showed a significant increase in both total cell and eosinophil infiltration in BALF (Fig. 37A, B). Furthermore, inhalation challenge with ovalbumin increased the mucin volume density in the airways of the β-arr2 KO mice compared to saline challenge (Fig. 37C, D). These studies suggest that our β-arr2 KO mice developed a robust asthma phenotype to ovalbumin sensitization and challenge irrespective of the protocol used.

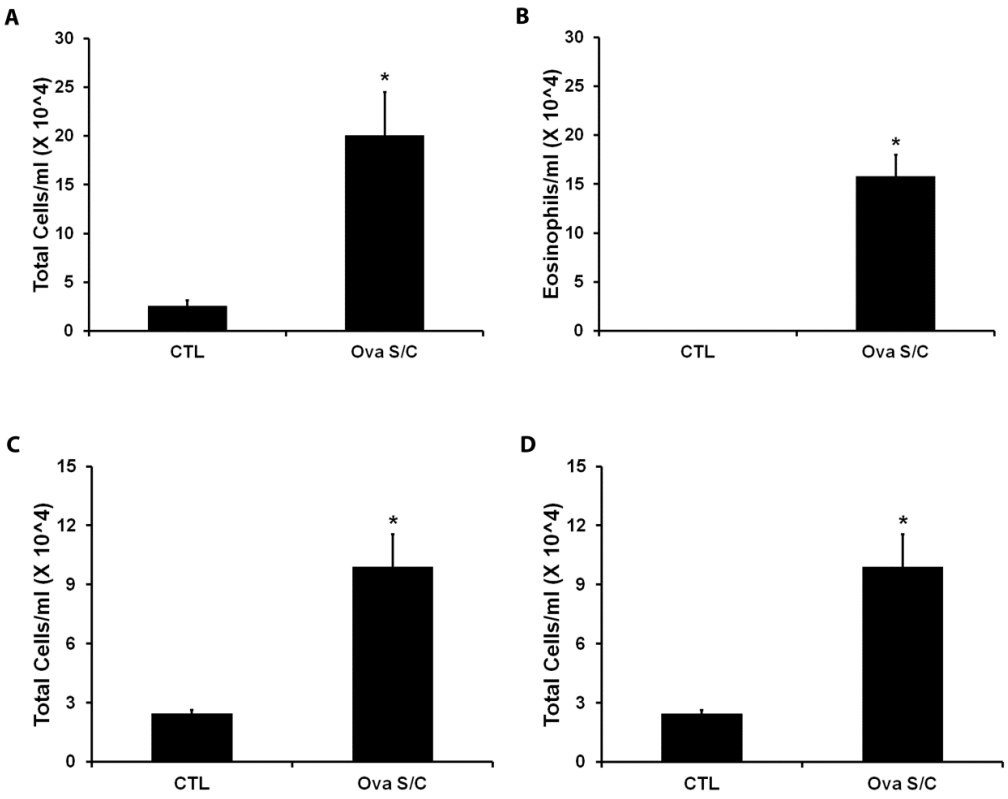

Figure. 36 Inflammatory Cellular Infiltration in BALF From Intranasal Ovalbumin Challenge in β-arr2 KO Mice The figure represents (A) total cells, and (B) eosinophils in BALF of β-arr2 KO mice sensitized to 0.4 mg/kg/day of ovalbumin followed by intranasal challenge with ovalbumin on days18-22 (acute protocol); (C) total cells, and (D) eosinophils in mice sensitized to 0.4 mg/kg/day of ovalbumin and challenged with ovalbumin by intranasal delivery on days 41-45 (chronic protocol), compared to the respective control mice challenged with saline. Data are plotted as mean ± SEM from 3-4 mice per group and analyzed by student's t-test. * represents statistical significance at $p < 0.05$ in reference to the respective CTL groups.

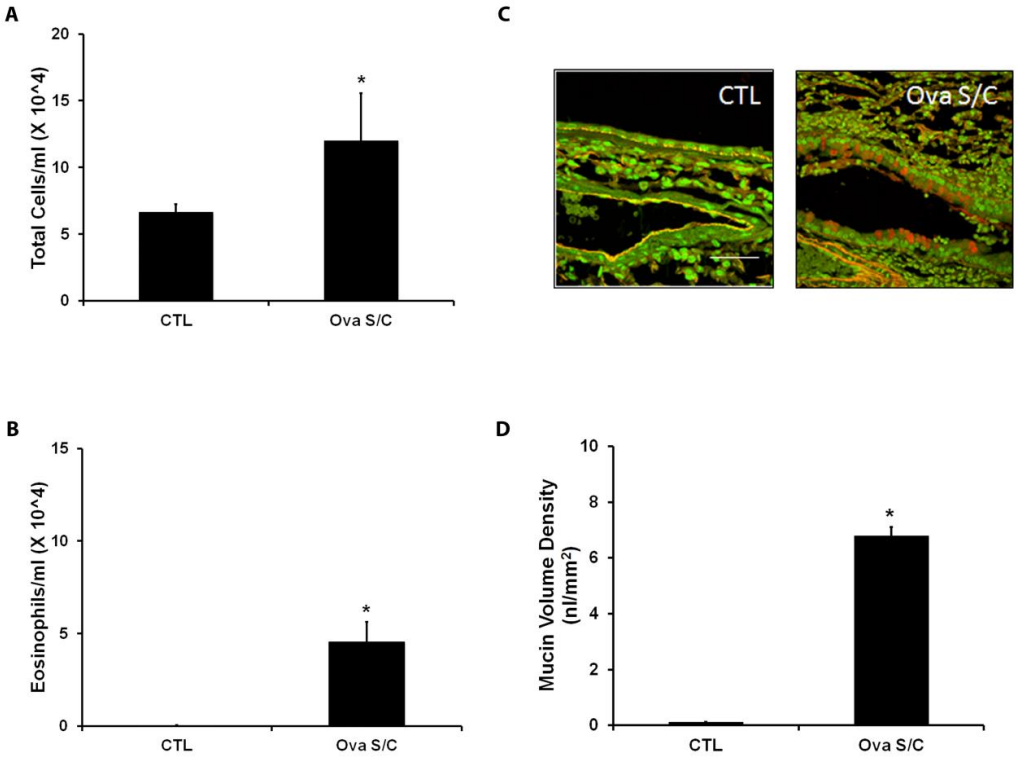

Figure. 37 Inflammatory Cellular Infiltration in BALF and Airway Mucous Metaplasia following Inhalation Challenge with Ovalbumin in β-arr2 KO Mice The figure represents BALF inflammatory cells (A, B), and mucous metaplasia (C, D) in mice subjected to ovalbumin sensitization and challenge by inhalation method. A. Total cells and B. eosinophil numbers in BALF of mice sensitized to 0.4 mg/kg/day of ovalbumin followed by challenge by inhalation of 1 %w/v ovalbumin on days 41-45 C. PAFS stained images showing mucin content (red) in airway epithelial cells (green), and D. morphometric quantification of mucin volume density in mice sensitized to ovalbumin followed by challenge by inhalation compared to saline challenged control mice. Data are plotted as mean ± SEM from 4-5 mice per group and analyzed by student's t test. * represents statistical significance at $p< 0.05$ in reference to the naïve control group.

6.3 Discussion

The current experiments were aimed to optimize the ovalbumin sensitization and challenge protocol to produce a robust asthma phenotype in mice. Previous studies in our laboratory had used a standard protocol for ovalbumin sensitization and challenge (Ova S/C) in the SvJ/129 mouse strain to produce the asthma phenotype. The protocol involved sensitization to 2 mg/kg/day of ovalbumin mixed with 2 mg of alum by three intraperitoneal (i.p.) injections over a period of two weeks, followed by intranasal challenge with 1mg/kg/day of ovalbumin administered on 5 consecutive days (Thanawala, Forkuo et al. 2013, Forkuo, Kim et al. 2016). The duration between sensitization and challenge phases of the protocol could vary depending on the requirements of the acute vs chronic models. However, when tested in C57BL/6 mice, the same protocol of Ova S/C failed to develop a significant phenotype in these mice, requiring us to optimize the protocol.

A previous study using the ovalbumin model of asthma in Balb/c mice had shown an inverse relation between the dose of ovalbumin for sensitization and the magnitude of asthma phenotype produced in mice (Sakai, Yokoyama et al. 1999). Based on this report, we decided to measure the effect of lowering the sensitization dose of ovalbumin from 2 mg/kg/day to 0.4 mg/kg/day on the asthma phenotype. The dose of the adjuvant alum was kept constant; however the ratio of ovalbumin to alum differed between the two protocols. Allergic

asthma involves a complex interplay between inflammatory cells leading to T helper cell differentiation to a Th2 phenotype and producing inflammatory cytokines such as IL-4, IL-5 and IL-13. The cytokine IL-4 stimulates B cells to produce allergen-specific immunoglobulin IgE, which further enhances the Th2 response (Fig. 38). The cytokines IL-5 and IL-13 produce eosinophilic recruitment in the airways, mucous metaplasia, and airway hyper-responsiveness (Lambrecht and Hammad 2012). Therefore, we measured the level of Ova-specific IgE in BALF as a magnitude of sensitization following intraperitoneal injections with the two different doses of ovalbumin.

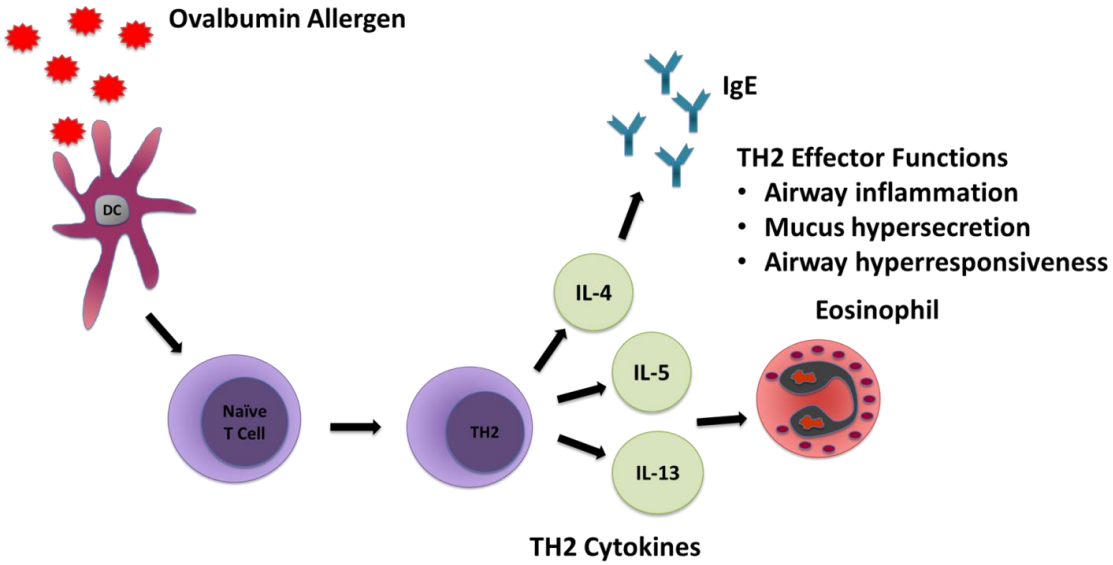

Figure. 38 Cellular Basis of Ovalbumin Sensitization and Challenge (Ova S/C) Model of Asthma The figure represents a schematic of the cellular mechanism driving the asthma phenotype in the ovalbumin model. Allergen sensitization promotes dendritic cell recruitment and maturation to activate differentiation of naïve T cells to Th2 phenotype. The Th2 cells produce inflammatory cytokines IL-4, IL-5 and IL-13 and promote IgE synthesis, and the development of the asthma phenotype.

Our results showed the lower dose of ovalbumin (0.4 mg/kg/day) for sensitization significantly increased the serum IgE levels, while the higher dose (2 mg/kg/day) did not alter serum IgE levels (Fig. 32). Upon subsequent challenge with ovalbumin, mice that had been sensitized with the lower and not the higher dose of ovalbumin produced a significant increase in eosinophilic inflammation in BALF (Fig. 33). Because of these results, we switched to using the lower sensitization dose of ovalbumin in all of our subsequent experiments. However, our β-arr2 KO mice produced anomalous results in the Ova S/C model of asthma as discussed in the previous chapter. Therefore, in addition to changing the sensitization protocol, we changed the method of ovalbumin challenge from intranasal delivery to inhalation exposure of mice to nebulized ovalbumin in a chamber. The change in the challenge protocol was made to be consistent with previous reports showing an attenuation of the asthma phenotype in β-arr2 KO mice when subjected to Ova S/C (Walker, Fong et al. 2003). We first tested the inhalation protocol (Fig. 31B) in C57BL/6 WT mice and found that the inhalation method of ovalbumin challenge produced a significant increase in eosinophilic inflammation in BALF (Fig. 34). Following the success of the new challenge protocol in WT mice, we tested the protocol in β-arr2 KO mice in subsequent studies. Despite these efforts to follow the same Ova S/C protocol as published in literature, our β-arr2 KO developed a significant increase in airway eosinophilia and mucous metaplasia (Fig. 36, 37)

in contrast with the previously published results (Walker, Fong et al. 2003, Deshpande, Theriot et al. 2008, Chen, Hegde et al. 2015).

6.4 Conclusion

The results discussed in this chapter suggested for the ovalbumin doses we used, there was an inverse relationship to the magnitude of sensitization and the degree of the asthma phenotype. However, even careful optimization of the ovalbumin sensitization and challenge protocols produced a significant increase in asthma phenotypes of airway eosinophilia and mucous metaplasia in our β-arr2 KO mice suggesting an alteration of the phenotype in our β-arr2 KO mouse colony from the original colony at Duke University.

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