# Activation of $\beta_2$-adrenoceptors is Required for Mucin Production in Airway Bronchial Epithelial Cells

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A Dissertation Submitted to

The Department of Pharmacological and Pharmaceutical Sciences

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Pharmacology

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By

Nour Al-Sawalha

August 2013

This work is dedicated to

*Mom and Dad*

*For their unconditional support and love…….*

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## Abstract

Asthma, a chronic inflammatory disease of the airways, is associated with mucus hypersecretion by airway epithelium. Accumulated mucus in the airways contributes to airway obstruction, airway hyperresponsiveness and sometimes death. In a murine model of asthma, chronically blocking $\beta_2$-adrenoceptors ($\beta_2$-ARs) or genetic ablation of $\beta_2$-ARs causes a reduction in mucus secretion, an index of inflammatory responses, in response to allergen. The detailed molecular components of these effects remain to be elucidated.

We examined the $\beta_2$-AR signaling pathways involved in mediating mucin production in response to IL-13 in airway epithelial cells. The expression of MUC5AC, the predominant mucin-producing gene in asthma, and mucin content were induced by IL-13 in normal human bronchial epithelial (NHBE) cells as compared to control cells only in the presence of epinephrine. Blocking $\beta_2$-ARs, but not $\beta_1$-ARs, attenuated the IL-13 effect. The three members of MAPK family, ERK1/2, p38 and JNK, were all involved in inducing mucin production in response to IL-13 in the presence of epinephrine.

Since $\beta_2$-ARs signal through the canonical Gs-adenylyl cyclase or β-arrestin signaling cascade, we examined each pathway separately. Elevating intracellular cAMP levels was associated with enhanced IL-13 response, either in the presence or absence of epinephrine and increased epithelial cell number. Moreover, inhibiting the activity of PKA and PDE4 resulted in attenuated IL-13

response. Stimulating β-arrestin-2 signaling did not alter the IL-13 response in NHBE cells, either in the presence or absence of epinephrine. However, the absence of β-arrestin-2 from mouse airway epithelial cells attenuated the response to IL-13 as compared to cells expressing β-arrestin-2.

$\beta_2$-ARs did not seem to affect IL-13 mediated mucuciliary shift from ciliated to goblet cells or cell proliferation. We conclude that, in human bronchial epithelial cells, epinephrine-induced $\beta_2$-AR signaling is required to enhance mucus production in response to IL-13. Moreover, MAPKs, cAMP and cAMP-dependent protein kinases are all involved components of $\beta_2$-AR signaling in mediating the effect of IL-13 in NHBE cells.

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## List of abbreviations

| | |
|---|---|
| 15-LO-1 | 15-Lipoxygenase-1 |
| AC | Adenylyl cyclase |
| AHR | Airway Hyper-responsiveness |
| AKAP | A kinase anchoring proteins |
| ALI | Air-liquid interface |
| AP-1 | Activator protein 1 |
| APCs | Antigen presenting cells |
| APRIL | A proliferation-inducing ligand |
| ASM | Airway smooth muscle |
| ATF | Activating transcription factor |
| ATP | Adenosine triphosphate |
| BAFF | B cell-activating factor of TNF family |
| BALF | Bronchoalveolar lavage fluid |
| BEBM | Bronchial epithelial basal media |
| BEGM | Bronchial epithelial growth medium |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CBF | Ciliary beat frequency |

| | |
|---|---|
| CBP | CREB-binding protein |
| CC10 | Clara cell 10 kDa protein |
| CCSP | Clara cell secretory protein |
| CF | Cystic fibrosis |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| cGMP | Cyclic guanosine monophosphate |
| CHF | Congestive heart failure |
| CLCA | Calcium-activated chloride channel |
| COPD | Chronic obstructive pulmonary disease |
| Cpla2 | Cytosolic phospholipase A2 |
| CRE | cAMP-response element |
| Ct | Threshold cycle |
| cγM | Common γ-chain |
| DCs | Dendritic cells |
| DMEM | Dulbecco’s modified Eagle medium |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| Epac | Exchange protein activated by cAMP |
| Epi | Epinephrine |
| ERK | Extracellular signal-regulated kinase |

| | |
|---|---|
| FBS | Fetal bovine serum |
| FEV1 | Forced expiratory volume in 1 second |
| FOXA2 | Forkhead box protein A2 |
| FOXJ1 | Forkhead box protein J1 |
| GATA3 | GATA-binding protein 3 |
| GDP | Guanosine diphosphate |
| GINA | The Global Initiative on Asthma |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GPCR | G-protein coupled receptors |
| Grb-2 | Factor receptor-bound protein-2 |
| GRE | Glucocorticoid response elements |
| GRK | G protein-coupled receptor kinase |
| GRO-α | Growth-related oncogene-α |
| GSK-3β | Glycogen synthase kinase-3β |
| GTP | Guanosine triphosphate |
| HB-EGF | Heparin-binding EGF-like growth factor |
| HFH-4 | Hepatocyte nuclear factor homolog |
| HIF-1 | Hypoxia-inducible factor-1 |
| HSP | Heat shock protein |

| | |
|---|---|
| IBMX | 1-Methyl-3-Isobutylxanthine |
| $I_{Ca}$ | L-type calcium current |
| ICS | Inhaled corticosteroids |
| IFN- γ | Interferon-γ |
| IgE | Immunoglobulin E |
| IL-13Rα1 | IL-13-receptor alpha 1 |
| IL-4Rα | IL-4-receptor alpha |
| IL | Interleukin |
| ILC2 | Group 2 innate lymphoid cells |
| IRS-1/2 | Insulin receptor substrate 1 and 2 |
| JAK | Janus tyrosine Kinase |
| JIP1/2 | JNK-interacting protein |
| JNK | c-Jun N-terminal kinase |
| KO | Knockout |
| Krt5 | Cytokeratins 5 |
| LABAs | Long acting $\beta_2$-AR agonists |
| LAR | Late asthmatic reaction |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen activated protein kinase |
| MAPKAP kinase 2 | MAP kinase activated protein kinase-2 |

| | |
|---|---|
| MAPKK | MAPK-kinase |
| MAPKKK | MAPK-kinase kinase |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MEK | Extracellular signal-regulated kinase (ERK)-kinases |
| MEK-1/2 | MAPK/ERK kinase-1/2 |
| MIP-α | Macrophage inflammatory protein 3 alpha |
| MLCP | Myosin light chain phosphatase |
| MLKC | Myosin light chain kinase |
| MLNs | Mediastinal lymph node |
| MMPs | Matrix metalloproteinases |
| MSK | Mitogen- and stress-activated kinase |
| mTECs | Mouse tracheal epithelial cells |
| NHBE cells | Normal human bronchial epithelial cells |
| NO | Nitric oxide |
| P70S6K | p70 ribosomal protein S6 kinase |
| PAMPs | Variety of pathogen-associated molecular patterns |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PCNA | Proliferating cell nuclear antigen |
| PDEs | Phosphodiesterases |

| | |
|---|---|
| PGE2 | Prostaglandin E2 |
| PAFS | Periodic acid fluorescent Schiff's |
| PFA | Paraformaldehyde |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLCβ1 | Phospholipase Cβ1 |
| PNMT | Phenylethanolamine N-methyltransferase |
| PVDF | Polyvinylidene difluoride |
| r | Radius |
| R | Resistance |
| RANTES | Regulated upon activation normal T cell expressed and presumably secreted |
| Rp-cAMPS | Adenosine-3`-5` cyclic monophosphorothioate, Rp isomer |
| RSK | 90 kDa ribosomal S6 kinase |
| SCF | Stem-cell factor |
| siRNA | Small interference RNA |
| Smad4 | Mothers against decapentaplegic homolog 4 |
| SOS | Son of sevenless |
| SP-1 | Specificity Protein 1 |
| STAT | Signal transducer and activator of transcription |

| | |
|---|---|
| TARC | Thymus and activation regulated chemokine |
| TCM | Central memory T |
| TEER | Transepithelial electrical resistance |
| TGF-α | Transforming growth factor-alpha |
| TGF-β | Transforming growth factor beta |
| $T_H1$ | T-helper-1 |
| $T_H2$ | T-helper-2 |
| TIR | Toll/IL-1 receptor |
| TLR | Toll-Like Receptor |
| TNF-α | Tumor necrosis factor- α |
| TR | Tandem repeats |
| Trp-63 | Transformation-related protein 63 |
| TSLP | Thymic stromal lymphopoietin |
| WT | Wild type |
| $\beta_1$-AR | $\beta_1$-adrenoceptors |
| $\beta_2$-AR | $\beta_2$-adrenoceptors |

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## 1. Introduction and statement of the problem

Approximately 300 million people worldwide currently suffer from asthma and the number may grow an additional100 million by 2025 (Masoli, Fabian et al. 2004; 2007). Despite advances in health care, asthma accounts for 250,000 death cases per year worldwide (2007).

The Global Initiative for Asthma ((GINA) 2012) defines asthma as:

"A chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment" ((GINA) 2012).

Understanding airway inflammation and establishing better treatment regimens have been the focus of research for decades (Djukanovic, Roche et al. 1990; Barnes 2008). Airway inflammation in asthma is characterized by an amplified T-helper-2 ($T_H2$) response at the expense of $T_H1$ responses (Kidd 2003). Therefore, there is an increase in $T_H2$ cytokines, such as interleukin (IL)-4, IL-5 and IL-13 in the airways (Kips 2001). To target the two main changes that are associated with asthma, inflammation and airway obstruction, inhaled

corticosteroids (ICS) and $\beta_2$-AR agonists (Fanta 2009) have been the drugs of choice for the last half-century .

ICS are the most effective anti-inflammatory medication used in asthma and they are effective in the majority of adults and children (Kupczyk, Dahlen et al. 2011; van Aalderen and Sprikkelman 2011). However, besides the existence of 'steroid-resistant' asthmatics, inhaled corticosteroids also have numerous adverse effects. Growth impairment in children (Dahl 2006; Kelly, Sternberg et al. 2012) and reduction in bone density (Kelly and Nelson 2003) are few of their side effects. Though these effects are usually dose related and more thus common when used at high doses in severe persistent asthma, they can be observed in what are typically moderate doses of ICS (Kelly, Sternberg et al. 2012).

All asthmatic patients are prescribed short acting $\beta_2$-AR agonists to provide acute bronchodilation for asthma attacks. Some patients develop a disease state that affects their daily life activities (moderate persistent asthma), and ICS are usually the first controller medications added in this circumstance. If additional control is needed long acting $\beta_2$-AR agonists (LABAs) are added (Fanta 2009). However, the use of $\beta_2$-AR agonists chronically is associated with increased risk of severe exacerbations and overall asthma related death (Nelson, Weiss et al. 2006). Several studies have shown that chronic use of $\beta_2$-AR agonists resulted in AHR exacerbation (van Schayck, Graafsma et al. 1990),

increased inhaled antigen load (Broadley 2006), increased allergen induced bronchial inflammation (Boulet, Chakir et al. 2001), enhanced sputum eosinophils (Gauvreau, Jordana et al. 1997) and eventually increased airway inflammation (Rider and Craig 2006). Therefore, according to the current asthma therapy guidelines, long acting $\beta_2$-AR agonists should never be used as monotherapy and should always be prescribed with ICS (Giembycz and Newton 2006). The restricted use resulting from the long term side effects of ICS and $\beta_2$-AR agonists creates a subset of patients who have uncontrolled disease, leading to learning disabilities and depression, in children and adults (O'Byrne, Pedersen et al. 2013). Several therapeutic agents have been developed recently to meet the need of patients with uncontrolled disease. Of these agents, leukotriene modifiers, mast cell stabilizers and a long list of monoclonal antibodies that neutralize several inflammatory mediators have been tested, and in some cases, marketed (Holgate and Polosa 2008; Holgate and Davies 2009). Unfortunately, these approaches are expensive and effective only in specific subpopulations. Therefore, there is a need to discover better therapeutic agents.

In summary, short-acting $\beta_2$-AR agonists are incredibly valuable bronchodilators acutely, but chronic use of LABAs can exacerbate airway inflammation, and cause a small but significant increase in asthma mortality. These agents should be used only if the addition of ICS still does not provide adequate asthma control. This scenario is analogous to congestive heart failure

(CHF) where the acute use of β-AR agonists improves the quality of life, but chronic use is associated with increased mortality (Weber, Likoff et al. 1982; Felker and O'Connor 2001). As 'beta-blockers' are negative inotropic agents, this class of drugs was contraindicated for many decades (Haber, Simek et al. 1993). However, after successful clinical studies, chronic administration of certain β-AR blockers became the gold standard medical therapy for CHF (Hunt, Baker et al. 2001). This temporal difference between acute and chronic use of agonists and antagonists in disease treatment may also be applicable to asthma. Acute administration of β-AR blockers can result in airway narrowing in asthmatic patients (Singh, Whitlock et al. 1976; Boskabady and Snashall 2000) and in an antigen-driven murine model of asthma (Callaerts-Vegh, Evans et al. 2004). However, chronically blocking $\beta_2$-AR signaling by nadolol or genetically ablating $\beta_2$-ARs results in reduction in airway hyperresponsiveness (AHR), inflammatory cell recruitment to the airways and mucin content of airway epithelium in allergen driven murine model of asthma (Callaerts-Vegh, Evans et al. 2004; Nguyen, Omoluabi et al. 2008; Nguyen, Lin et al. 2009).

Airway smooth muscle (ASM) cells control the airway caliber and hence contribute to AHR. Constriction of ASM results in airway narrowing in response to nonspecific stimuli or pharmacological agonist. Because of this, ASM cells are the predominant cell type studied for control of AHR. ASM cells can also contribute to AHR and inflammation through releasing different inflammatory

mediators (Johnson, Roth et al. 2001; Borger, Tamm et al. 2006; Hershenson, Brown et al. 2008; Zuyderduyn, Sukkar et al. 2008). The role of airway epithelial cells, the second most predominant parenchymal lung cell, in asthma has been the subject of less intense investigation and initially considered primarily as mechanical barrier to maintain the integrity of inhaled substances from entering the systemic circulation. However, several recent studies have shown an emerging role for epithelium as a tissue orchestrating a sophisticated set of responses (review by Holgate). These include a critical role in initiating the immune response to inhaled allergens (Lambrecht and Hammad 2012) and skewing the inflammatory response toward $T_H2$ (Bulek, Swaidani et al. 2010). Moreover, airway epithelial cells, through producing mucus and forming mucus plugs, cause airway obstruction. AHR and mucous plugs are the cause of death in almost all asthma-related fatalities (Aikawa, Shimura et al. 1992; Fahy 2002).

Chronic $\beta_2$-AR activation can result in increased airway inflammation and airway epithelial cells respond to inflammatory mediators by increasing mucus production (Kuperman, Huang et al. 2002; Evans and Koo 2009). However, studies have shown that chronic inhibition of $\beta_2$-AR signaling can rescue airway inflammation and hence attenuate mucus production asthma (Nguyen, Omoluabi et al. 2008; Nguyen, Lin et al. 2009). Therefore, studying the $\beta_2$-AR signaling mechanism mediating mucous production and the disease pathophysiology is critical in our understanding of asthma. Due to the complexity of the signaling

pathways that are involved in mediating mucous production and the involvement of diverse inflammatory and parenchymal cells in whole animal models, we undertook to study cultured human airway epithelial cells. Many studies have established that *in vitro* cell culture using human airway epithelial cells share many morphological and functional distinctive properties with *in vivo* airway epithelial cells (Lin, Li et al. 2007; Stewart, Torr et al. 2012). In our studies we investigated the requirement of agonist induced $\beta_2$-AR signaling for the expression MUC5AC, the major mucin producing gene, and intracellular mucin in response to IL-13, a $T_H2$ cytokine, in human bronchial epithelial cells. Additionally, we investigated the involvement of various potential IL-13 signaling components including mitogen-activated protein kinases (MAPKs), canonical Gs-adenylyl cyclase/protein kinase A (PKA) signaling and β-arrestin-2 signaling in IL-13 induced mucus changes. Furthermore, we examined the source of goblet cell formation and the involvement of $\beta_2$-AR signaling in mediating the mucociliary shift induced by IL-13.

The results from these studies contribute key findings in our understanding of the mechanisms by which chronic use of $\beta_2$-AR agonists deteriorate the disease condition. Also these results influence a change in treatment paradigms and may enhance acceptance of chronic use of a subset of β-blockers, such as nadolol, in the treatment of asthma. This dramatic departure

from the current paradigm, if validated, may help overcome the difficulties in managing asthma in general and mucous hypersecretion in particular.

## 2. Literature Survey

### 2.1 Asthma:

Asthma is a chronic inflammatory disorder of the airways (Busse and Lemanske 2001; Lemanske and Busse 2003) and is characterized by airway hyperresponsiveness (AHR), airway obstruction, airway inflammation and mucus metaplasia. Asthmatic patients usually present with shortness of breath, cough and wheezing (Busse 2011). Asthma affects around 300 million people worldwide and it is estimated that this number will keep growing to reach around 400 million by year 2025 (Masoli, Fabian et al. 2004; 2007). The increase in asthma prevalence is associated with increased financial burden. This economic cost is resulted from the direct cost of hospitalization and medications and the indirect cost because of work and school absenteeism (Bahadori, Doyle-Waters et al. 2009). Despite the presence of different treatment modalities, asthma accounts for 250,000 death cases per year (2007).

#### 2.1.1 Airway inflammation in asthma:

Several inflammatory cells and mediators play a role in asthma pathogenesis as outlined in Figure 1. Asthma is characterized by T-helper-2 ($T_H2$) response. This preferential skewing toward $T_H2$ is results from different factors, in particular, the cytokine environment that promotes the expression of transcription factors that drives the differentiation of the naïve T cells to $T_H2$ (Ho

and Pai 2007). GATA-binding protein 3 (GATA3) and T-bet are the transcription factors that drive $T_H2$ and $T_H1$ differentiation respectively (Barnes 2008). Moreover, these two responses downregulate each other. The major biological function of $T_H1$ and $T_H2$ responses is to eradicate intracellular pathogens (cellular immunity) and extracellular pathogens (humoral immunity) respectively (Kidd 2003). Based on the $T_H1/T_H2$ model, the "Hygiene hypothesis" was introduced proposing that lack of exposure to infection early in life alters the $T_H1/T_H2$ balance and causes the preferential programming of naïve T cells to $T_H2$ (Sheikh and Strachan 2004). However, hygiene hypothesis is not the only explanation of asthma development and prevalence (Maziak 2003; Ramsey and Celedon 2005). Moreover, the description of asthma as solely a $T_H2$ response is too simple and raised too many concerns that have been reviewed systematically (Salvi, Babu et al. 2001; Ngoc, Gold et al. 2005). The involvement of several types of inflammatory cells, structural cells and inflammatory mediators are summarized below.

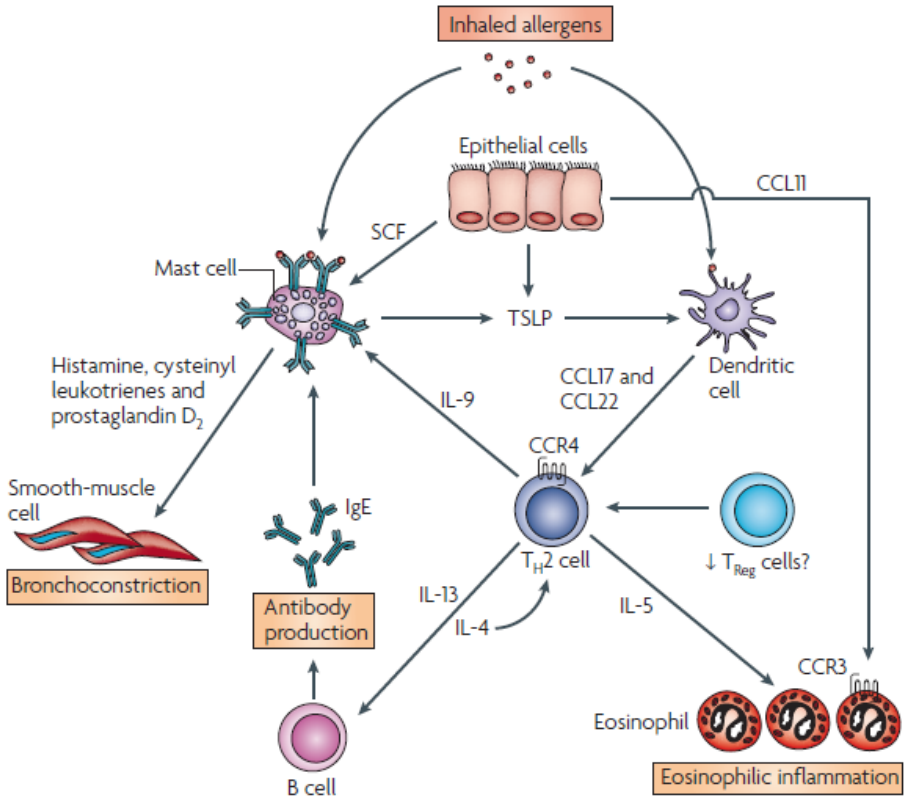



**Figure 1. Overview of the inflammatory cells that are involved in asthma**: Different inflammatory and structural cells play a role in asthma pathogenesis. Mast cells trigger airway smooth muscle bronchoconstriction through releasing histamine and cysteinyl leukotrienes. Epithelial cells, through releasing stem-cell factor (SCF) and thymic stromal lymphopoietin (TSLP), recruit mast cells to the surface of airways and initiate $T_H2$ inflammation respectively. The released $T_H2$ cytokines (IL-13, IL-4, IL-5 and IL-9) induce eosinophilic inflammation, IgE production from B cells and mast cell proliferation as described in the text. Adapted from Barnes (2008).

#### 2.1.1.1 Inflammatory cells:

Different inflammatory cells are recruited to the airways and contribute to the disease development. Eosinophilic inflammation is a major characteristic of asthma (Watt, Schock et al. 2005) and the levels of eosinophils in blood is correlated with the disease severity (Griffin, Hakansson et al. 1991). Eosinophils contribute to late asthmatic reaction (LAR) (Bradding 2008) through releasing pro-inflammatory proteins such, as major basic protein, and several leukotriens and cytokines such as cysteinyl leukotriene C4 and IL-4 and 5 (Busse and Lemanske 2001; Broadley 2006). The granular proteins that are released by eosinophils contribute to epithelial shedding and damage while chemokines and cytokines recruit more inflammatory cells and augment the inflammation (Broadley 2006). Mast cells contribute to immediate response to allergen through releasing their preformed content of histamine (Barnes 2008) upon Immunoglobulin E (IgE) cross-linking on the high affinity receptors that present on mast cells (Robinson 2004). Moreover, mast cells also release several newly synthesized cytokines such as interleukin (IL)-4, IL-5, IL-13 and tumor necrosis factor-α (TNF-α) (Bradding 2008). Basophils also possess the high affinity IgE receptors, release the stored histamine upon IgE binding and secrete IL-4 and IL-13 (Hamid, Tulic et al. 2003). Analyzing the sputum of asthmatic patients reveals the presence of basophils and mast cells (Gauvreau, Lee et al. 2000). Neutrophil numbers are increased in severe asthma and severe sudden exacerbations

(Ennis 2003; Macdowell and Peters 2007; Monteseirin 2009). Macrophages also contribute to airway inflammation (Hamid, Tulic et al. 2003). Beside $T_H2$ cells, regulatory T cells, Th17 (Vock, Hauber et al. 2010) and B cells also play a role in asthma (Barnes 2008).

#### 2.1.1.2 Airway Structural cells:

Airway smooth muscle (ASM) cells are the major structural cell that have been studied thoroughly due to their role in controlling airway caliber. ASM cells are increased in mass in asthma due to hypertrophy (Benayoun, Druilhe et al. 2003) or hyperplasia (Woodruff, Dolganov et al. 2004). ASM cells contribute to AHR, which is the increase in airway narrowing in response to nonspecific stimuli or pharmacological agonist. The contractility of ASM cells from asthmatic patients is higher than controls and this results from increased expression of myosin light chain kinase (MLCK) (Ma, Cheng et al. 2002). Moreover, ASM cells contribute to the inflammatory circuit in asthma by releasing different chemokines such as eotaxin, RANTES, IL-8 and cytokines such as interleukin-1β (IL-1β) and GM-CSF (Hershenson, Brown et al. 2008). Of interest, pretreating ASM cells with IL-1β caused a decrease in relaxation in response to isoproterenol due to β-adrenoceptor and Gs-adenylyl cyclase uncoupling (Shore, Laporte et al. 1997). Thus, ASM cells released inflammatory mediators that eventually impair their response to bronchodilators.

Airway epithelial cells also contribute to asthma pathophysiology by preferentially initiating $T_H2$ response (Bulek, Swaidani et al. 2010), releasing different chemokines and cytokines (Barnes 2008) and increasing mucus production and secretion (Bai and Knight 2005). The involvement of airway epithelial cells in asthma will be discussed in more details in section 2.2.

#### 2.1.1.3 Cytokines and inflammatory mediators:

In asthma, there is an increase in $T_H2$ cytokines (IL-4, 5, 9 and 13) (Kips 2001) and each of these cytokines play a major role in orchestrating the inflammation. IL-5 mediates eosinophil differentiation from precursor cells and their survival (Barnes 2001) while IL-4, but not IL-13, is involved in the differentiation of $T_H2$ cells (Barnes 2008). Moreover, IL-4 promotes the production of IgE by B-cells which mediates mast cell degranulation upon allergen exposure (Gould and Sutton 2008). IL-13 alone is sufficient to induce AHR, mucus metaplasia and airway inflammation in mice (Grunig, Warnock et al. 1998; Kuperman, Huang et al. 2002; Wills-Karp 2004). Additionally, several other cytokines are released in the airways, including TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-17, IL-11 and IL-1β (Kips 2001; Hamid, Tulic et al. 2003; Atamas, Chapoval et al. 2013). Several chemokines are secreted from structural and inflammatory cells that contribute in recruiting inflammatory cells to the airways (Lukacs, Oliveira et al. 1999; Lukacs 2001).

#### 2.1.1.4 Airway remodeling in asthma:

In asthma, the airways undergo several changes (Chetta, Foresi et al. 1997; Homer and Elias 2000; Bergeron, Al-Ramli et al. 2009), summarized in Figure 2, such as thickening of the basement membrane (Shifren, Witt et al. 2012), mucous metaplasia (Aikawa, Shimura et al. 1992; Fahy 2001; Ordonez, Khashayar et al. 2001), increased airway smooth muscle mass (Lambert, Wiggs et al. 1993), increased airway vascularity and permeability (Li and Wilson 1997; Freyer, Johnson et al. 2001; Tanaka, Yamada et al. 2003), subepithelial fibrosis (Roche, Beasley et al. 1989; Minshall, Leung et al. 1997; Hoshino, Nakamura et al. 1998) and recruitment of inflammatory cells and the release of different cytokines, especially from $T_H2$ cells (Bhakta and Woodruff 2011). Collectively these changes lead to AHR and airway obstruction.

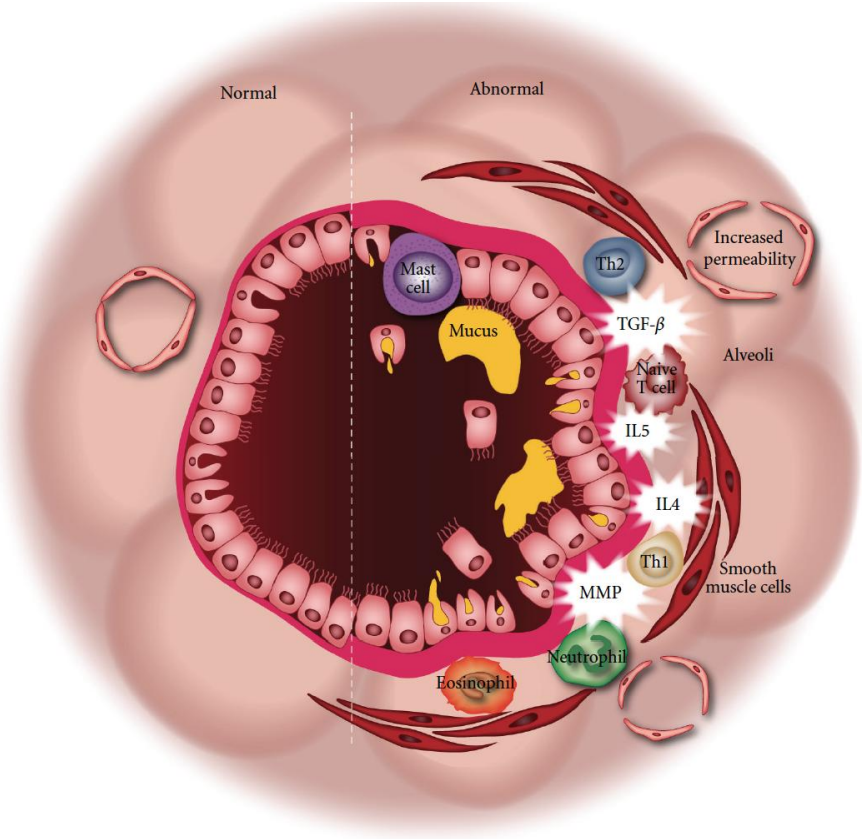



**Figure 2. Airway remodeling associated with asthma**: Several changes in all structural cells of the airways are induced by the released inflammatory mediators from inflammatory cells, as discussed in the text. Adapted from Shifren, Witt, et al (2012).

### 2.1.2 Therapeutic approaches:

Patients with asthma are diagnosed with intermittent or persistent mild, moderate and severe disease, based different parameters such as the frequency of the symptoms and the use of short acting bronchodilators as well as lung

function (National Asthma and Prevention 2007). The approach to the treatment of asthma is based on the severity of the disease. Short acting $\beta_2$-Adrenoceptors ($\beta_2$-ARs) agonists are the most effective bronchodilators that are used to control acute asthma attacks (Fanta 2009). Inhaled corticosteroids and long acting $\beta_2$-AR agonists are among the widely used medications for long term disease control (Fanta 2009). Inhaled corticosteroids (ICS) are effective anti-inflammatory medications that are used in asthma. However, ICSs, especially at high doses in severe persistent asthma, result in serious systemic side effects such as growth impairment in children (Dahl 2006) and reduction in bone density (Kelly and Nelson 2003). Chronic use of $\beta_2$-AR agonists is also associated with serious consequences such as increased risk of severe asthma exacerbations and possible death (Currie, Lee et al. 2006; Salpeter, Buckley et al. 2006). The increase in death associated with $\beta_2$-AR agonists was first observed with regular use of the newly introduced inhaled bronchodilator fenoterol in New Zealand (Crane, Pearce et al. 1989). This drug, when used chronically, results in disease exacerbation and worsening of lung function (Taylor, Sears et al. 1993). In another study, regular use of salbutamol was associated with disease deterioration and AHR exacerbation (van Schayck, Graafsma et al. 1990). Even though the acute bronchodilatory effect of $\beta_2$-AR agonists is lifesaving, their regular use increases the inhaled antigen load (Broadley 2006) and provokes more inflammation. This is reflected by the increased allergen induced bronchial

inflammation (Boulet, Chakir et al. 2001) and sputum eosinophils (Gauvreau, Jordana et al. 1997) in response to regular use of salmeterol and salbutamol respectively. However, a recent review reported that the use of salmeterol together with ICS did not result in serious adverse events, either fatal or non-fatal (Cates, Jaeschke et al. 2013). In another meta-analysis, salmeterol resulted in increased mortality only when it was used as monotherapy while this risk was reduced when ICS were added to the treatment regimen (Weatherall, Wijesinghe et al. 2010). However, it was not possible to conclude that formoterol in combination with ICS did not have any increase in mortality (Cates, Lasserson et al. 2009). The recent analysis by FDA demonstrated that the risk asthma related deaths of formoterol monotherapy was not reduced by adding ICS (Rodrigo 2010). Therefore, FDA recommends physician in practice not to use long acting $\beta_2$-AR agonists as monotherapy and it should be combined with ICS (Giembycz and Newton 2006).

The fear of long term side effects of ICS and $\beta_2$-AR agonists creates a subset of patients who have uncontrolled disease. Uncontrolled disease results in several negative consequences such as learning disabilities and depression, in children and adults (O'Byrne, Pedersen et al. 2013). Additionally, another subset of asthmatic patients, (~5% to 10%), are not responsive to conventional treatment approaches and exhibit severe disease manifestations (Busse, Banks-Schlegel et al. 2000; Papiris, Kotanidou et al. 2002). A better understanding of

the disease pathophysiology and the critical role of inflammatory mediators in disease progression has resulted in the development of a variety of promising therapeutic interventions such as leukotriene modifiers, mast cell stabilizers, monoclonal antibodies that target IL-13 and TNF-α (Holgate and Polosa 2008; Holgate and Davies 2009). These therapeutic approaches are expensive, effective only in a specific subset of asthmatic patients and their regular use is not risk-free. Thus, more research is required to improve the pharmacological approaches in asthma treatment.

The detrimental effects with long term use of $\beta_2$-AR agonists in asthma is similar to that previously observed in congestive heart failure (CHF), where chronic use of β-AR agonists was associated with increased mortality (Weber, Likoff et al. 1982; Felker and O'Connor 2001). β-AR blockers resulted acutely in serious detrimental effects and thus these agents had been contraindicated in patients with CHF for many decades (Haber, Simek et al. 1993). However, after successful clinical studies, chronic administration of certain β-AR blockers became the gold standard medical therapy for CHF (Hunt, Baker et al. 2001). A similar picture is also perceived with β-AR blockers in asthma. These agents are associated with airway narrowing when administered acutely (Singh, Whitlock et al. 1976; Boskabady and Snashall 2000). However, chronically treatment with the β-blocker nadolol, or genetically ablating $\beta_2$-ARs resulted in reductions in AHR, inflammatory cell recruitment to the airways and mucus metaplasia in an allergen

driven murine model of asthma (Nguyen, Omoluabi et al. 2008; Nguyen, Lin et al. 2009). The anti-inflammatory effect of nadolol not only resulted in additive effect when combined with glucocorticosteroid at the level of airway inflammation and mucus metaplasia, but also had "glucocorticoid-sparing" effect (Nguyen, Singh et al. 2012). Moreover, chronic nadolol administration to mild asthmatic patients reduced AHR (Hanania, Singh et al. 2008) and these patients still respond to salbutamol to reverse bronchoconstriction provoked by methacholine (Hanania, Mannava et al. 2010). Therefore, β-blockers are potentially a promising therapeutic approach to treat asthma and reduce the adverse effects that are associated with chronic use of ICS.

### 2.2 Airway epithelium:

Airway epithelium is first line of defense against foreign particles through providing functional and physical barriers, initiating immune responses and removing inhaled particles (Thompson, Robbins et al. 1995). The barrier function is created by the continuous layer of polarized epithelial cells that are connected by tight junctions (Vareille, Kieninger et al. 2011). Also, airway epithelial cells are covered by a layer of mucus that contains mucins and other proteins, such as antimicrobial proteins and cytokines (Nicholas, Skipp et al. 2006). Antimicrobial proteins include, but are not limited to, secretory immunoglobulin A, lysozyme, lactoferrin and defensins (Eisele and Anderson 2011; Li, Wang et al. 2012).

Inhaled particles that are trapped in the viscous mucus layer are cleared from the airways by the action of the cilia and cough reflex (Thompson, Robbins et al. 1995). In addition, airway epithelial cells secrete different cytokines and mediators that recruit inflammatory cells to the airway to clear the invading agents. These mediators include thymus and activation regulated chemokine (TARC), eotaxin-1 and interleukin-8 (IL-8) (Bloemen, Verstraelen et al. 2007) that play a role in recruiting T-cells (Panina-Bordignon, Papi et al. 2001), eosinophils (Ying, Robinson et al. 1997) and neutrophils (Nocker, Schoonbrood et al. 1996) respectively.

Airway epithelial cells play a major role in initiating the immune response and in connecting both innate and adaptive immunity. Upon allergen exposure, allergens are processed primarily by dendritic cells (DCs). Activated DCs migrate to mediastinal lymph node (MLNs) and present processed allergen to T cells (Lambrecht and Hammad 2009). These T cells become either central memory T (TCM) cells or effector T cells and then migrate to non-draining lymph nodes or to the lung respectively (Lambrecht and Hammad 2003). In ongoing inflammation, naïve T cells preferentially develop into T-helper-2 ($T_H2$) cells (Lambrecht and Hammad 2003). Airway epithelial cells play a role in skewing the inflammatory response toward $T_H2$ by producing different mediators that are called "$T_H2$-driving cytokines" that create a microenvironment that favors $T_H2$ response (Bulek, Swaidani et al. 2010) such as TSLP (thymic stromal

lymphopoietin) (Schleimer, Kato et al. 2007) that activates DCs to regulate the release of $T_H2$ cytokines from naïve T helper cells (Bulek, Swaidani et al. 2010), including interleukin-25 (IL-25) (Hammad and Lambrecht 2011) and interleukin-33 (IL-33) (Holgate 2013) that contribute to $T_H2$ cell polarization (Bulek, Swaidani et al. 2010). Moreover, these $T_H2$ driving cytokines trigger group 2 innate lymphoid cells (ILC2 cells) to secrete $T_H2$ cytokines (Licona-Limon, Kim et al. 2013).

The effect of epithelial cells is not restricted to DCs: epithelial cells though the release of different mediators also affect and recruit T and B cells. For example, TARC recruits $T_H2$ to the airways (Schleimer, Kato et al. 2007) while B cell-activating factor of TNF family (BAFF) and a proliferation-inducing ligand (APRIL) affect B cell activation and switching to IgE production (Kato, Truong-Tran et al. 2006).

### 2.2.1 Types of epithelial cells:

The epithelial layer is composed of different cell types that vary in their distribution along the respiratory tract, as shown in Figure 3. To simplify the description of different airway epithelial cells, the airway is divided into two zones; the conducting zone that only conducts air, and the respiratory zone where gas exchange takes place.

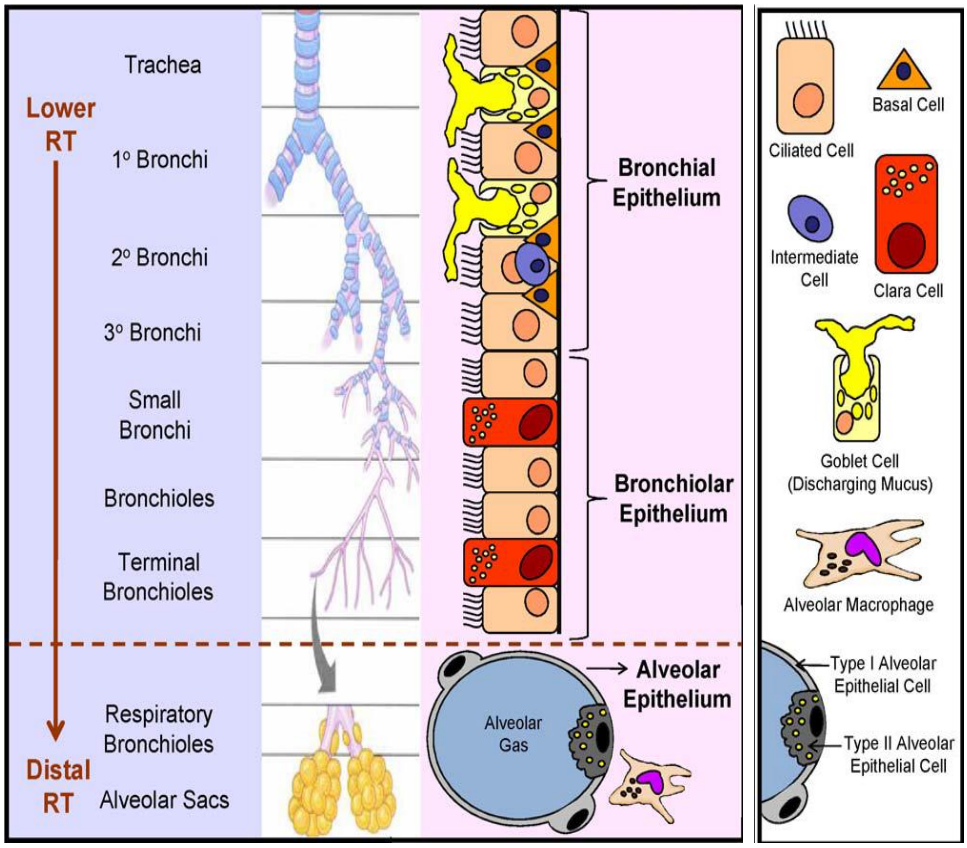



**Figure 3. The principal cell types comprising the airway epithelium in the human respiratory tract**: Lower respiratory tract (RT) represents the conducting zone and the distal RT represents the respiratory zone. The conducting zone is characterized by the presence of goblet, ciliated, basal and Clara cells (club cells). The respiratory zone is comprised of type I and II alveolar airway epithelial cells. Adapted from Berube, Prytherch et al. (2010).

The distribution of epithelial cell types along the conducting airways varies between species, as summarized in the table (Table 1) below.

| Species | Basal | Intermediate | Goblet | Serous | Clara | NCC | Ciliated | SMG |
|---|---|---|---|---|---|---|---|---|
| Mouse | T,B | – | –[a] | – | T,B,Br | – | T,B,Br | T |
| Rat | T,B | T,B | –[a] | T,B | Br | T,B | T,B,Br | T |
| Monkey | T,B | T,B | T,B | NA | NA | T,B | T,B,Br | T,B |
| Human | T,B | T,B | T,B | – | Br | T,B | T,B,Br | T,B |

**Table 1. Comparison of the distribution of different types of airway epithelial cells in rodents and primates:** NA: unknown or under investigation; T: trachea; B: bronchi; Br: bronchioles; NCC: nonciliated, nonsecretory columnar cells; SMG: submucosal glands. "a" For pathogen-free animals; in infection or cytokine stimulation the abundance of goblet cells is increased. Adapted from Liu, Driskell et al. (2006).

#### 2.2.1.1 Basal cells:

Basal cells are attached to the basement membrane through hemidesmosomes (Mary Mann-Jong Chang 2008). Basal cells connect different cells of the pseudostratified layer to the basement membrane, control the inflammatory response and regulate water trans-epithelial movement (Evans, Van Winkle et al. 2001; Knight and Holgate 2003; Mary Mann-Jong Chang 2008).

Moreover, basal cells are described as progenitor or stem cells in the airways because of their ability to self-renew and differentiate into columnar airway epithelial cells (Evans, Van Winkle et al. 2001; Hong, Reynolds et al. 2004; Liu, Driskell et al. 2006; Rock, Onaitis et al. 2009). Other evidence suggests that basal cells differentiate first to mucous cells and then to ciliated cells (Ayers and Jeffery 1988). Regardless which step occurs first, basal cells have the ability to differentiate to ciliated and mucous cells. Different markers are used to distinguish basal cells such as transcription factor transformation-related protein 63 (Trp-63) and cytokeratins 5 (Krt5) and Krt14 (Rock, Onaitis et al. 2009; Rock, Randell et al. 2010).

#### 2.2.1.2 Ciliated cells:

Ciliated cells are characterized by their columnar shape and the their apical cilia, numbering about 200–250 cilia/cell (Mary Mann-Jong Chang 2008). The cilia extends from the inner liquid mucus layer, that is composed mainly of water (Cohn 2006), into the outer viscous gel layer (Evans, Kim et al. 2009; Fahy and Dickey 2010). These cells are present throughout the conducting zone of the airways (Berube, Prytherch et al. 2010). The cilia of neighbor cells share a common orientation and their beating rate is highly coordinated (Thompson, Robbins et al. 1995). Ciliated cells can be characterized by the presence of protein such as tektin, hepatocyte nuclear factor homolog (HFH-4)/ forkhead box

protein J1 (FOXJ1) and β-tubulin IV (Blatt, Yan et al. 1999; Yoshisue, Puddicombe et al. 2004).

#### 2.2.1.3 Non-ciliated secretory cells:

The major secretory cells in human are goblet cells, while club cells are the predominant secretory cells in mice and rabbits (Liu, Driskell et al. 2006; Mary Mann-Jong Chang 2008).

##### 2.2.1.3.1 Goblet cells:

Goblet cells are characterized by the presence of mucus granules in their apical compartment (Rogers 1994). Mucin glycoproteins are the main component of these mucus granules. Mucin contributes to the viscoelastic properties of the mucus layer and traps inhaled particles that are subsequently removed by the action of cilia (Kim, McCracken et al. 1997; Rogers 2002; Fahy and Dickey 2010). Goblet cells are identified by immunohistochemical detection of mucin 5AC or MUC5AC expression, the main mucin-producing gene in goblet cells (Atherton, Jones et al. 2003; Yoshisue, Puddicombe et al. 2004).

Goblet cell hyperplasia and metaplasia are two different terms that are commonly used to describe hypersecretory diseases, such as asthma and chronic obstructive pulmonary disease (COPD). Briefly, goblet cell hyperplasia is defined by the increase in the goblet cell numbers in areas where goblet cells are

usually present (Rogers 2003; Mary Mann-Jong Chang 2008). On the other hand, metaplasia describes the appearance of these goblet cells in areas where these cells are not commonly present (Rogers 2003; Mary Mann-Jong Chang 2008). Goblet cells may develop from ciliated cells by the process of transdifferentiation (Tyner, Kim et al. 2006; Turner, Roger et al. 2011).

#### 2.2.1.3.2 Club cells:

Club cells (known formally as Clara cells) are the main non-ciliated secretory cells throughout mouse airways but in humans are only found in bronchioles (Table 1). These cells play an important role in xenobiotic metabolism (Knight and Holgate 2003; Reynolds and Malkinson 2010), secretion of antiproteases (De Water, Willems et al. 1986) and of Clara cell protein (CC10), also called Clara cell secretory protein (CCSP) (Singh and Katyal 1997), which has anti-inflammatory and anti-oxidant roles (Broeckaert, Clippe et al. 2000). This important role of club cells is noted in asthmatic patients, where the reduction in CC10 levels is associated with increased mast and T cells numbers in small airways (Shijubo, Itoh et al. 1999). Moreover, analysis of protein content in bronchoalveolar lavage fluid (BALF) from asthmatic patients revealed low levels of CC10 content compared to normal controls (Van Vyve, Chanez et al. 1995).

Club cells also have the capacity to serve as precursor for ciliated and mucus secreting cells (Knight and Holgate 2003; Reynolds and Malkinson 2010).

Several studies have shown that club cells are the origin of goblet cells in murine model of asthma (Reader, Tepper et al. 2003; Evans, Williams et al. 2004; Hayashi, Ishii et al. 2004; Chen, Korfhagen et al. 2009). It has been suggested that “metaplasia” term should be used carefully, because club cells are not replaced by goblet cells and mucin granules are exclusively present in CCSP-positive cells in allergen induce murine model of asthma (Evans, Williams et al. 2004).

#### 2.2.1.4 Alveolar epithelium:

The two types of alveolar epithelial cells, type I and type II, are distinguished by their different physiological roles. The majority of the surface area of the lung alveoli is covered by type I cells, while type II cells cover only 5% of the lung surface area (Thompson, Robbins et al. 1995; Mason 2006). Type I cells form an essential component in gas-exchange barrier (Mary Mann-Jong Chang 2008) and enhance innate immunity during infection (Yamamoto, Ferrari et al. 2012). Type II cells play a major role in the production of surfactant (Fehrenbach 2001), synthesis and secretion of anti-inflammatory and anti-microbial agents (Mason 2006) and are the progenitor cells (stem cells) for type I cells (Mason 2006). Moreover, type II cells have the capacity to proliferate and differentiate when type I cells are exposed to injury and death (Liu, Driskell et al. 2006).

### 2.2.2 Changes of epithelial cells in asthma:

Besides all the changes that has been described earlier, transcription factors that regulate inflammatory genes are upregulated in asthmatic airway epithelial cells (Knight and Holgate 2003). Damaged epithelial cells tend to desquamate and appear in the sputum and BALF (Naylor 1962; Wardlaw, Dunnette et al. 1988) as well as in airways biopsies from asthmatic patients (Laitinen, Heino et al. 1985). Epithelial cell shedding also causes the decline in epithelial derived relaxing factors and hence could contribute the increase in airway hyper-reactivity (Vanhoutte 1989). However, the epithelial cell desquamation observed in asthmatic biopsies could be an artifact caused by endobronchial sampling processes (Ordonez, Ferrando et al. 2000). Furthermore, the barrier integrity of the epithelial layer is also altered in asthma (Lambrecht and Hammad 2012).

## 2.3 Airway Mucus:

The apical surface of the respiratory tract is covered by mucus. The mucus layer protects the respiratory tract from inhaled particles and pathogens (Rogers 2002). Mucus is mainly composed of water and a mixture of numerous substances such as lipid, proteins and the highly glycosylated mucin particles (Rogers 2002; Fahy and Dickey 2010). Mucin is a heavily glycosylated, high molecular weight protein and is responsible for the viscoelastic properties of

mucus (Kim, McCracken et al. 1997; Rogers 2002; Fahy and Dickey 2010). Structurally, as outlined in Figure 4, mucin is composed of a peptide backbone, called apomucin (Rogers 2002), which is encoded by MUC genes (Rose and Voynow 2006). Mucins are glycosylated on serine/threonine residues of the tandem repeats (TR) domains (Kim, McCracken et al. 1997; Rogers 2002; Rose and Voynow 2006; Lai and Rogers 2010). Mucins are negatively charged proteins because of their terminal carboxyl or sulfate groups (Fahy and Dickey 2010).

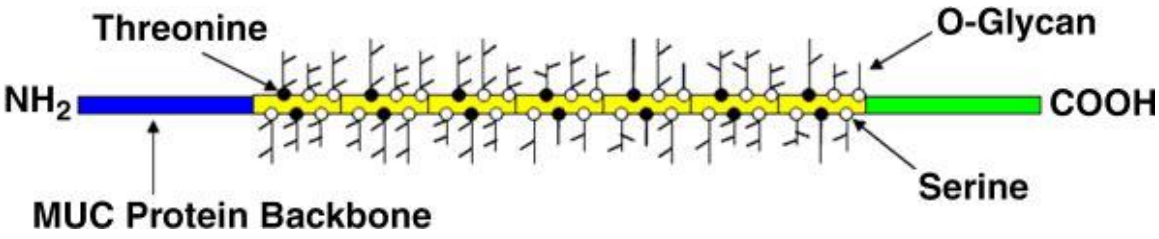



**Figure 4**. **Structure of mucin**: Mucin is composed of a protein backbone that is highly glycosylated as described in the text. Adapted from Rose and Voynow (2006).

### 2.3.1 Airway Mucin genes:

Seventeen MUC genes are present in the human genome (Thornton, Rousseau et al. 2008), and based on a backbone that is encoded by these genes, mucins are categorized into:

#### 2.3.1.1 Secreted gel forming mucins:

MUC5AC, MUC5B, MUC2, MUC6 and MUC19 (Fahy 2002; Rose and Voynow 2006; Thornton, Rousseau et al. 2008; Evans and Koo 2009; Voynow and Rubin 2009) are examples of this category. These secreted polymerized mucins contribute to mucus viscosity and form a gel layer that coats the airway epithelium (Evans, Kim et al. 2009). Secreted gel forming mucins are characterized by their high molecular weight and water absorbent properties (Rogers 2002; Evans and Koo 2009). MUC5AC and MUC5B are the main mucins present in mucus and sputum (Thornton, Rousseau et al. 2008). MUC5AC is produced by goblet cells located on the surface epithelium while submucosal glands secrete MUC5B (Thornton, Rousseau et al. 2008; Evans, Kim et al. 2009). Under normal conditions, MUC5AC is constitutively expressed in proximal human airways while muc5ac is not detectable in mice airways (Fahy and Dickey 2010). MUC5AC is the most abundant secreted mucin in asthma and muc5ac is upregulated by antigen challenge in murine model (Evans and Koo 2009). MUC5B and muc5b are both constitutively expressed in the airways

(Evans, Kim et al. 2009) and the sputum level of MUC5B is increased in patients with asthma as compared to normal individuals (Kirkham, Sheehan et al. 2002). Endobronchial biopsy from patients with asthma revealed that MUC2 and 4 are also significantly elevated as compared to normal individuals (Fahy 2002).

#### 2.3.1.2 Secreted nonpolymerizing mucins:

MUC7 (Rose and Voynow 2006; Thornton, Rousseau et al. 2008; Voynow and Rubin 2009), MUC8 (Rose and Voynow 2006; Thornton, Rousseau et al. 2008) and MUC9 (Rose and Voynow 2006) are examples of this family of mucins. Due to the absence of cysteine rich domains in their tandem repeats, these secreted mucins tend not to polymerize (Rose and Voynow 2006; Thornton, Rousseau et al. 2008).

The secreted mucins are packed into the intracellular granules by virtue of high calcium concentrations (Fahy 2002). Following stimulation, the release of calcium from the granules upon membrane fusion causes the entrance of extracellular water. Consequently, the condensed mucins are no longer packed and the large hydrated repulsive macromolecules explode from the cell (Fahy 2002).

#### 2.3.1.3 Membrane associated mucins:

These mucins include MUC1, MUC4, MUC11, MUC13, MUC15, MUC12, MUC3A & B, MUC16, MUC17 and MUC20 (Rose and Voynow 2006; Voynow and Rubin 2009). Of these, MUC1, 4 and 16 are known to be expressed constitutively in the lung (Evans, Kim et al. 2009). These mucins are present at the plasma membrane of the surface epithelium (Evans, Kim et al. 2009) and they determine the physical characteristics of the inner liquid layer that covers airway epithelium (Fahy and Dickey 2010). Furthermore, membrane associated mucins participate in cell signaling either through their cytoplasmic domain or via interaction with other surface receptors (Evans and Koo 2009).

### 2.3.2 Effect of mucus overproduction:

Decades ago, physicians underestimated the critical role of mucus plugs in asthma due to the absence of phlegm in asthmatic cough (Fahy and Dickey 2010). Autopsies of patients who died of severe asthma attacks revealed goblet cell hyperplasia and mucus accumulation in peripheral airways when compared to asthmatic patients who did not die of acute attacks (Aikawa, Shimura et al. 1992; Fahy 2002). Mucus plugs from patients who died of asthma are rich in mucin content in contrast to mucus from normal individual (Sheehan, Richardson et al. 1995). Moreover, there is an increase in the goblet cell number and intracellular mucin in patients with mild-moderate asthma (Fahy 2002). Mucus

composition varies between health and disease states. In healthy individuals, mucus contains mucin glycoproteins and small amount of inflammatory cells and proteins with no RNA, actin or bacteria (Fahy and Dickey 2010). In asthmatic individuals, mucus viscosity is increased due to elevated levels of mucin, proteins and inflammatory cells combined with DNA and actin (Fahy and Dickey 2010; Lai and Rogers 2010). Different cytokines that are released in asthma induce the expression of MUC genes, goblet hyperplasia and mucus secretion (Rogers 2004).

The accumulation of mucus leads to:

**A)** Airway obstruction: Excessive mucus plugs enhances dyspnea as a result of ventilation-perfusion mismatch (Cohn 2006).

**B)** Airflow resistance: The reduction in the airway's radius, due to bronchonstriction, combined with mucus accumulation in the airway results in dramatic increase in airway resistance, as summarized in Figure 5 below (Lai and Rogers 2010).

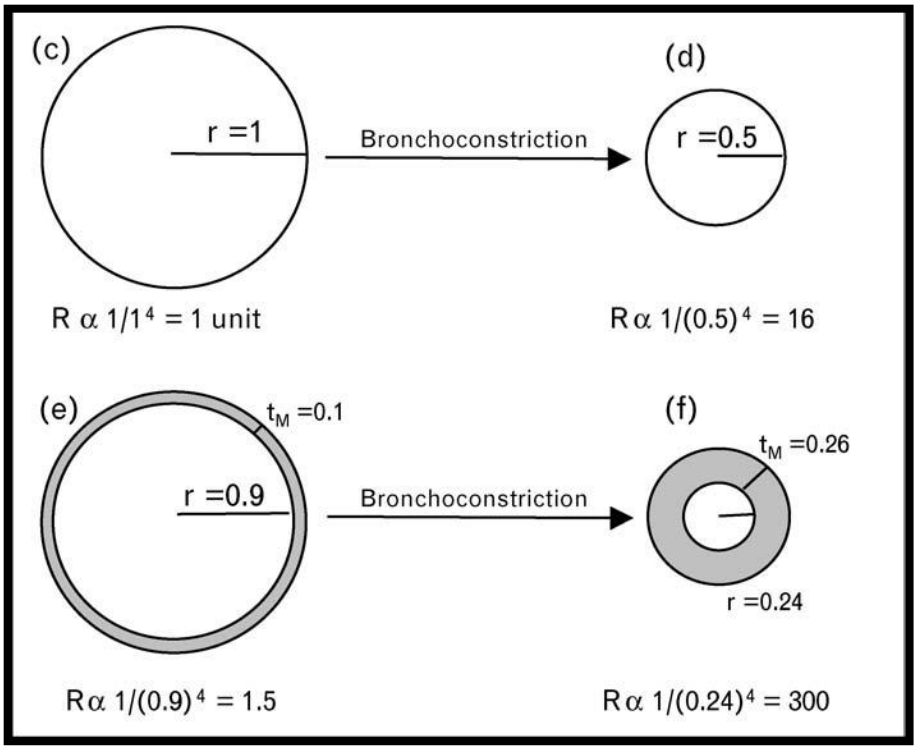



**Figure 5. The effect of airway mucus accumulation on airway resistance:** Based on Poiseuille's law, resistance can be calculated as $1/r^4$, where r is the airway radius. If r is set to one, then the resistance (R) will be 1 unit (**c**). When the radius is reduced by 50% because of bronchoconstriction, then there is 16-fold increase in the airway resistance (**d**). Accumulation of mucus in the airway causes a small increase in airway resistance (**e**), but when bronchoconstriction takes place on the top of mucus layer, a dramatic increase in the resistance results (**f**). Adapted from Lai and Rogers (2010).

### 2.3.3 MUC5AC and asthma:

Endobronchial biopsy from asthmatic patients reveals 60% higher levels of MUC5AC expression as compared to unaffected individuals (Fahy 2002). In healthy and asthmatic individuals, the MUC5AC gene product is restricted to airway goblet cells (Rose and Voynow 2006). MUC5B is expressed in submucosal glands in healthy individuals and is secreted from goblet and glandular neck cells in asthma patients (Kirkham, Sheehan et al. 2002). The sputum levels of both MUC5AC and MUC5B are increased in patients with asthma as compared to normal individuals (Kirkham, Sheehan et al. 2002).

The MUC5AC and muc5ac genes are highly regulated at the level of transcription. MUC5AC transcription is induced in response to different cytokines as demonstrated by increased MUC5AC promoter activity following the transfection of cells with a MUC5AC promoter-luciferase construct (Rose and Voynow 2006; Fujisawa, Velichko et al. 2009). Examples of these mediators are TNF-α (Lai and Rogers 2010), EGF (Zhen, Park et al. 2007), IL-1β (Gray, Nettesheim et al. 2004) and most importantly IL-13, which is discussed further in the following section. Moreover, other inflammatory mediators such as IL-8 (Bautista, Chen et al. 2009) and TNF-α (Borchers, Carty et al. 1999) regulate MUC5AC expression at posttranscriptional level through increasing mRNA stability (Rose and Voynow 2006).

## 2.4 Interleukin-13 (IL-13):

IL-13 is a major $T_H2$ cytokine that is released in response to allergen from $CD4^+$ and $CD8^+$ T cells, non-T cells such as natural killers and eosinophils (Schmid-Grendelmeier, Altznauer et al. 2002; Akbari, Stock et al. 2003) and ILC2 cells in response to $T_H2$-driving cytokines released from airway epithelial cells (Licona-Limon, Kim et al. 2013). There is substantial experimental evidence that IL-13 is involved in asthma pathogenesis. In the allergen driven murine model of asthma, elevated levels of IL-13 are detected in BALF (Taube, Duez et al. 2002) and neutralizing IL-13 antibodies attenuates the asthma phenotype (Grunig, Warnock et al. 1998). Asthmatic patients have higher levels of IL-13 in plasma, sputum, broncheoalveolar lavage fluid and bronchial biopsy as compared to normal individuals (Naseer, Minshall et al. 1997; Wong, Ho et al. 2001; Brightling, Symon et al. 2003; Berry, Parker et al. 2004; Park, Jangm et al. 2005). Moreover, IL-13 levels are raised in patients with severe refractory disease (Saha, Berry et al. 2008) and during acute exacerbations (Lee, Lee et al. 2001). Higher IL-13 levels in sputum are associated with lower concentration of the constrictor agent that causes a 20% fall in forced expiratory volume in 1 second (FEV1) (Park, Jangm et al. 2005). Additionally, IL-13 may be responsible for the elevated levels of produced IgE in asthma patients (Van der Pouw Kraan, Van der Zee et al. 1998).

### 2.4.1 IL-13 signaling pathways:

IL-13 binds to IL-13-receptor alpha 1 (IL-13Rα1) at low affinity and this binding recruits IL-4-receptor alpha (IL-4Rα) to form a high affinity complex (type II IL-4Rα/IL-13Rα1 receptor) (Wills-Karp 2004; Kelly-Welch, Hanson et al. 2005; Izuhara, Arima et al. 2006). Each of these receptor chains is composed of extracellular, transmembrane and intracellular domains. The intracellular domain has several regions that participate in the phosphorylation and activation of different signaling molecules that mediate the effect of IL-13 (Izuhara, Arima et al. 2006) as shown in Figure 6 below.

IL-13 also binds to IL-13-receptor alpha 2 (IL-13Rα2) chain, which is known as a decoy receptor, at a high affinity (Izuhara, Arima et al. 2006). IL-13Rα2 has a short cytoplasmic domain and it blocks signaling induced by IL-13 (Izuhara, Umeshita-Suyama et al. 2000; Andrews, Nordgren et al. 2009). However, membrane bound IL-13Rα2 may contribute to cell signaling. Binding of IL-13 to IL-13Rα2 in macrophages enhances the release of transforming growth factor-beta (TGF-β), (Fichtner-Feigl, Strober et al. 2006), which causes lung fibrosis. Moreover, the presence of soluble IL-13Rα2 in interstitial spaces sequesters IL-13 and diminishes IL-13 availability (Andrews, Nordgren et al. 2009).

IL-13Rα2, IL-4Rα (Lordan, Bucchieri et al. 2002) and IL-13Rα1 (Wills-Karp 2004) are expressed on the surface of airway epithelial cells. Moreover, the IL-13

receptor complex is also expressed on airway smooth muscle (ASM) cells (Izuhara, Umeshita-Suyama et al. 2000).

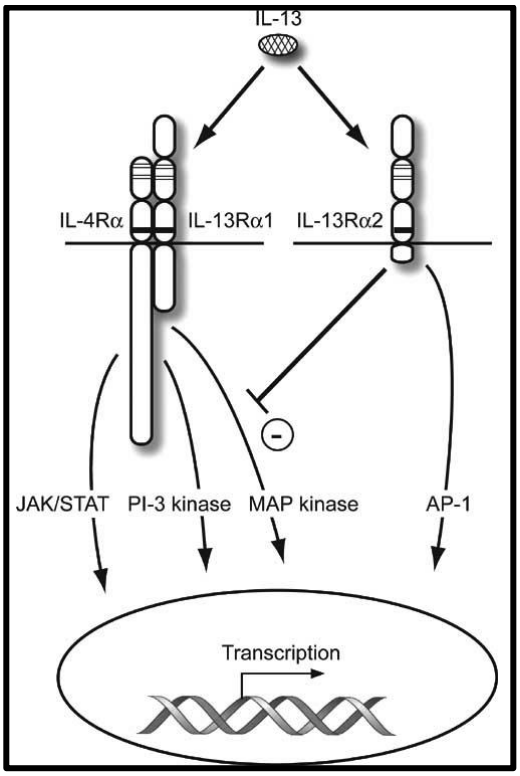



**Figure 6. Schematic presentation of IL-13 receptor chains and the activation of downstream signaling cascade**: IL-13 modulates gene transcription through activation of several downstream signaling cascades, such as JAK/STAT, PI3 kinase and MAPK pathways. However, binding of IL-13 to IL-13Rα2 activates AP-1 family of transcription factors and inhibits the aforementioned signaling cascades. Adapted from Izuhara, Arima et al. (2006).

The binding of IL-13 to IL-4Rα/IL-13Rα1 activates Janus tyrosine Kinase (JAK) / signal transducer and activator of transcription-6 (STAT6) signaling cascade (Izuhara, Umeshita-Suyama et al. 2000; Wills-Karp 2004). Once STAT6 is phosphorylated, it translocates to the nucleus as a dimer and binds to specific binding sites on target gene promoters to stimulate transcription (Wills-Karp 2004; Goenka and Kaplan 2011). Many IL-13 effects are mediated by the STAT6 signaling cascade, such as $T_H2$ cell differentiation and IgE synthesis (Pernis and Rothman 2002). In the antigen driven murine model of asthma, STAT6 Knockout (KO) mice failed to develop AHR, mucus metaplasia and $T_H2$ response (Tomkinson, Kanehiro et al. 1999). Signal transducer and activator of transcription-3 (STAT3) is also activated by IL-13 IL-13Rα1 chain (de Vries 1998) but its biological contribution is yet to be determined.

IL-13 binding to type II receptor complex phosphorylates tyrosine residues of the IL-4Rα chain, which in turn activates and phosphorylates insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) (Jiang, Harris et al. 2000; Hershey 2003). Phosphorylated IRS1/2 activates mitogen activated protein kinases (MAPKs) through growth factor receptor-bound protein-2 (Grb-2)/ son of sevenless (SOS)/ Ras pathway (Jiang, Harris et al. 2000; Hershey 2003). Moreover, activated IRS1/2 activates phosphoinositol-3 (PI3) kinase signaling pathway (Jiang, Harris et al. 2000; Hershey 2003). Activated PI3 kinase signals through Akt (survival

signaling) (Downward 1998) or p70 ribosomal protein S6 kinase (p70S6K) (Alessi, Kozlowski et al. 1998).

### 2.4.2 Role of IL-13 in asthma pathogenesis:

IL-13 transgenic mice that overexpress IL-13 in the airways develop airway inflammation, AHR and airway remodeling (Zhu, Homer et al. 1999). Interestingly, IL-13 KO mice are incapable of developing AHR in response to allergen despite the presence of eosinophilic inflammation and other $T_H2$ cytokines (Walter, McIntire et al. 2001). The development of AHR by IL-13 treatment appears at earlier time point as compared to the development of inflammation (Venkayya, Lam et al. 2002) and is independent of eosinophils (Yang, Hogan et al. 2001). Thus, the effect of IL-13 on AHR appears to be mediated through airway resident cells. IL-13 affects different cells that are involved in asthma pathogenesis, as summarized in Figure 7. In cultured human ASM, IL-13 increases contractility and reduces β-AR responsiveness (Laporte, Moore et al. 2001; Tliba, Deshpande et al. 2003). IL-13 transgenic mice that express STAT6 in airway epithelial cells develop AHR and mucus metaplasia, independent of eosinophilic inflammation (Kuperman, Huang et al. 2002). Additionally, IL-13 reduces the release of nitric oxide (NO), through inhibiting inducible nitric oxide synthase and inducing arginase I (Wills-Karp 2004).

IL-13 induces airway remodeling by different mechanisms. It stimulates subepithelial fibrosis by upregulating arginase I synthesis, which contributes to collagen synthesis by fibroblasts (Hesse, Modolell et al. 2001), and enhances the release of pro-fibrotic TGF-β from epithelial cells (Wen, Kohyama et al. 2002) and monocytes/ macrophages (Lee, Homer et al. 2001). Moreover, IL-13 promotes mucus metaplasia (Atherton, Jones et al. 2003; Fujisawa, Ide et al. 2008).

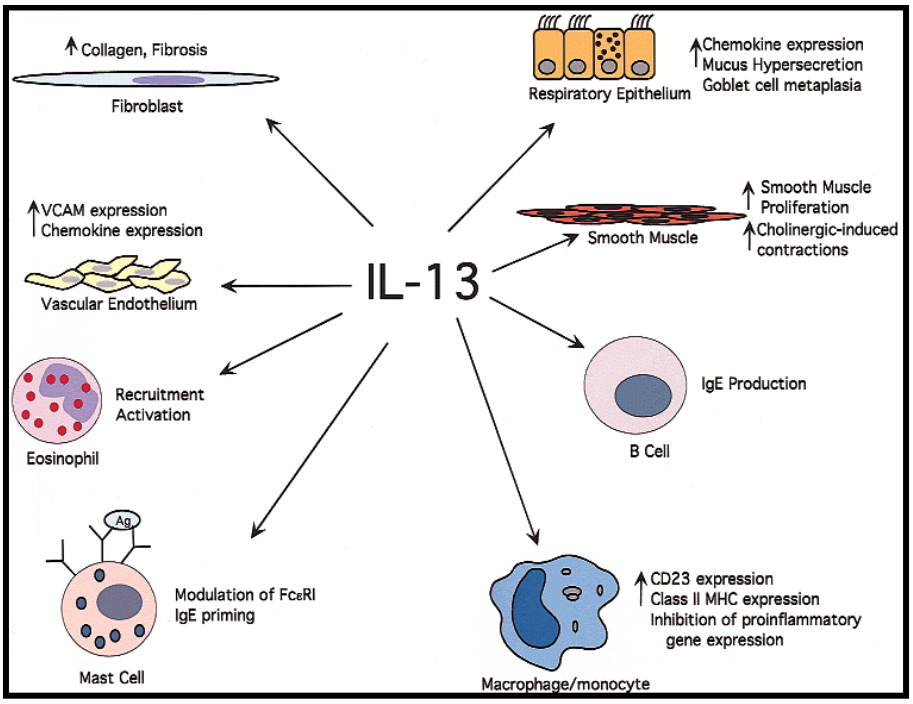



**Figure 7: Schematic presentation of IL-13 effects on different airway cells**. IL-13 affects lung parenchymal cells as well as the inflammatory cells that are involved in asthma pathogenesis as described in the text. Adapted from Hershey (2003).

### 2.4.3 Effects of IL-13 on airway epithelium:

IL-13 induces the release of several chemokines from airway epithelial cells, including macrophage inflammatory protein 3 alpha (MIP-3α) (Reibman, Hsu et al. 2003), IL-8 and RANTES (Fujisawa, Kato et al. 2000), MCP-1 (Lee, Homer et al. 2001; Oeckinghaus and Ghosh 2009), eotaxins 1,2 and 3 (Matsukura, Stellato et al. 2001; Komiya, Nagase et al. 2003) and growth-related oncogene-α (GRO-α) (Meyer-Hoffert, Lezcano-Meza et al. 2003). These factors contribute to airway inflammation through recruiting different inflammatory cells to the airways.

IL-13 drives MUC5AC expression and mucin content in cultured differentiated normal human bronchial epithelial (NHBE) cells (Laoukili, Perret et al. 2001; Atherton, Jones et al. 2003; Zhen, Park et al. 2007; Kono, Nishiuma et al. 2010; Tanabe, Kanoh et al. 2011) through different mechanisms that eventually activates transcription factors to translocate to the nucleus and binds to MUC5AC promoter region. A detailed study of human MUC5AC promoter region revealed the presence of different binding sites for transcription factors as outlined in Figure 8.

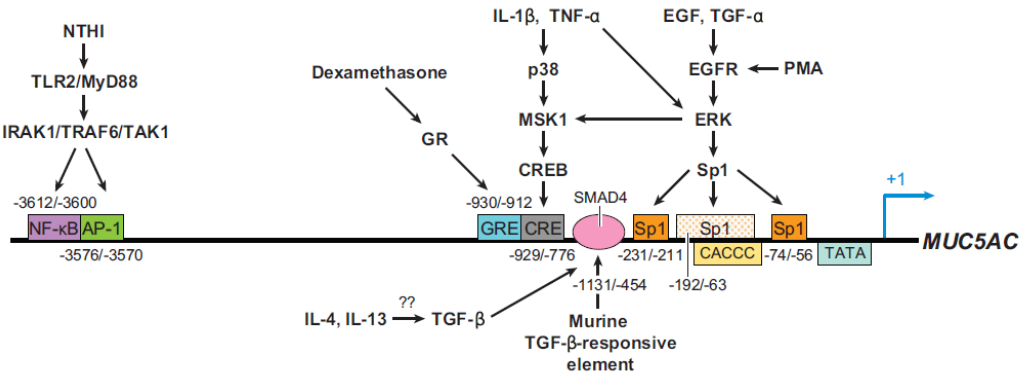



**Figure 8. 5' flanking region of MUC5AC:** Activation of different signaling pathways causes activation or translocation of transcription factors that bind to specific binding motifs in human MUC5AC promoter. NF-κB, activator protein 1 (AP-1), glucocorticoid response elements (GRE), cAMP-response element (CRE), Smad4, and Specificity Protein 1 (SP1) are examples of the binding sites of transcription factors. Adapted from Thai, Loukoianov et al. (2008).

IL-13 induces the release of TGF-β2 from airway epithelial cells which in turn increases MUC5AC expression through SMAD4 dependent mechanisms (Chu, Balzar et al. 2004). Moreover, IL-13 stimulates the release of heparin-binding EGF-like growth factor (HB-EGF) from airway epithelial cells, which activates epidermal growth factor receptor (EGFR) (Allahverdian, Harada et al. 2008). EGFR signaling is involved in IL-13 induced mucin production (Shim, Dabbagh et al. 2001; Kim, Shim et al. 2002). This effect is attributed to the reduction in forkhead box protein A2 (FOXA2) expression, which is a negative

regulator of MUC5AC expression (Zhen, Park et al. 2007). Moreover, EGFR signaling enhances ciliated cell survival, through PI3 kinase, and IL-13 induces the transdifferentiation of ciliated cells to goblet cells (Tyner, Kim et al. 2006). IL-13 also reduces the expression and the apical translocation of ezrin, a protein that is involved in basal body attachment to the apical surface of ciliated cells (Laoukili, Perret et al. 2001). IL-13 upregulates 15-Lipoxygenase-1 (15-LO-1) in human airway epithelial cells leading to increased MUC5AC expression (Zhao, Maskrey et al. 2009) via the Raf-1/MEK/ERK signaling cascade (Zhao, O'Donnell et al. 2011). Moreover, elements of IL-13 signaling pathways, specifically STAT6 and IL-4R, play a major role in the development of mucus production in murine model of asthma (Gavett, O'Hearn et al. 1997; Kuperman, Schofield et al. 1998).

IL-13 also provokes the secretory phenotype of airway epithelial cells through increasing the apical calcium activated anion conductance (Danahay, Atherton et al. 2002). This effect is due to increased expression of calcium-activated chloride channel, mCLCA3 (in mice) and hCLCA1 (in humans) (Zhou, Dong et al. 2001). The expression level of hCLCA1 is upregulated in airway epithelial cells from asthmatic patients (Hoshino, Morita et al. 2002). Additionally, introducing mCLCA3 / hCLCA1 into airway epithelial cells increases mucus production and MUC5AC expression (Nakanishi, Morita et al. 2001), pointing toward the important role of hCLCA1 in MUC5AC expression. It is important to note that IL-13 regulates hCLCA1 and MUC5AC expression at different time

points. After 24 hours of IL-13 treatment, hCLCA1, but not MUC5AC, expression level is increased through JAK/STAT6 dependent pathway (Thai, Chen et al. 2005). It is thus possible that MUC5AC expression is regulated by STAT6 independent pathways (Thai, Chen et al. 2005). In support of this notion, muc5ac (and MUC5AC) 5' flanking region lacks STAT6 consensus motif, as outlined in Figure 5, so STAT6 may indirectly contribute to MUC5AC expression through activation of other transcription factors such as hypoxia-inducible factor-1 (HIF-1) and SMAD4 (Young, Williams et al. 2007; Thai, Loukoianov et al. 2008).

IL-13 also initiates airway epithelial cell proliferation through the release of transforming growth factor-alpha (TGF-α) and activation of EGFR signaling cascade (Booth, Adler et al. 2001).

## 2.5 β-Adrenoceptor in airway cells:

### 2.5.1 $\beta_2$-AR signaling pathway:

$\beta_2$-ARs belong to the G protein-coupled superfamily of receptors (GPCRs), which are characterized by their seven transmembrane domains. These receptors transduce their effect through heterotrimeric G protein that consists of α, β, and γ subunits (Gilman 1987). There are at least 23 Gα subunits grouped into four main classes ($G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12}$), 5 types of Gβ and 11 types of Gγ (Hermans 2003). When the receptor is not stimulated by a ligand, the guanosine diphosphate (GDP) bound-α subunit is associated with βγ

subunits and the heterotrimer is coupled to the receptor. Receptor activation by agonist binding stimulates the release of GDP, and guanosine triphosphate (GTP) subsequently binds the α subunit, which dissociates from βγ subunits. Each of the dissociated subunits (α and βγ) activate downstream signaling cascades. The GTPase activity of α subunit eventually hydrolyses the terminal phosphate of GTP and causes termination of the signaling cascade, re-association of α and βγ, and recoupling of the heterotrimer to receptors (Neer 1995).

The Gαs subunit released upon $\beta_2$-AR activation activates adenylyl cyclase (AC), which increases intracellular cyclic adenosine monophosphate (cAMP), consequently activating cAMP dependent protein kinase A (PKA) and Exchange Protein directly Activated by cAMP (Epac) (Pierce, Premont et al. 2002). In mammalian cells, there are at least 9 membrane bound isoforms of AC and one soluble AC (Hanoune and Defer 2001; Pierre, Eschenhagen et al. 2009). In the lung, AC2, 6, 8 and 9 are highly expressed while AC3, 4, 5 and 7 are expressed at a low level and AC1 is not detected (Pierre, Eschenhagen et al. 2009). GTP-bound Gαs activates the enzymatic catalytic activity of AC (Pierre, Eschenhagen et al. 2009) while Gαi inhibits the catalytic activity (Pierre, Eschenhagen et al. 2009). The intracellular level of cAMP is regulated at the synthesis level, through AC, and degradation level, through phosphodiesterases

(PDEs), which hydrolyze the 3`cyclic phosphate bond in cAMP (Bender and Beavo 2006).

PKA, a downstream target of cAMP, is composed of two regulatory subunits bound to two catalytic subunits (Taylor, Kim et al. 2008; Pidoux and Tasken 2010). Binding of four cAMP molecules to the regulatory subunits induces the dissociation of the regulatory subunits from the catalytic subunits (Builder, Beavo et al. 1980). This dissociation activates the catalytic subunits that phosphorylate serine threonine residues in numerous cellular substrates. Figure 9 summarizes cAMP/PKA-signaling pathways downstream of activated GPCR.

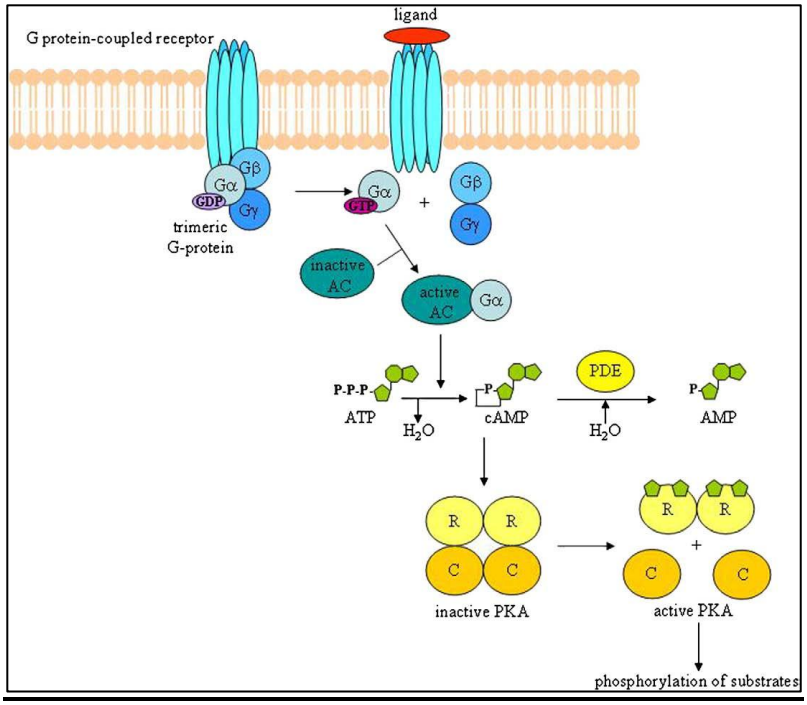



**Figure 9: Schematic diagram of the cAMP/PKA signaling pathway**. Activation of the G-protein coupled receptors increase intracellular cAMP levels through activating adenylyl cyclase. cAMP binds to and activates PKA which in turn phosphorylates several downstream substrates. However, intracellular cAMP is degraded by phosphodiesterases (PDEs). Adapted from Gerits, Kostenko et al. (2008)**.**

Additionally, PKA phosphorylates the third intracellular loop of $\beta_2$-AR and causes a partial uncoupling of the receptor from Gs (desensitization) (Benovic, Bouvier et al. 1988) and switches the receptor to a form that couples to Gi (Daaka, Luttrell et al. 1997; Zamah, Delahunty et al. 2002)**.** Released βγ subunits

bind to G protein-coupled receptor kinase (GRK) 2/3 and induces its translocation to the plasma membrane (Penela, Ribas et al. 2003). GRKs then phosphorylate agonist activated $\beta_2$-AR at serine residues different from those phosphorylated by PKA (Ferguson 2001). Activated and phosphorylated receptors bind to β-arrestin-2, which fully uncouples receptors and attenuates their signaling through a steric effect (Noor, Patel et al. 2011) or by increasing cAMP degradation via recruiting PDEs to the activated $\beta_2$-AR (Perry, Baillie et al. 2002). Moreover, β-arrestins function as an adaptor protein that bring activated receptor into clathrin-coated pits and thus facilitates internalization (Sibley, Strasser et al. 1986; Krupnick, Goodman et al. 1997; Pierce and Lefkowitz 2001), β-arrestin-2 binds transiently and weakly to the receptor and rapidly dissociates after targeting the receptors to clathrin-coated pits (DeWire, Ahn et al. 2007). Receptor internalization completes desensitization, and enables receptors to be dephosphorylated by intracellular phosphatases before returning to the cell surface (Ferguson 2001). The roles of β-arrestins are summarized in Figure 10 below.

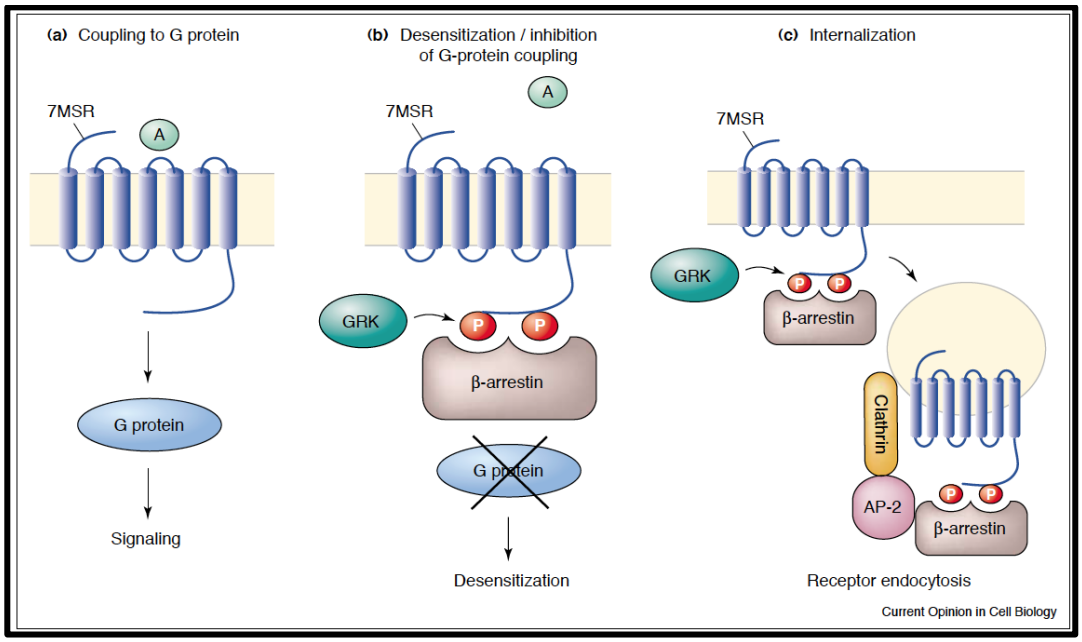



**Figure 10: The classical roles of β-arrestins in receptor desensitization and internalization.** Binding of a ligand to the receptor initiates signaling waves through G-protein (a). However, β-arrestin recruitment to phosphorylated ligand-occupied receptor inhibits the G-protein coupling and causes desensitization (b). Moreover, β-arrestin also causes receptor internalization through targeting the receptors to clathrin-coated pits (c). Adapted from Lefkowitz and Whalen (2004).

Beside the aforementioned classical functions of β-arrestins, these molecules initiate a second wave of signaling pathways through their scaffold properties, as demonstrated in Figure 11. β-arrestins activate ERK1/2, JNK3 (Lefkowitz and Shenoy 2005), p38 and Akt (DeWire, Ahn et al. 2007). Activated

ERK1/2 by G-protein dependent pathways is translocated to the nucleus while it is retained in the cytosol if it is activated through β-arrestins (Defea 2008). The activation of ERK1/2 by Gs is rapid whereas β-arrestin mediated activation is slow and sustained (Shenoy, Drake et al. 2006). ERK1/2 is activated by β-arrestins through recruiting Src, a non-receptor tyrosine kinase, to the active $\beta_2$-AR (Luttrell, Ferguson et al. 1999). Besides activating ERK1/2, Src transactivates EGFR through the release of HB-EGF by matrix metalloproteinase (MMP) dependent pathways (Prenzel, Zwick et al. 1999).

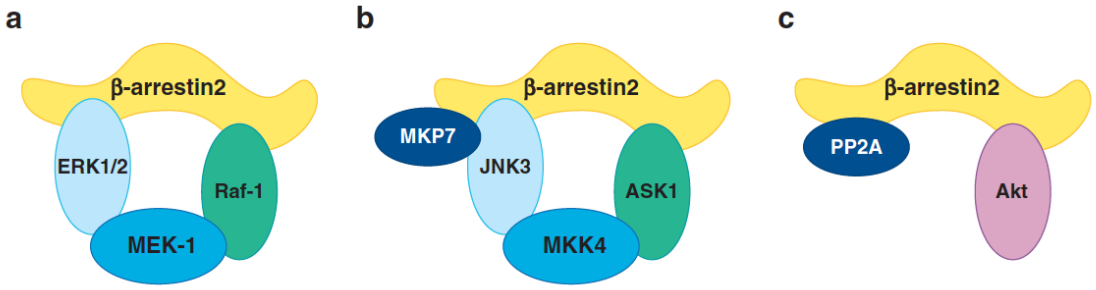



**Figure 11: Scaffold properties of β-arrestin-2 and their role in initiating a second wave of signaling pathways.** β-arrestin-2 regulates the activity of ERK1/2 (a), JNK3 (b) and Akt (c), through its scaffold properties, and activates their downstream signaling cascades. Adapted from DeWire, Ahn et al (2007).

### 2.5.2 Role of $\beta_2$-AR signaling in airway cells:

Activation of airway $\beta_2$-ARs causes several important physiological changes in different airway cells. Chronic activation of $\beta_2$-ARs, by agonists, results in several detrimental consequences that will be discussed in conjunction with the physiological roles of $\beta_2$-AR signaling for each cell type.

#### 2.5.2.1 Role of $\beta_2$-AR signaling in inflammatory cells:

Several inflammatory cells express $\beta_2$-ARs, such as mast cells (Chong, Chess-Williams et al. 2002), macrophages (Schenkelaars and Bonta 1984), eosinophils (Yukawa, Ukena et al. 1990), neutrophils (Galant and Allred 1980) and lymphocytes (Brodde, Brinkmann et al. 1985). There is some evidence that $\beta_2$AR-signalling may promote allergic inflammation.

Stimulation of $\beta_2$-ARs on peripheral blood mononuclear cell (PBMC) by epinephrine or terbutaline resulted in skewing the $T_H1/T_H2$ balance toward $T_H2$ where there is an increase in IL-4, IL-5 and IL-10 besides a reduction in interferon-γ (IFN-γ) levels (Agarwal and Marshall 2000). $\beta_2$-ARs are expressed on activated $T_H1$ cells, but not activated $T_H2$ cells, and activation of these receptors by terbutaline reduces the release of IL-2. This reduction in IL-2 disturbs the inhibitory effect of $T_H1$ cytokines on $T_H2$ cell (Ramer-Quinn, Baker et al. 1997) and enhances the $T_H2$ response. Moreover, salbutamol and fenoterol enhance the production of IgE induced by IL-4 in cultured PBMCs (Coqueret,

Dugas et al. 1995). Salbutamol, by shifting the immune response toward $T_H2$, potentiated IgE production in response to $T_H2$ cytokines (Fedyk, Adawi et al. 1996).

$\beta_2$-AR agonists also reduce the release of some inflammatory mediators (Barnes 1999). Salbutamol inhibits histamine release from mast cells (Chong, Chess-Williams et al. 2002) and salmeterol reduces the release of histamine and leukotrienes from human lung (Butchers, Vardey et al. 1991). Histamine and leukotrienes are immediate inducers of airway smooth muscle contraction. $\beta_2$-AR agonists inhibit lipopolysaccharide (LPS) induced TNF-α and IL-8 production from human monocyte cells via cAMP/PKA signaling (Farmer and Pugin 2000), suggesting a reduction in $T_H1$ responses that would promote $T_H2$-mediated allergy. In addition, the reduction in heparin sulphate release from mast cells by $\beta_2$-AR agonists may contribute to several detrimental effects. Heparin sulphate is normally released from mast cells and neutralizes cationic proteins that are produced by eosinophils (Broadley 2006). Cationic proteins, such as major basic protein, have the potential to damage airway epithelial cells (Motojima, Frigas et al. 1989). Moreover, heparin inhibits ASM proliferation (Kanabar, Hirst et al. 2005). Finally, regular use of salbutamol causes an increase in late asthmatic response and eosinophil influx into the airways (Gauvreau, Jordana et al. 1997).

#### 2.5.2.2 Role of $\beta_2$-AR signaling in airway smooth muscle (ASM) cells:

$\beta_2$-AR signaling affects ASM and enhances airway relaxation through numerous mechanisms. For example, potassium channels phosphorylated by PKA tend to open more frequently and thus reduce cell excitability (Giembycz and Newton 2006). PKA also inactivates myosin light chain kinase (MLCK) (Miller, Silver et al. 1983) while it activates myosin light chain phosphatase (MLCP) (Janssen, Tazzeo et al. 2004) leading collectively to muscle relaxation (Kassel, Wyatt et al. 2008).

In addition, activation of $\beta_2$-AR signaling has anti-inflammatory effects through reducing the release of different chemokines from ASM such as RANTES (Hallsworth, Twort et al. 2001; Lazzeri, Belvisi et al. 2001), GM-CSF (Hallsworth, Twort et al. 2001) and eotaxin (Hallsworth, Twort et al. 2001; Pang and Knox 2001), in contrast to the $\beta_2$-AR effects on leucocytes described above.

With chronic use of $\beta_2$-AR agonists, their ability to enhance bronchodilation is reduced (Giembycz and Newton 2006). Several mechanisms have been proposed to explain this reduction in response, including receptor desensitization via uncoupling of $\beta_2$-AR from Gs, β-arrestin binding followed by receptor internalization, sequestration and downregulation (Johnson 1998), downregulation of Gαs (Finney, Donnelly et al. 2001) and upregulation of PDEs (Torphy, Zhou et al. 1992; Seybold, Newton et al. 1998). The bronchodilatory

effect of $\beta_2$-AR agonists is relatively resistant to desensitization in contrast to other responses such as the anti-inflammatory effects (Broadley 2006).

The loss of bronchodilation with chronic $\beta_2$-AR agonist is also due to upregulation of phospholipase Cβ1 (PLCβ1) (McGraw, Almoosa et al. 2003), the signaling molecule that mediates the bronchoconstriction response induced by agents such as methacholine and acetylcholine, as outlined in Figure 12. Overexpression of $\beta_2$-AR in ASM cells enhanced acetylcholine-induced bronchoconstriction by upregulating PLCβ1 expression (McGraw, Almoosa et al. 2003). However, the bronchoconstriction response induced by methacholine and Gq-coupled receptor agonists was reduced in $\beta_2$-AR KO (McGraw, Almoosa et al. 2003). This mechanism provides an explanation for the AHR induced by bronchoconstrictors after chronic administration of $\beta_2$-AR agonists (Barnes 2011). Moreover, chronic $\beta_2$-AR activation increases cytokine release from ASM and consequently airway remodeling via ERK dependent mechanisms (Shore and Drazen 2003).

The inflammatory milieu reduces $\beta_2$-AR responsiveness due to several mechanisms. For example, IL-1β reduces ASM relaxation in response to $\beta_2$-AR stimulation through increasing prostaglandin E2 (PGE2) release in ASM cells. PGE2 increased cAMP levels and PKA activation resulted in heterologous phosphorylation of $\beta_2$-AR (Laporte, Moore et al. 1998) and consequently uncoupling of $\beta_2$-AR from Gs (Shore, Laporte et al. 1997; Laporte, Moore et al.

1998). TNF-α and IL-1β combination also decreased ASM response to $\beta_2$-AR agonist via the same mechanism (Moore, Lahiri et al. 2001). Moreover, IL-13 and IL-4, as a part of $T_H2$ cytokines, attenuate ASM relaxation in response to $\beta_2$-AR agonists (Shore 2002).

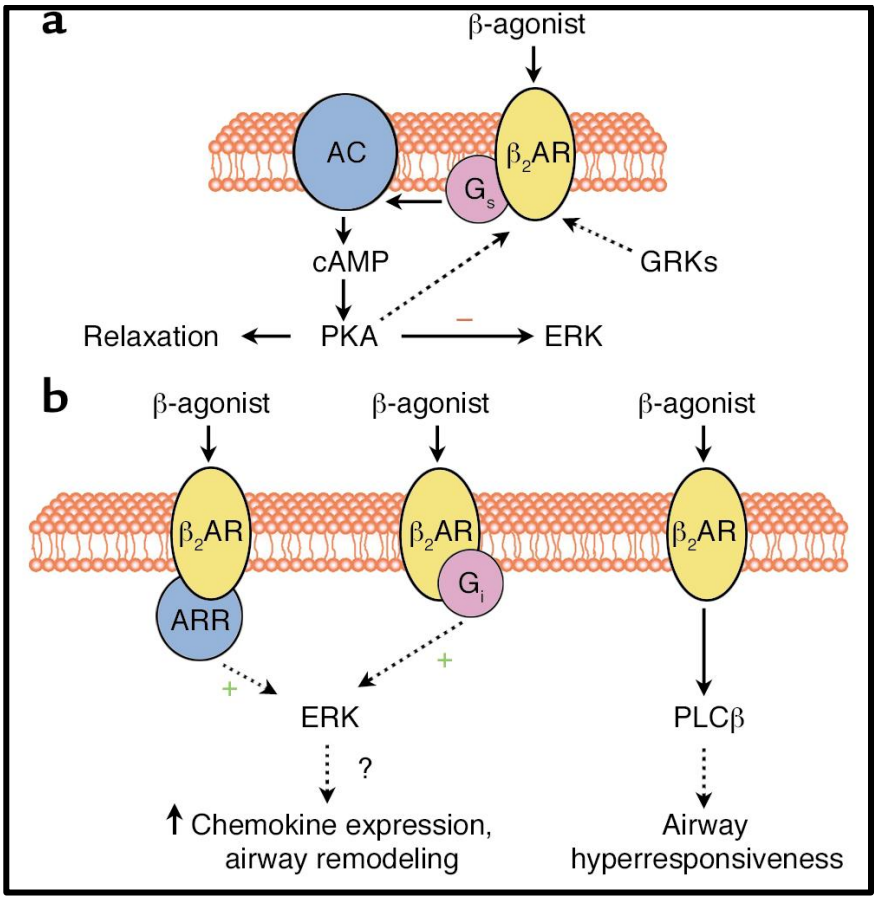



**Figure 12: Detrimental effects of chronic $\beta_2$-AR agonist stimulation.** Binding of agonist to $\beta_2$-AR induces airway relaxation through increasing PKA activity and inhibiting ERK signaling (a). However, regular activation of $\beta_2$-AR enhances the expression of chemokines and airway remodeling through increasing ERK activation by Gi/β-arrestin coupling (b). In addition, regular activation of $\beta_2$-AR also induces AHR through increasing PLC-β levels. Adapted from Shore and Drazen (2003).

### 2.5.2.3 Role of $\beta_2$-AR signaling in airway epithelial cells:

The coordinated beating of cilia is necessary for mucociliary clearance. β-agonists increase the ciliary beat frequency (CBF) (Verdugo, Johnson et al. 1980; Yanaura, Imamura et al. 1981; Lopez-Vidriero, Jacobs et al. 1985; Devalia, Sapsford et al. 1992; Tamaoki, Chiyotani et al. 1993; Wyatt, Spurzem et al. 1998). The effect $\beta_2$-AR agonist on CBF is mediated by cAMP/PKA signaling (Di Benedetto, Manara-Shediac et al. 1991; Salathe 2002). Interestingly, cAMP enhances calcium release from intracellular stores and thus stimulates nitric oxide induced CBF (Salathe 2002). In support to this notion, acute β-agonist inhalation increases the rate of mucociliary clearance in both normal individuals (Lafortuna and Fazio 1984) and in patients with chronic bronchitis (Fazio and Lafortuna 1981; Lafortuna and Fazio 1984).

In many studies, $\beta_2$-AR agonist causes changes in the release of inflammatory mediators. Formoterol, a long acting $\beta_2$-AR agonist, increases IL-8 release from airway epithelium and decreases GM-CSF level in response to TNF-α (Korn, Jerre et al. 2001). Moreover, salbutamol, salmeterol and formoterol enhance the release of IL-8 and IL-6 from airway epithelium in response to IL-1β through cAMP-PKA signaling (Holden, Rider et al. 2010). In contrast, another study has shown that formoterol did not affect IL-8 or GM-CSF levels in airway epithelium from asthmatic patients (Wilson, Wallin et al. 2001). It is noteworthy to mention that the inflammatory cytokines that are released in asthma also play a

role in altering $\beta_2$-AR/AC system. For example, IL-1β increases $\beta_2$-AR levels in airway epithelium but also elevates Gαi levels, thus leading to dichotomous effect on functional coupling of $\beta_2$-AR to cAMP production (Kelsen, Anakwe et al. 1997).

### 2.5.3 β-Blockers in asthma:

Based on all of the above-mentioned evidence for the detrimental effect of chronically activating $\beta_2$-ARs, blocking these receptors might have beneficial effects. β-AR blockers are contraindicated in asthma because acute administration of these agents is associated with airway narrowing (Singh, Whitlock et al. 1976; Boskabady and Snashall 2000). Chronic, but not acute, administration of nadolol reduces AHR in murine model of asthma (Callaerts-Vegh, Evans et al. 2004). The decreased AHR after blocking $\beta_2$-AR is partially due to reduction in the levels of PLC-β1, PDE4D and Gi (Lin, Peng et al. 2008). Blocking $\beta_2$-AR chronically, by nadolol administration, or by genetically ablating $\beta_2$-AR reduces AHR, eosinophil recruitment to airways and mucus metaplasia (Nguyen, Omoluabi et al. 2008; Nguyen, Lin et al. 2009). These lines of evidence point toward the detrimental effect of activating $\beta_2$-ARs and their pro-inflammatory role and suggest that blocking these receptors might result in attenuating asthma phenotype.

## 3. Methods

### 3.1 Cell culture:

Normal human bronchial epithelial (NHBE) cells were obtained from Lonza (Walkersville, MD). Cells were cultured in bronchial epithelial growth medium (BEGM; Lonza) and grown in 5% $CO_2$ and 95 % air at 37°C. At 80–90% confluence, cells (2 x $10^4$ cells/ $cm^2$) were seeded onto Transwell-culture inserts (24.5 mm diameter with 0.45 µm pore size) in a media composed of 50% of bronchial epithelial basal media (BEBM) and 50% of Dulbecco's modified Eagle medium (DMEM) high glucose and supplemented with 30 µg/ml bovine pituitary extract, 0.5 µg/ml bovine serum albumin (BSA), 0.5 µg/ml epinephrine, 50 µg/ml gentamycin, 50 ng/ml amphotericin B, 0.5 ng/ml human EGF, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 7 ng/ml triiodothyronine, 10 µg/ml transferrin and 0.1 ng/ml retinoic acid. This media will be referred as "differentiation media" throughout the text. Cells were cultured with epinephrine for ~8 days until they reached *confluence*, then the apical medium was removed and air-liquid interface (ALI) was established. After establishing ALI, the medium was changed every other day.

In some experiments, cells were grown in the absence of epinephrine for 72 hours before reaching ALI and throughout the ALI period. This condition will be referred as "absence of epinephrine". The cells were then treated with 20 ng/ml of IL-13 combined with different agonist/antagonist or inhibitors at the start

of ALI for 14 days as demonstrated in Figure (13). All experiments were repeated with three independent IL-13-responsive donor cell samples (N=3).

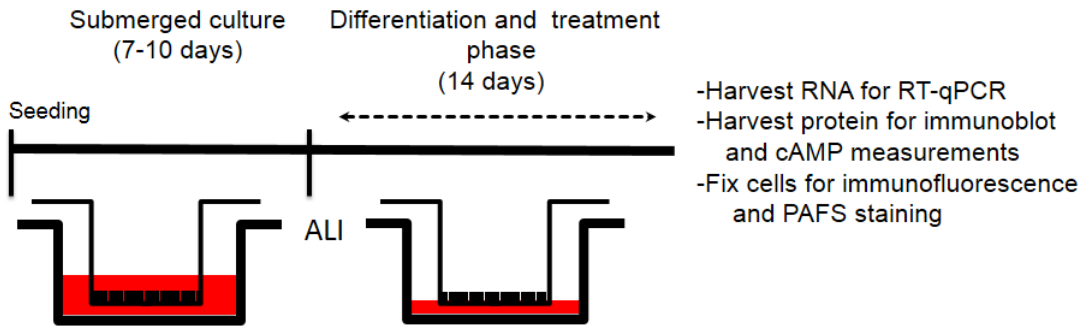



**Figure 13:** NHBE cells culture and treatment protocol.

### 3.2 NHBE cell treatment:

Cells were treated with IL-13 (20 ng/ml) combined with one of these additions for 14 days after establishing ALI: 1μM ICI-118,551 (selective $\beta_2$AR antagonist), 3μM CGP-20712A ( selective $\beta_1$AR antagonist), 10 μM Nadolol or alprenolol, 3μM FR180204 (ERK1/2 inhibitor), 3μM SB203580 (p38 inhibitor), 3μM SP600125 (JNK inhibitor), 3μM H89 (PKA inhibitor), 50 or 100 μM Rp-cAMPS (cAMP-dependent protein kinases inhibitor), 10 μM forskolin with 100 μM IBMX, 100 nM roflumilast (PDE4 inhibitor) or 100 nM carvedilol. These reagents were applied to the basal media at their final concentrations. The basal media was replaced with new media to which was added the reagents at their final concentrations every 48 hours. Compound-related toxicity was assessed by

observing the dryness of the apical surface of the cultured NHBE cells (Atherton, Jones et al. 2003).

### 3.3 Transepithelial Electrical Resistance (TEER) measurements:

The transepithelial electrical resistance (TEER) was measured using epithelial ohmvolt meter EVOM voltohmmeter device (WPI, Sarasota, FL) throughout the culture period to evaluate the monolayer integrity. Briefly, the medium was added to the apical compartment and resistance was measured from 3 inserts for each treatment group from each donor. Apical medium was then removed to maintain ALI conditions. TEER values were then subtracted from TEER of an empty insert. The values were then multiplied by the surface area and the data presented in units of $\Omega \cdot cm^2$.

### 3.4 Real-Time quantitative PCR Analysis:

Total RNA was extracted from cells using Trizol® (Invitrogen, CA), according to manufacturer's protocol. cDNA was generated from 5µg of total RNA using SuperScript III RT (Invitrogen, CA). MUC5AC, FOXJ1 and 18s mRNA was quantified using the Taqman Gene Expression Assay® (Applied Biosystems, CA) and analyzed by real time quantitative PCR (ABI PRISM 7000 Sequence Detection System, Applied Biosystems, CA). The threshold cycle (Ct) of treated

groups was compared to control group and normalized to 18s. Relative MUC5AC expression was calculated using Delta-Delta CT method.

**3.5 Immunoblotting:**

NHBE cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$ and 1 µg/ml leupeptin (Cell Signaling, Danvers, MA) combined with protease inhibitor cocktail tablet (Roche Applied Sciences, Indianapolis, IN). Total protein concentration was determined by BCA Protein Assay Kit® (Thermo Fisher Scientific Inc, Rockford, IL), according to the manufacture's protocols. Protein extracts were subjected to SDS-PAGE using 10% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA) and transblotted to polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA). Membranes were blocked in 3% BSA for 1 hour at room temperature and then incubated with primary antibodies, at the concentrations shown in Table 2, overnight at $4^oC$ followed by treatment for 1 hour with HRP conjugated antibodies against secondary antibody. The protein bands were developed using SuperSignal West Pico chemiluminescent substrate® (Thermo Fisher Scientific) according to manufacturer's recommendations. A CCD camera (Fluorochem 8800™) was used to collect the digital images and AlphaEase software (ProteinSimple, Santa Clara, CA) to quantify band density. The

membranes were then stripped and probed with GAPDH antibody or total-ERK1/2. The signal density of the phosphorylated proteins was normalized with that of GAPDH or total-protein. The data was presented as fold change as compared to control untreated cells to account for donors profile variations.

| Primary antibody | Source | Dilution |
|---|---|---|
| Phospho-ERK1/2, | Cell Signaling | 1:1000 |
| Total-ERK1/2 | Cell Signaling | 1:1000 |
| Phospho-CREB | Cell Signaling | 1:250 |
| Total-CREB | Cell Signaling | 1:1000 |
| Phospho-p38 | Santa Cruz Biotechnology | 1:1000 |
| Phospho-c-Jun | Millipore | 1:1000 |
| Phospgo-STAT6 | Cell Signaling | 1:1000 |
| GAPDH | Millipore | 1:1000 |

**Table 2: List of primary antibodies used for immunoblotting**.

### 3.6 Periodic acid fluorescent Schiff's (PAFS) stain:

The apical surfaces of NHBE cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) and permeabilized with Triton X-100. The inserts were stained with PAFS as described previously (Piccotti, Dickey et al. 2012). Red fluorescence of mucin was detected when the slides were excited at 380–580 nm and observed at 600–650 nm while the nucleic acid and cytoplasm of the cells fluorescence green when observed at a lower

wavelength (380–500 nm and 450–475 nm excitation and emission wavelengths respectively) (Piccotti, Dickey et al. 2012). Images were captured using an Olympus DUS spinning disc confocal microscope maintained in the College of Pharmacy Imaging Core. To correct for insert background, empty inserts were stained and used as a negative controls. To maintain consistency in subsequent image analysis, we used the same channel-specific threshold when capturing all images. The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated using Image J software (NIH). The data are represented as fold change compared to the correspondent control cells.

**3.7 Immunofluorescence labeling:**

The apical surfaces of NHBE cells were washed with PBS, fixed in 4% paraformaldehyde (PFA) and permeabilized with Triton X-100. The inserts were incubated for 15 minutes with 10% normal goat serum at room temperature followed by rabbit polyclonal mucin 5AC antibody (H-160, Santa Cruz) (Fujisawa, Velichko et al. 2009) or ZO-1 antibody (Invitrogen) (Stewart, Torr et al. 2012) at dilutions of 1:200 overnight at 4$^{o}$C. After washing the inserts with PBS, they were incubated with Alexa 594 goat anti-rabbit secondary antibody at 1:200 for 1 hour at room temperature. DAPI, at final concentration of 1 µg/ml, was used to counterstain the nuclei for 15 minutes at room temperature. Slides incubated with primary antibody diluents were used as negative controls. Images were captured

by confocal microscopy and the same channel-specific threshold was maintained when capturing the images. The ratio of integrated mucin 5AC density of each group to the integrated mucin 5AC density of the corresponding control group was calculated using Image J software (NIH).

### 3.8 Chemokine release:

Basolateral medium from the correspondent treatment groups were sampled at day 5 of ALI. Samples were flash frozen and sent to Aushon Biosystems (Billerica, MA) for assay of selected chemokines by indirect ELISA.

### 3.9 cAMP measurements:

Intracellular cAMP levels were measured by cAMP complete ELISA kit® (Ezo life sciences, NY) competitive immunoassay according to the manufacture's protocols. Briefly, NHBE cells were lysed in 0.1 M HCl to inhibit the activity of endogenous phosphodiesterases. The samples were then centrifuged and cell suspensions were stored at -80$^{o}$C. The standards and the samples were run in duplicate and incubated with alkaline phosphatase conjugated cAMP and cAMP antibody. After incubating the plate with the substrate, the absorbance was measured at 405 nm and cAMP concentration was calculated as pmol/ml. The cAMP concentration was then divided by the protein concentration (mg/ml) to normalize for protein content. Data are represented as pmol/mg of protein.

### 3.10 mouse tracheal epithelial cells (mTECs) Isolation:

Wild type and β-arrestin-2 knockout mice were provided and genotyped by Garbo Mak from Dr. David Corry's lab (Baylor College of Medicine, Houston, TX). Xiaoyi Yuan from Dr. Farrah Kheradmand 's lab (Baylor College of Medicine, Houston, TX) provided the technical assistance for mTECs isolation.

mTECs were isolated as described previously (You and Brody 2013). The resected trachea was opened longitudinally and rinsed with cold calcium and magnesium-free PBS. Then it was kept in cold DMEM/Ham's F-12 medium supplemented with 25 units-µg/ml penicillin/streptomycin. To isolate the mTECs, the trachea was incubated for 18-24 hours at $4^{o}$C in DMEM/Ham's F-12 penicillin/streptomycin media containing 0.15% pronase. Tracheal epithelial cells were detached from the trachea by gentle inversion in DMEM/Ham's F-12 medium supplemented with 25 units-µg/ml penicillin/streptomycin and 10% fetal bovine serum (FBS). After centrifugation, the cells were suspended in 'mTEC Basic' medium and incubated at $37^{o}$C 5% $CO_2$ for ~6 hours in primary culture dishes to remove adherent fibroblasts. Non-adherent cells were collected, centrifuged and suspended in 'mTEC/Plus' medium.

### 3.11 *In vitro* culture of mTEC:

After isolating mTECs, the cells were seeded into collagen-coated inserts and kept submerged in 'mTEC/Plus' medium. After 7 days, the medium from the

apical compartment was removed and ALI was established. During ALI, ‘mTEC/ NuSerum’ medium was added to the basolateral compartment. Fourteen days after establishing ALI, the cells were treated with 20 ng/ml mouse recombinant IL-13 and 3 µM epinephrine for 14 days. IL-13 and epinephrine were added to ‘mTEC/ NuSerum’ medium. The compositions of different culture media that were used for mTECs are summarized in the table 3. Figure 14 below summarizes the culture and treatment protocol.

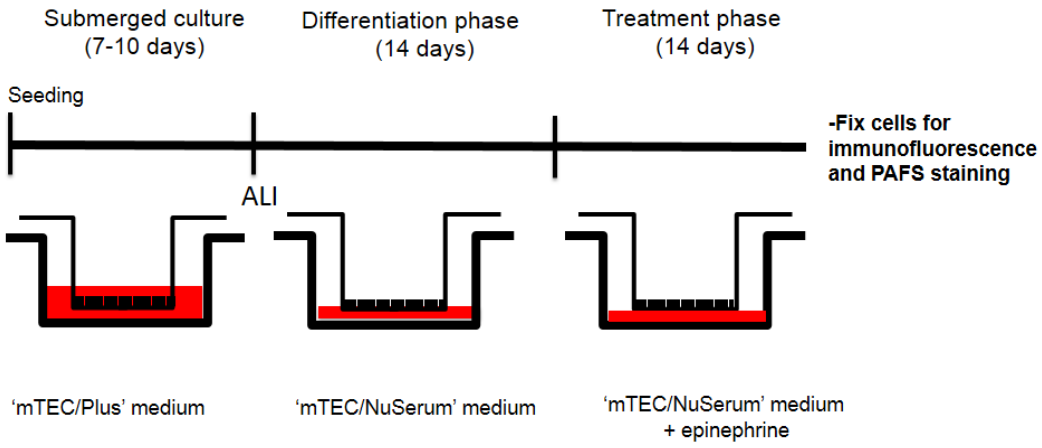



**Figure 14:** mTECs culture and treatment protocol

| mTEC medium | Composition |
|---|---|
| **'mTEC Basic'** | DMEM/Ham's F-12 (1:1 v/v) supplemented with 4 mM glutamine, 0.25 µg/ml amphotericin B, 50 µg/ml gentamicin and 10 units-µg/ml penicillin-streptomycin |
| **'mTEC/Plus'** | DMEM/Ham's F-12 (1:1 v/v) supplemented with 5% FBS, 4 mM glutamine, 0.25 µg/ml amphotericin B, 50 µg/ml gentamicin, 10 units- µg/ml penicillin-streptomycin, 5 mg/ml insulin, 30 µg/ml bovine pituitary extract, 5 µg/ml human transferrin, 0.1 µg/ml cholera toxin, 25 ng/ml epidermal growth factor and $10^{-8}$ M retinoic acid. |
| **'mTEC/NuSerum'** | 'mTEC basic' medium supplemented with 2% NuSerum and $10^{-8}$ M retinoic acid. |

**Table3: Composition of different culture media used for mTECs.**

### 3.12 Statistical Analysis:

Data are presented as means ± SEM. All experiments were done with NHBE cells from 3 donors (N=3). One-way ANOVA followed by Tukey's multicomparison test for multiple group statistical analysis was performed using GraphPad Prism 4® software. $p<0.05$ was considered statistically significant.

## 4. Results:

### 4.1 Epinephrine is required for mucin production in response to IL-13 in NHBE cells

In many published studies, epinephrine is routinely added to the medium of cultured NHBE cells (Atherton, Jones et al. 2003; Fulcher, Gabriel et al. 2005; Zhen, Park et al. 2007). We thus evaluated the requirement of epinephrine in inducing mucus production in response to IL-13. When NHBE cells were grown in the absence of epinephrine 72 hours before the cells reached ALI and 14 days after establishing AL, MUC5AC was expressed at low levels. Treating these cells with 20 ng/ml IL-13 for 14 days after establishing ALI did not induce MUC5AC expression (Figure 15A).

However, in the presence of epinephrine, treating NHBE cells with 20 ng/ml IL-13 for 14 days increased MUC5AC expression by ~ 15 fold as compared to control cells (-IL-13) (Figure 15A). To correlate the expression level with intracellular mucin 5AC and mucin glycoprotein, NHBE cells were incubated with primary antibody that recognize mucin 5AC of human origin or with PAFS staining respectively. A similar trend was also observed at the level of intracellular content of mucin 5AC and mucin glycoprotein (Figure 15B and Figure 16).

To determine whether growing NHBE cells in the absence of epinephrine compromised their integrity, we evaluated tight-junction formation between the cells by measuring transepithelial electrical resistance (TEER) throughout the culture period. TEERs increased during ALI, peaked on ~ALI day 7 and then decreased. The presence or absence of IL-13 or epinephrine did not greatly reduce TEERs values below untreated cells (Figure 17A). The formation of tight junctions was also confirmed by showing the presence of ZO-1 on the cell peripheries by immunofluorescence on ALI day 14 (Figure 17B).

We examined if the absence of epinephrine affected cell differentiation and whether this is the underlying mechanism for not responding to IL-13 stimulation. The expression of FOXJ1, a transcription factor expressed early in ciliogenesis, and MUC5AC were similar in the absence or presence of epinephrine (Figure 18A). The secretion of a well-studied epithelial cytokine, thymic stromal lymphopoietin (TSLP), and other cytokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 3 alpha (MIP3$\alpha$), were also not affected by the presence or absence of epinephrine. (Figure 18C).

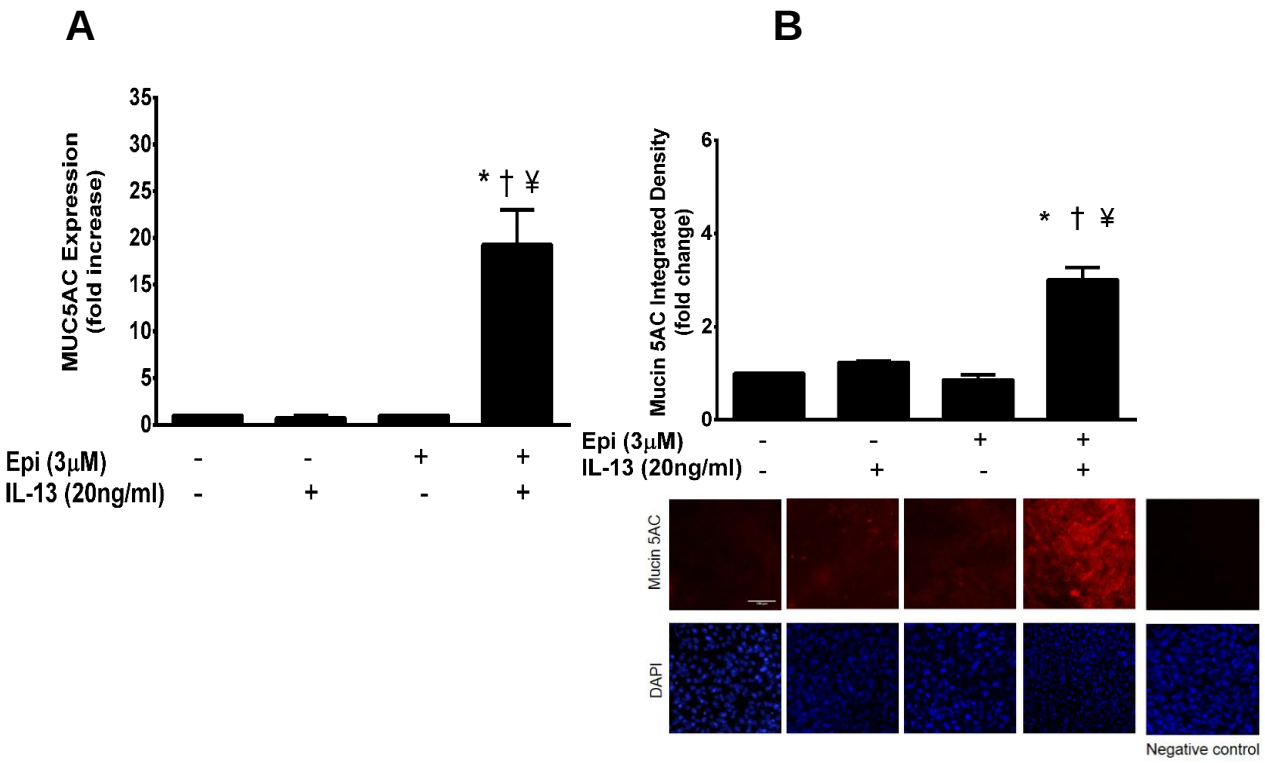



**Figure 15.** ***Epinephrine is required for mucin 5AC production in response to IL-13 in NHBE cells:*** **A)** NHBE cells were grown in the presence or absence of 3 μM epinephrine. At ALI, the cells were treated with 20 ng/ml IL-13 for 14 days, total RNA was harvested and then MUC5AC transcripts were measured by qRT-PCR. Data are presented as fold change compared to the corresponding treatment control (in the absence of IL13). **B)** The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. *, † and ¥ indicate $p<0.05$ significance as compared to +epi, -epi and -epi + IL-13 treated cells respectively.

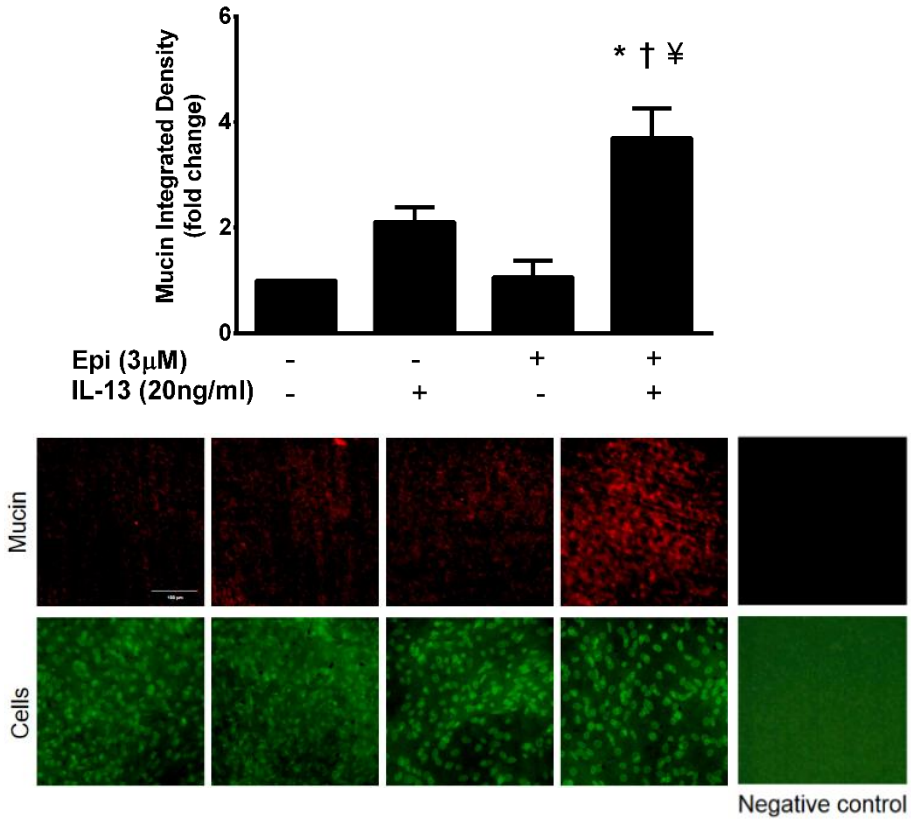



**Figure 16.** ***Epinephrine is required for mucin production in response to IL-13 in NHBE cells:*** NHBE cells were grown in the presence or absence of 3 μM epinephrine. At ALI, the cells were treated with 20 ng/ml IL-13 for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative image is the inset. Data are presented as means ± SEM from three donors. *, † and ¥ indicate $p<0.05$ significance as compared to +epi, -epi and -epi +IL-13 treated cells respectively.

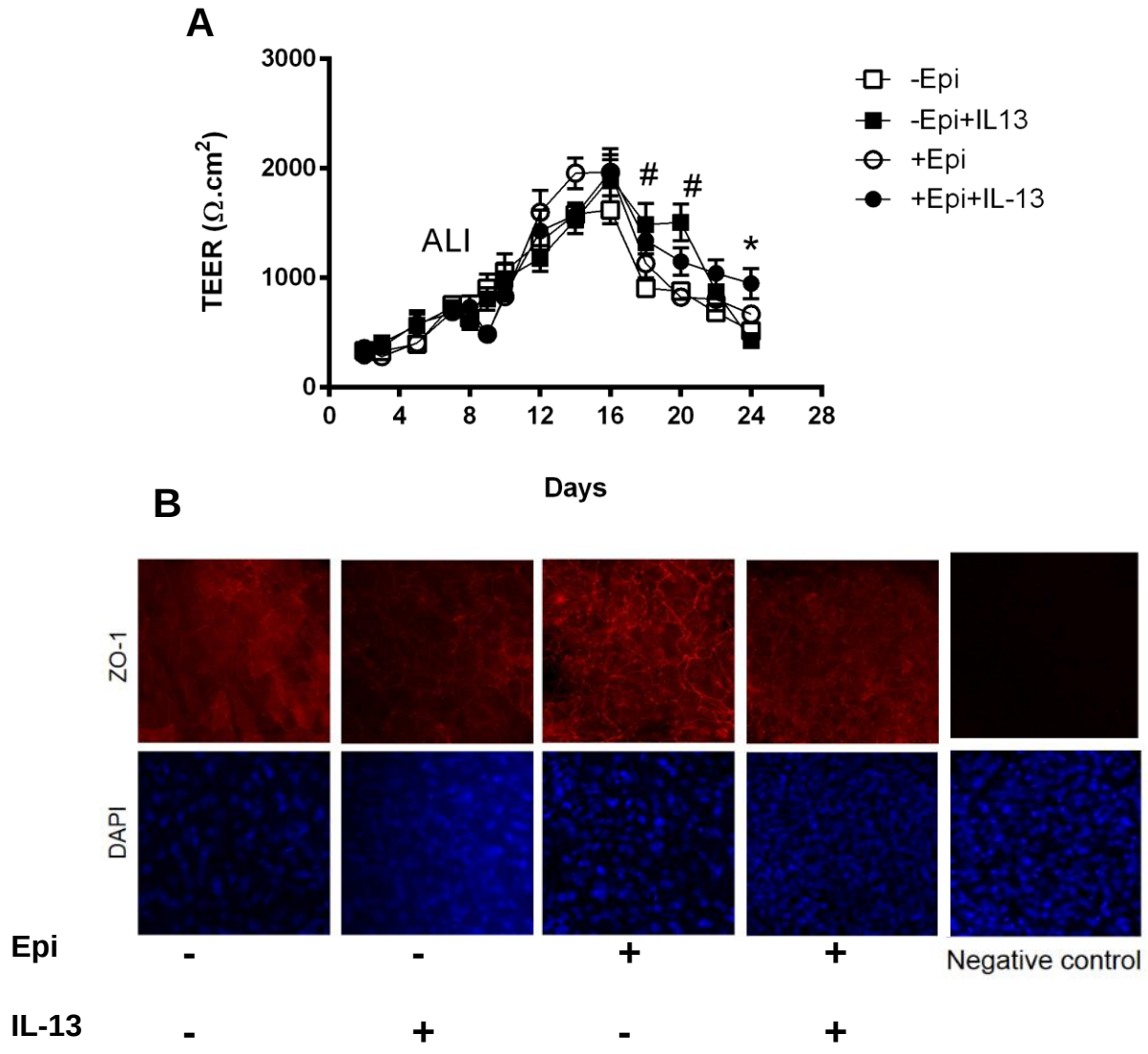



**Figure 17.** ***Role of epinephrine in the integrity of NHBE cells monolayer in response to IL-13:*** NHBE cells were grown in the presence or absence of 3 μM epinephrine. At ALI, the cells were treated with 20 ng/ml IL-13 for 14 days. **A)** Trans-epithelial electrical resistance (TEER) was measured throughout the culture period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance between –epi+IL-13 and +epi+IL-13 and –epi and -epi +IL-13 treated cells respectively. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 μm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Incubation with antibody diluent showed no

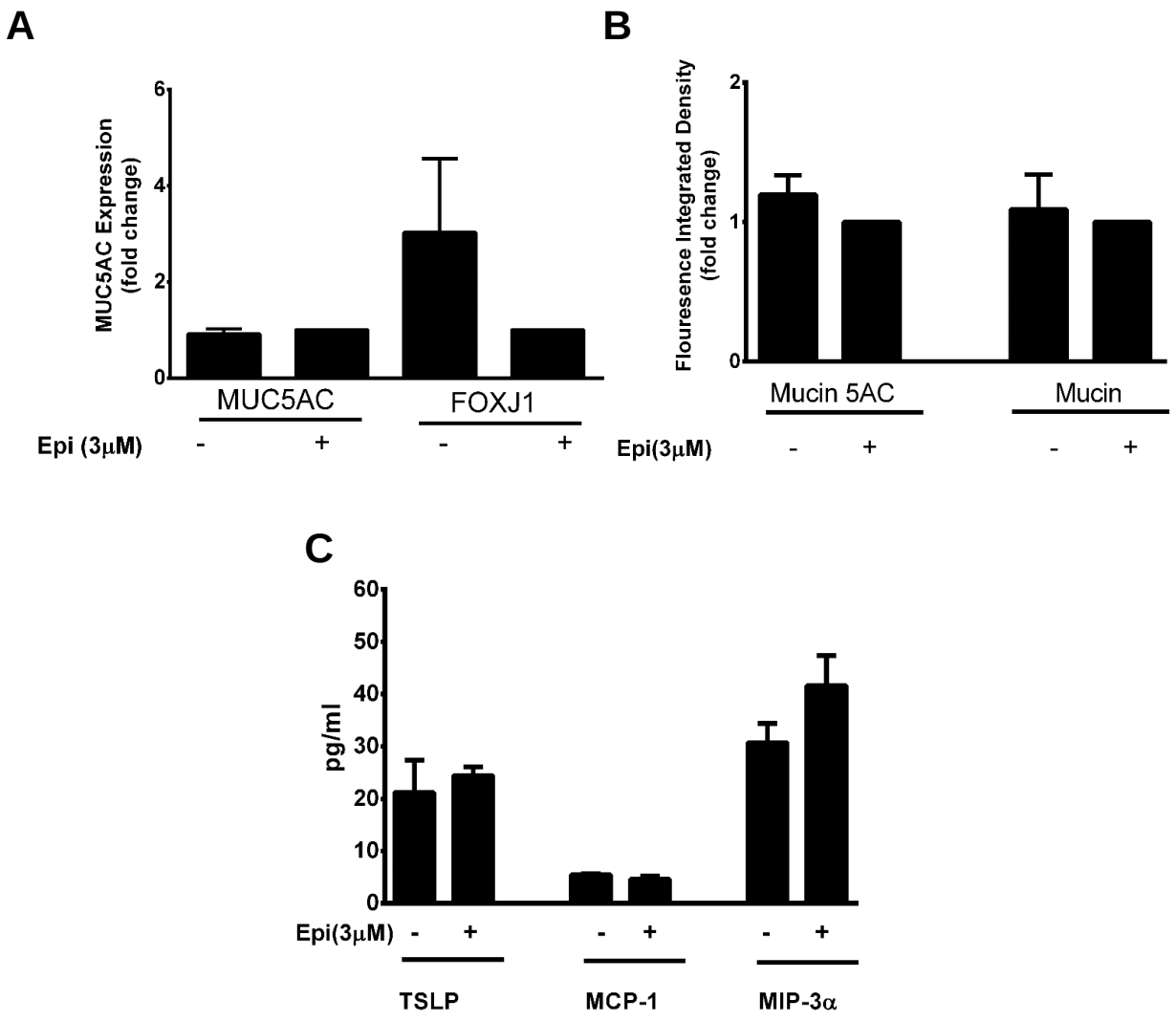



**Figure 18.** ***Effect of epinephrine on NHBE cell differentiation****:* NHBE cells were grown in the presence or absence of 3 μM epinephrine after establishing ALI. For cells grown in the absence of epinephrine, epinephrine was removed from the differentiation media 72 hours before ALI. **A)** After 14 days of growth at ALI, total RNA was harvested and then FOXJ1 and MUC5AC transcripts were measured by qRT-PCR. Data are presented as fold change compared to cells grown in the presence of epinephrine. **B)** The ratio of integrated fluorescence density (mucin 5AC and mucin glycoproteins) of each group to the integrated fluorescence density of the corresponding control group was calculated and expressed as fold change compared to cells grown in the presence of epinephrine. **C)** Secretion of thymic stromal lymphopoietin (*TSLP*), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 3α (MIP3α) from cells grown in the presence or absence of epinephrine (Indira Pokkunuri et al., BPS winter meeting, 2011). Data are presented as means ± SEM from three donors.

### 4.2 Epinephrine induced-$\beta_2$-AR signaling is required for mucin production in response to IL-13 in NHBE cells

To determine the β-AR subtype involved in MUC5AC expression in response to IL-13 in the presence of epinephrine, NHBE cells were incubated with either a selective $\beta_2$-AR antagonist (1 μM ICI-118,551) or a selective $\beta_1$-AR antagonist (3 μM CGP-20712A). ICI-118,551 completely abolished (>99%) IL-13 induced MUC5AC expression (0.039 ± 0.038 fold *vs* 15.99 ± 1.48 fold increase by IL-13. $p<0.05$). On the other hand, CGP-20712A did not affect the MUC5AC expression level (14.75 ± 0.96 fold *vs* 15.99 ± 1.48 fold increase by IL-13, $p>0.05$) (Figure 19A). CGP-20712A did not affect the intracellular mucin levels induced by IL-13 while ICI-118,551 brought the levels back to baseline (Figure 19B and Figure 20). Neither CGP-20712A nor ICI-118,551 affected the integrity of the monolayer as evaluated by TEERs measurement and ZO-1 localization (Figure 21).

We next asked if the increase in MUC5AC expression in response to IL-13 is due to agonist induced or constitutive $\beta_2$-AR signaling. NHBE cells were treated with 10 μM nadolol, a non-selective β-AR ligand that has inverse agonist activity on $\beta_2$-ARs and blocks both constitutive and agonist-induced receptor activity, or with 10 μM alprenolol, a non-selective β-AR antagonist which does not have inverse agonist activity, for 14 days in combination with IL-13 and in the presence of epinephrine. Treatment with nadolol reduced IL-13 induced

MUC5AC expression (3.36 ± 4.10 fold *vs* 25.37 ± 16.30 fold increase by IL-13, $p<0.05$) (Figure 22A), intracellular mucin 5AC protein and mucin content (Figure 22B and Figure 23). Treatment with alprenolol reduced IL-13-induced MUC5AC expression to a similar extent (3.19 ± 3.73 fold *vs* 25.37 ± 16.30 fold increase by IL-13, $p<0.05$) as well as intracellular mucin 5AC and mucin content.

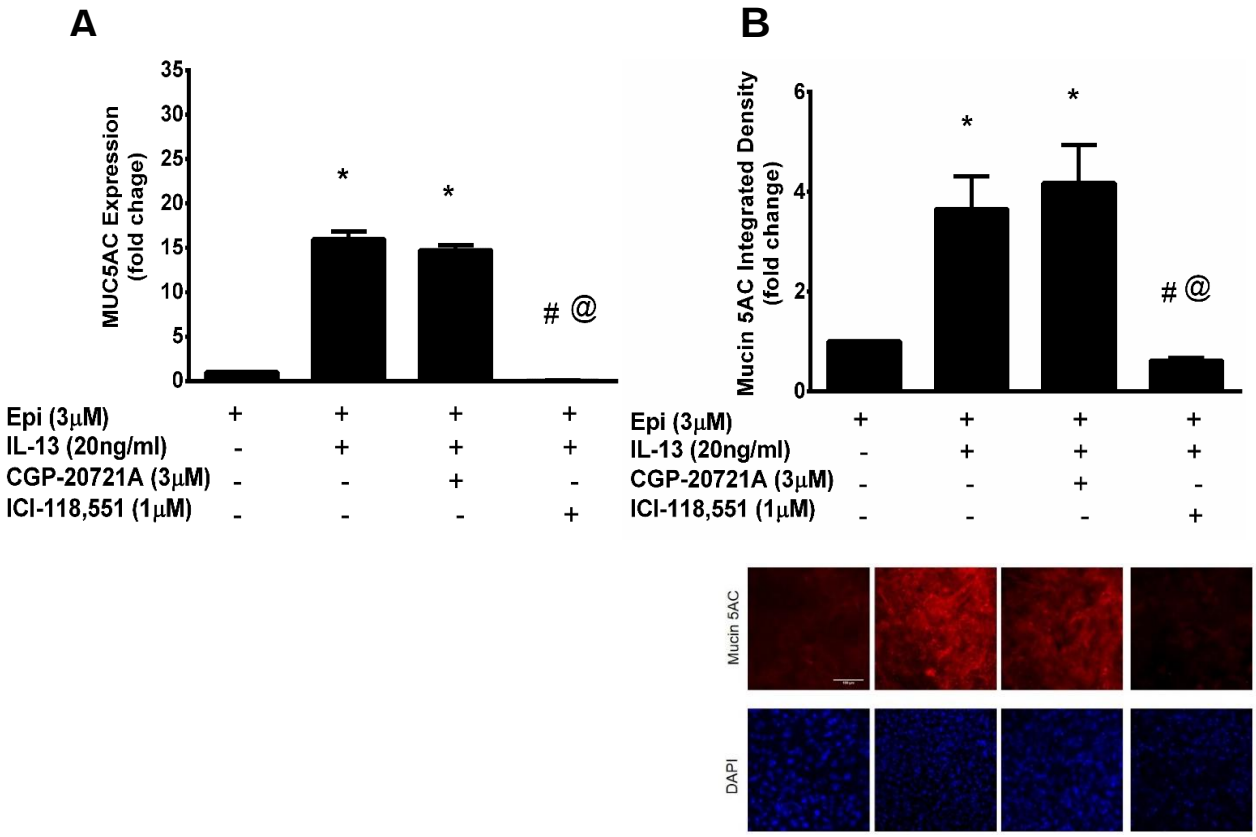



**Figure 19.** ***$\beta_2$-ARs are required for mucin 5AC production in response to IL-13 in NHBE cells****:* NHBE cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM CGP-20712A (a preferential $\beta_1$-AR antagonist) or 1 μM ICI-118,551 (a preferential $\beta_2$-AR antagonist) for 14 days. **A**) MUC5AC transcripts were quantified from extracted total RNA by qRT-PCR. Data are presented as fold change compared to cells grown in the presence of epinephrine only. **B**) The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. *, # and @ indicate $p<0.05$ significance as compared to +epi, +epi+IL-13 and +epi+IL-13+CGP-20721A treated cells respectively.

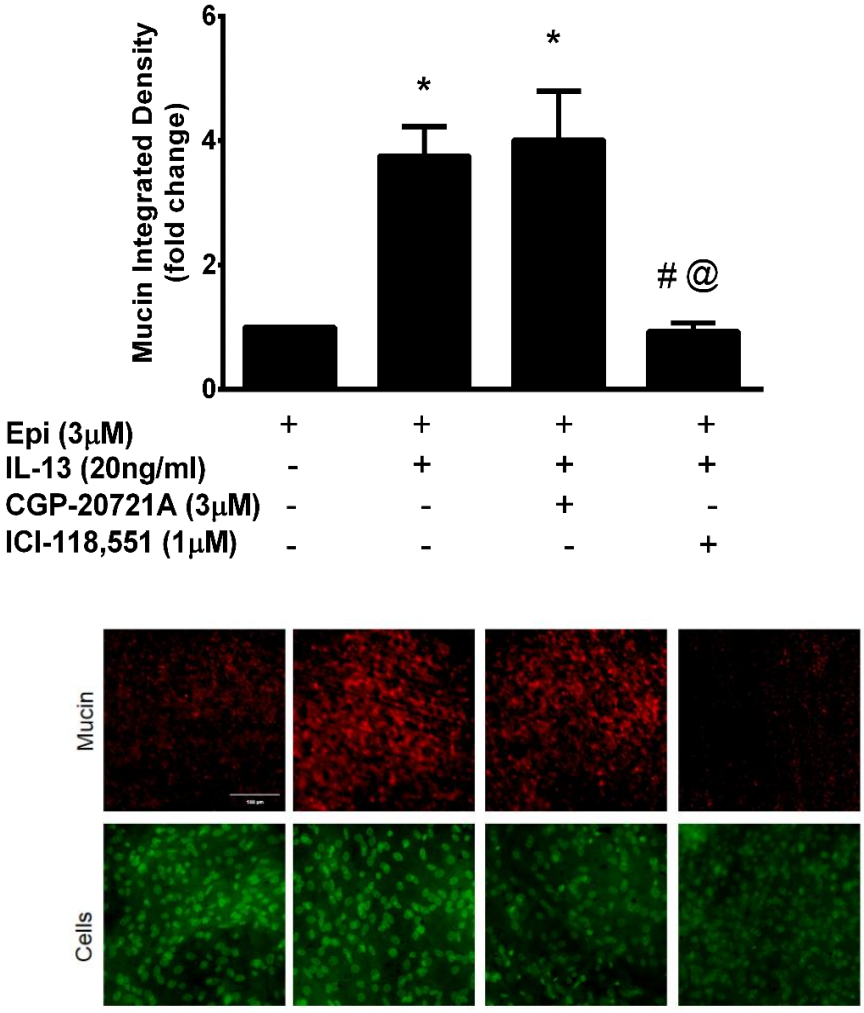
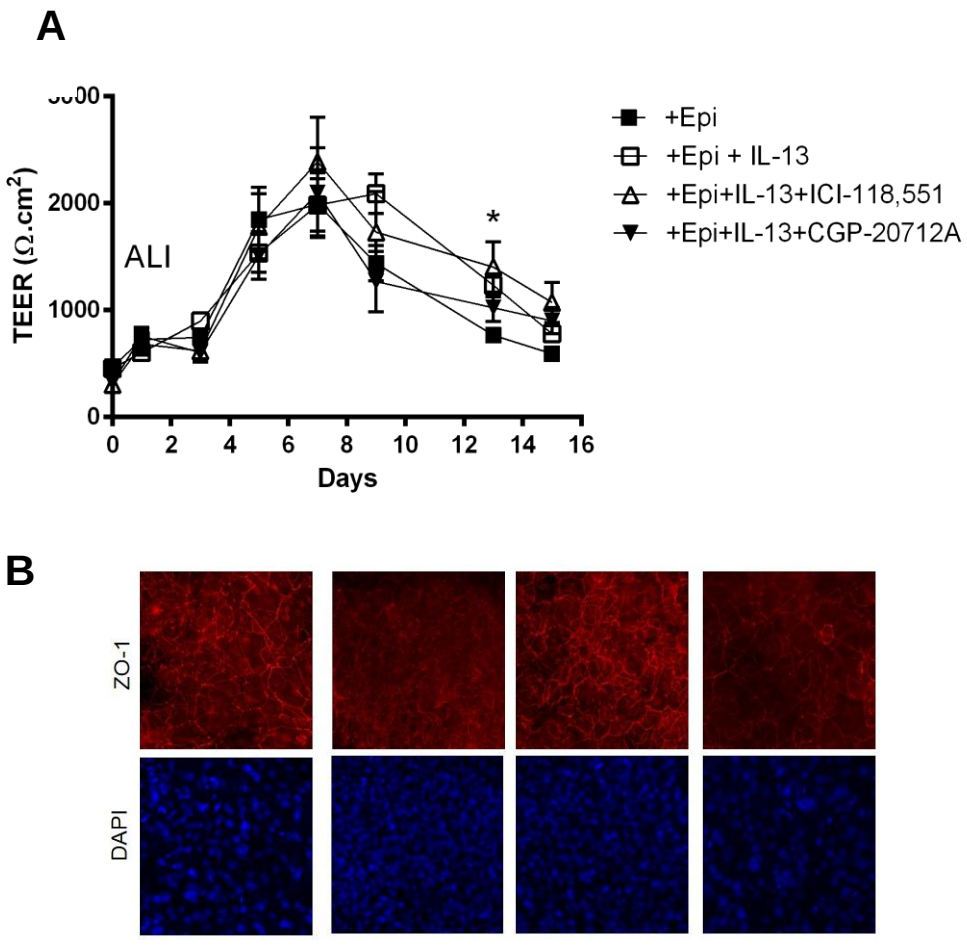



**Figure 20. *$\beta_2$-ARs are required for mucin production in response to IL-13 in NHBE cells:*** NHBE cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM CGP-20712A (a preferential $\beta_1$-AR antagonist) or 1 μM ICI-118,551 (a preferential $\beta_2$-AR antagonist) for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. *, # and @ indicate $p<0.05$ significance as compared to +epi, +epi+IL-13 and +epi+IL-13+CGP-20721A treated cells respectively.




| | | | | |
|---|---|---|---|---|
| **Epi (3μM)** | **+** | **+** | **+** | **+** |
| **IL-13 (20ng/ml)** | **-** | **+** | **+** | **+** |

**Figure 21.** ***Role of $\beta_2$-ARs in the integrity of NHBE cells monolayer in response to IL-13:*** NHBE cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with CGP-20712A or ICI-118,551 for 14 days. NHBE cells were grown in the presence or absence of 3 μM epinephrine. At ALI, the cells were treated with 20 ng/ml IL-13 for 14 days. **A**) Trans-epithelial electrical resistance (TEER) was measured throughout the culture period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 μm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Data are presented as means ± SEM from three donors. * indicates $p<0.05$ significant difference between +epi and +epi+IL-13.

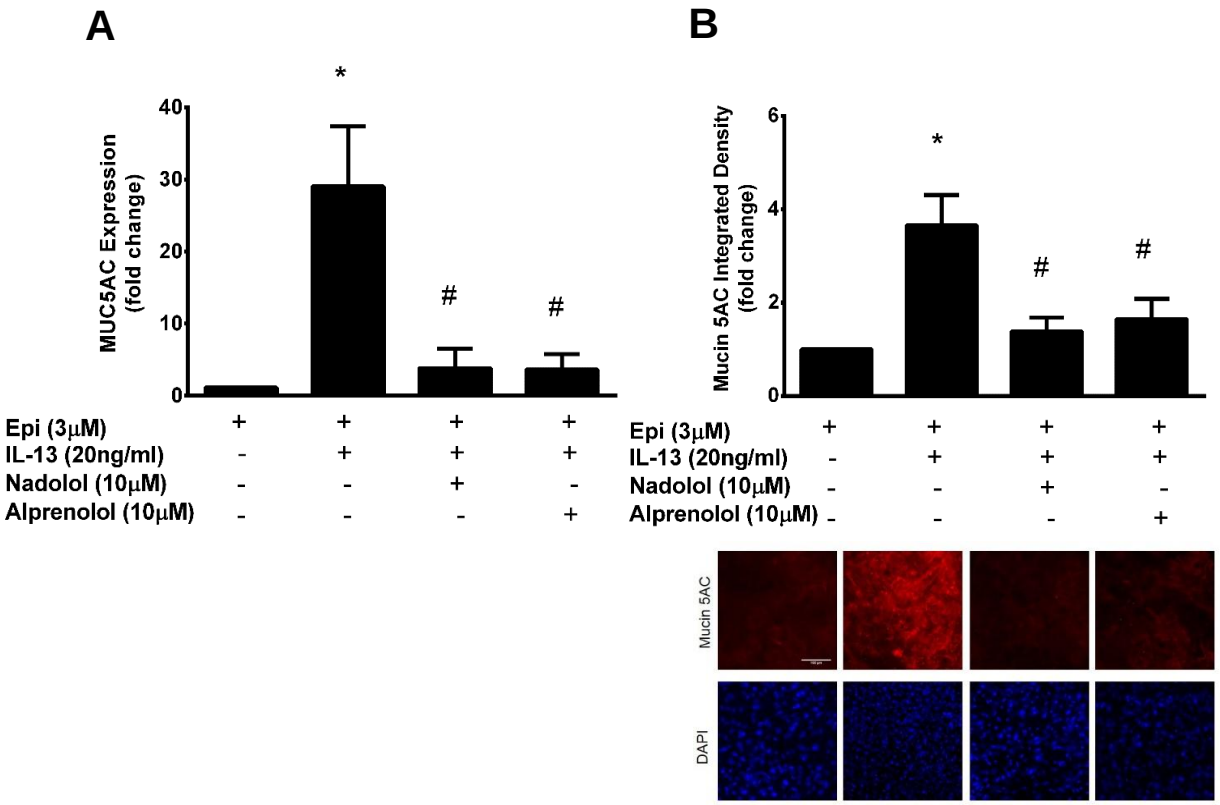



**Figure 22.** ***Agonist induced $\beta_2$-AR signaling is required for mucin 5AC production in response to IL-13 in NHBE cells****:* NHBE cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 10 μM nadolol (a non-selective β-AR blocker with inverse agonist of $\beta_2$-ARs) or 10 μM alprenolol (a non-selective β-AR blocker with no inverse agonist activity) for 14 days. **A)** MUC5AC transcripts were measured by qRT-PCR. Data are presented as fold change compared to cells grown in the presence of epinephrine only. **B)** The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

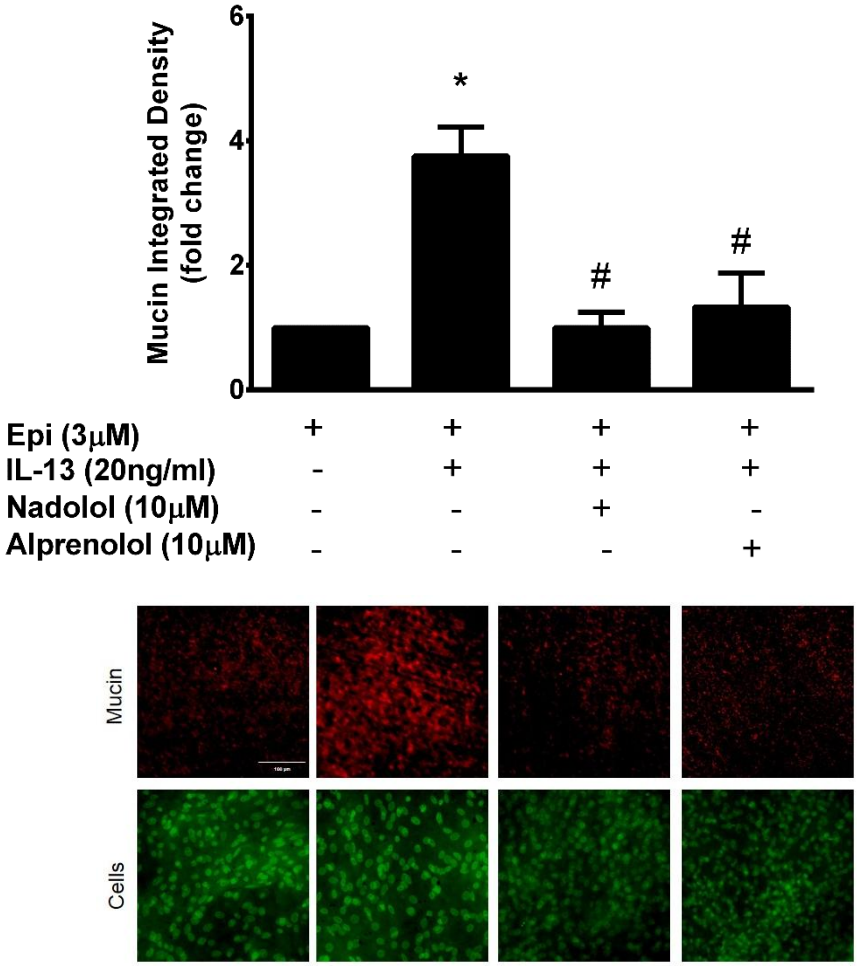



**Figure 23.** ***Agonist induced $\beta_2$-AR signaling is required for mucin production in response to IL-13 in NHBE cells****:* NHBE cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 10 μM nadolol (a non-selective β-AR blocker with inverse agonist of $\beta_2$-ARs) or 10 μM alprenolol (a non-selective β-AR blocker with no inverse agonist activity) for 14 days.. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

### 4.3 Mitogen activated protein kinase (MAPK) signaling is involved in mucin production in response to IL-13 in NHBE cells

MAPKs play an important role in inflammation by transmitting inflammatory signals from the cell surface into the nucleus (Garrington and Johnson 1999). In addition, MAPKs are involved in asthma pathogenesis (Pelaia, Cuda et al. 2005). To investigate the role of MAPKs in IL-13 induced mucin production in NHBE cells, we evaluated the activation of ERK1/2, JNK and p38 in response to IL-13 and whether epinephrine modulated this effect. In the absence of epinephrine, IL-13 did not affect the phosphorylation of ERK1/2, c-Jun or p38 as compared to their corresponding controls (Figure 24A, B and C). When epinephrine was included in the medium, IL-13 induced an approximately 3-fold increase in the phosphorylation of ERK1/2 and c-Jun when compared to their corresponding controls (Figure 24A and B). However, phosphorylation of p38 was still unaffected by IL-13 even in the presence of epinephrine (Figure 24 C).

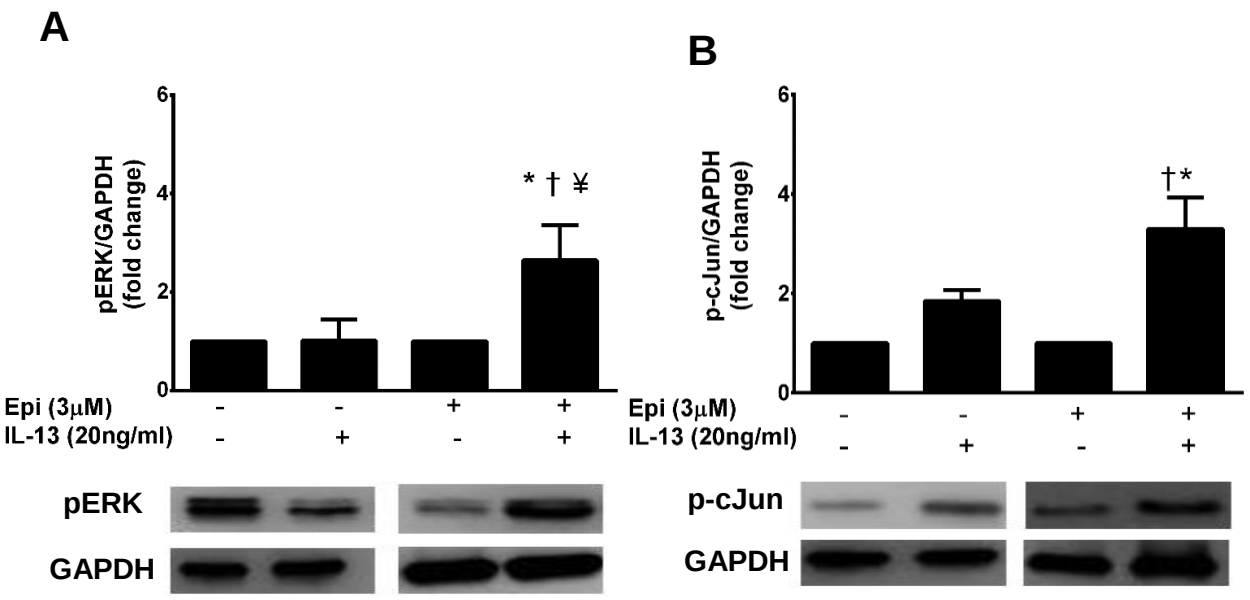
#### 4.3.1 Role of ERK1/2 signaling in mucin production in response to IL-13 in NHBE cells

We treated the cells with 3 μM U0126, an inhibitor of MEK1/2 (which activates ERK1/2), for 14 days in combination with IL-13. U0126 reduced MUC5AC expression significantly in response to IL-13 (17.74± 7.55 fold increase by IL-13 vs 2.51 ± 1.67 fold by U0126, $p<0.05$) (Figure 25A). The effect of U1026

was also associated with reduction in ERK1/2 phosphorylation in response to IL-13 (Figure 25B).

To further confirm the involvement of ERK1/2, we treated NHBE cells with 3 μM FR180204, a direct ERK1/2 inhibitor, in combination with IL-13 and epinephrine for 14 days. FR180204 significantly attenuated MUC5AC gene expression (15.18 ± 3.76 fold increase by IL-13 vs 1.82 ± 0.68 fold by FR180204, $p<0.05$) (Figure 26A). Moreover, mucin 5AC and mucin glycoproteins were also reduced significantly with FR180204 when compared to IL-13 treated cells (Figure 26B and Figure 27). The concentration of FR180204 that we used did not alter the integrity of NHBE cell monolayer (Figure 28).

We then tested if FR180204 affected the activation of other MAPKs; ERK1/2, p38 and cJun, a marker of JNK activity, in response to IL-13. FR180204 did not affect ERK1/2 or p38 activation whereas it significantly attenuated cJun phosphorylation in response to IL-13 (Figure 29). Furthermore, phosphorylation of RSK-1, a substrate of ERK1/2, and CREB transcription factor were not affected by IL-13 or by ERK1/2 inhibitor (Figure 30).



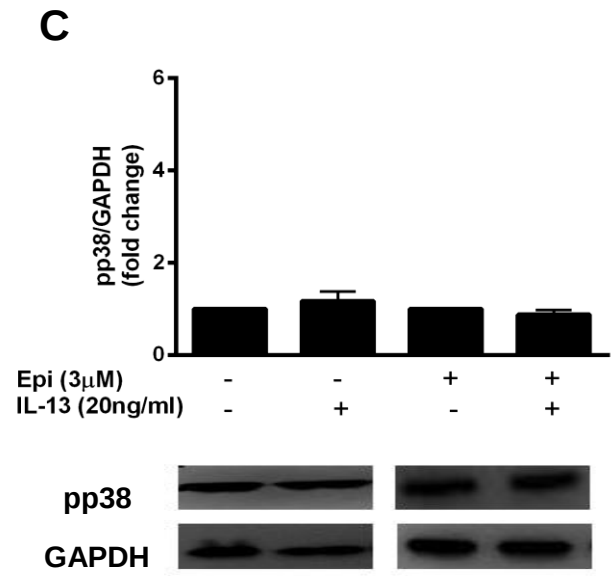




**Figure 24.** ***Effects of epinephrine on MAPKs activation in response to IL-13 in NHBE cells:*** The cells were grown in the presence or absence of epinephrine or IL-13 as indicated for 14 days after ALI before harvesting for total proteins. Immunoblots were performed using antibodies to the indicated phosphorylated MAP-kinases. The signal densities of the phosphorylated proteins were normalized to GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation. **B)** cJun phosphorylation. **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. *, † and ¥ indicate $p<0.05$ significance as compared to +epi, -epi and -epi +IL-13 treated cells respectively.

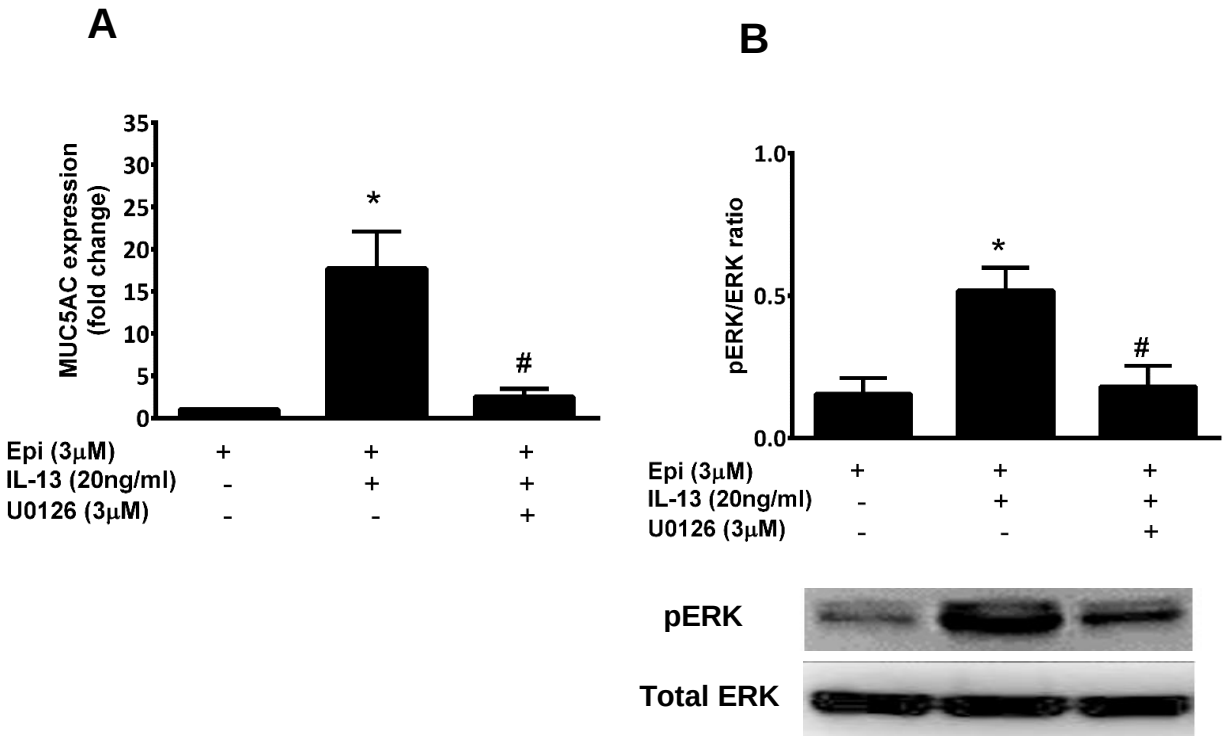



**Figure 25.** ***Role of MEK1/2 in MUC5AC expression in response to IL-13 in NHBE cells:*** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM U0126 (MEK1/2 inhibitor) for 14 days. **A)** MUC5AC transcripts were measured by qRT-PCR, and the data presented as fold change compared to cells grown in the presence of epinephrine only. **B)** Immunoblots were performed using antibodies to the indicated phosphorylated ERK1/2. The signal densities of the phosphorylated proteins were normalized to total ERK1/2 protein density and the relative intensities were reported as the degree of activation of the protein. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi+IL-13 treated cells respectively.

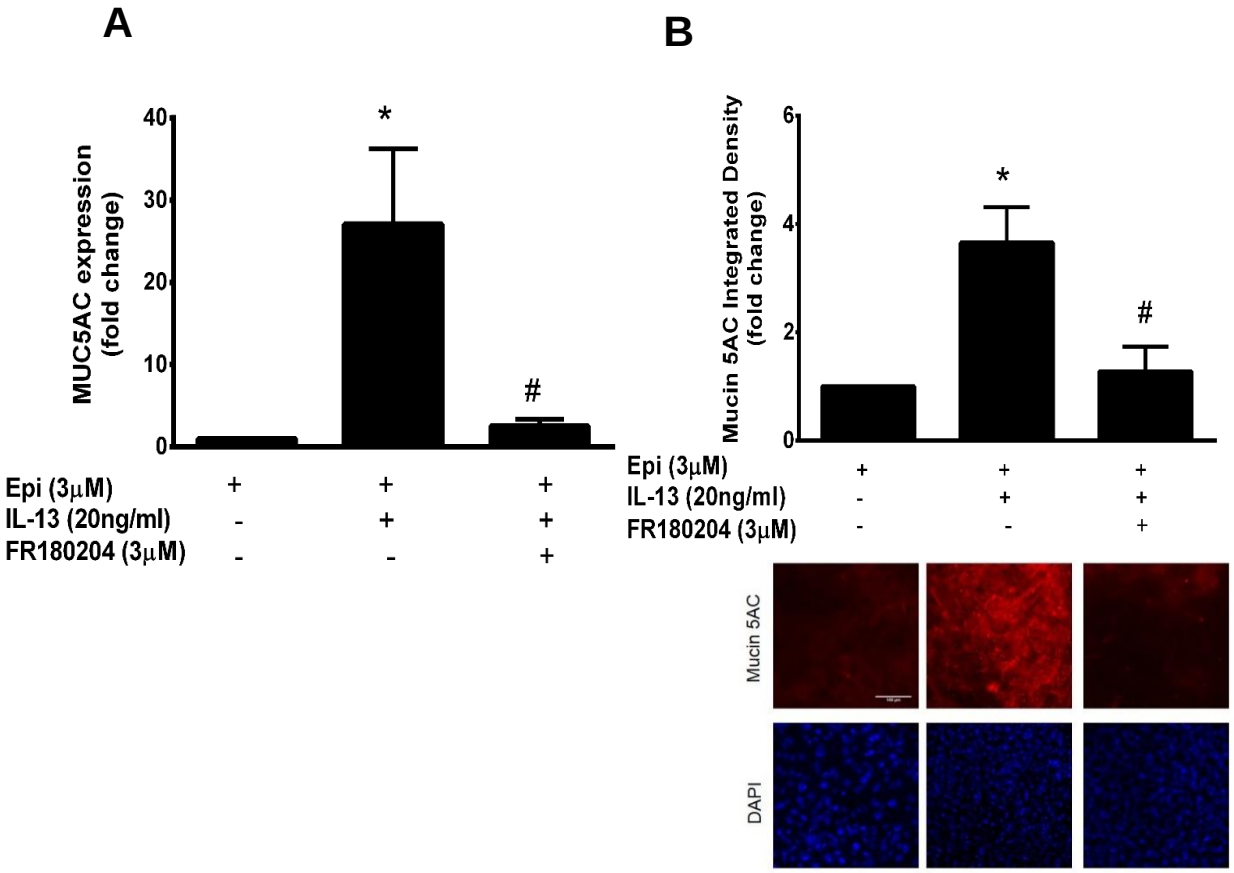



**Figure 26.** ***Role of ERK1/2 signaling in mucin 5AC production in response to IL-13 in NHBE cells*****:** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM FR180204 (ERK1/2 inhibitor) for 14 days. **A)** MUC5AC transcripts were measured by qRT-PCR, and the data presented as fold change compared to cells grown in the presence of epinephrine only. **B)** The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi+IL-13 treated cells respectively.

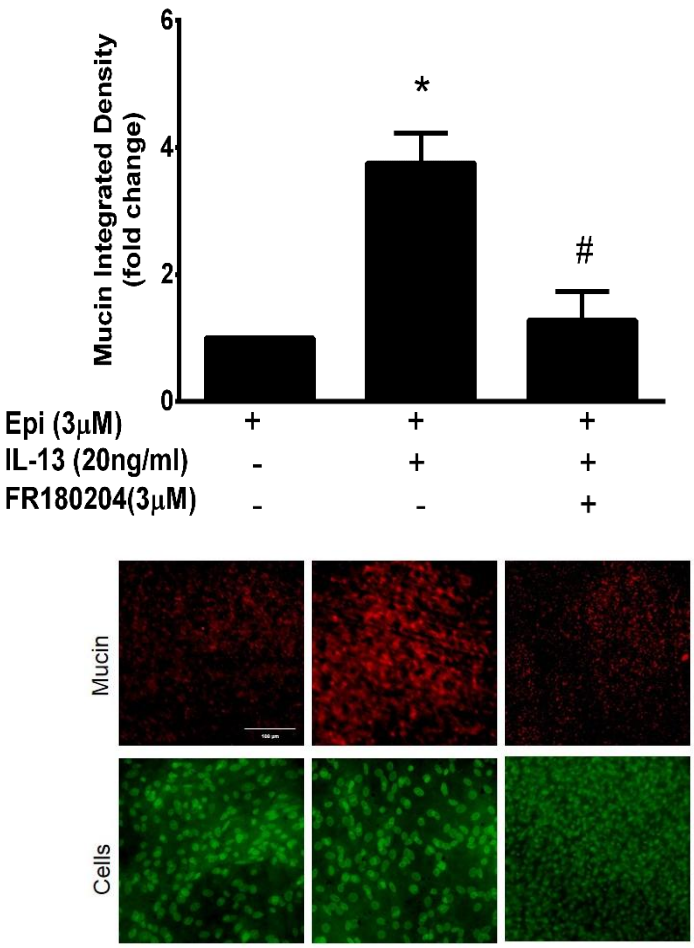



**Figure 27.** ***Role of ERK1/2 signaling in mucin production in response to IL-13 in NHBE cells*****:** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM FR180204 (ERK1/2 inhibitor) for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

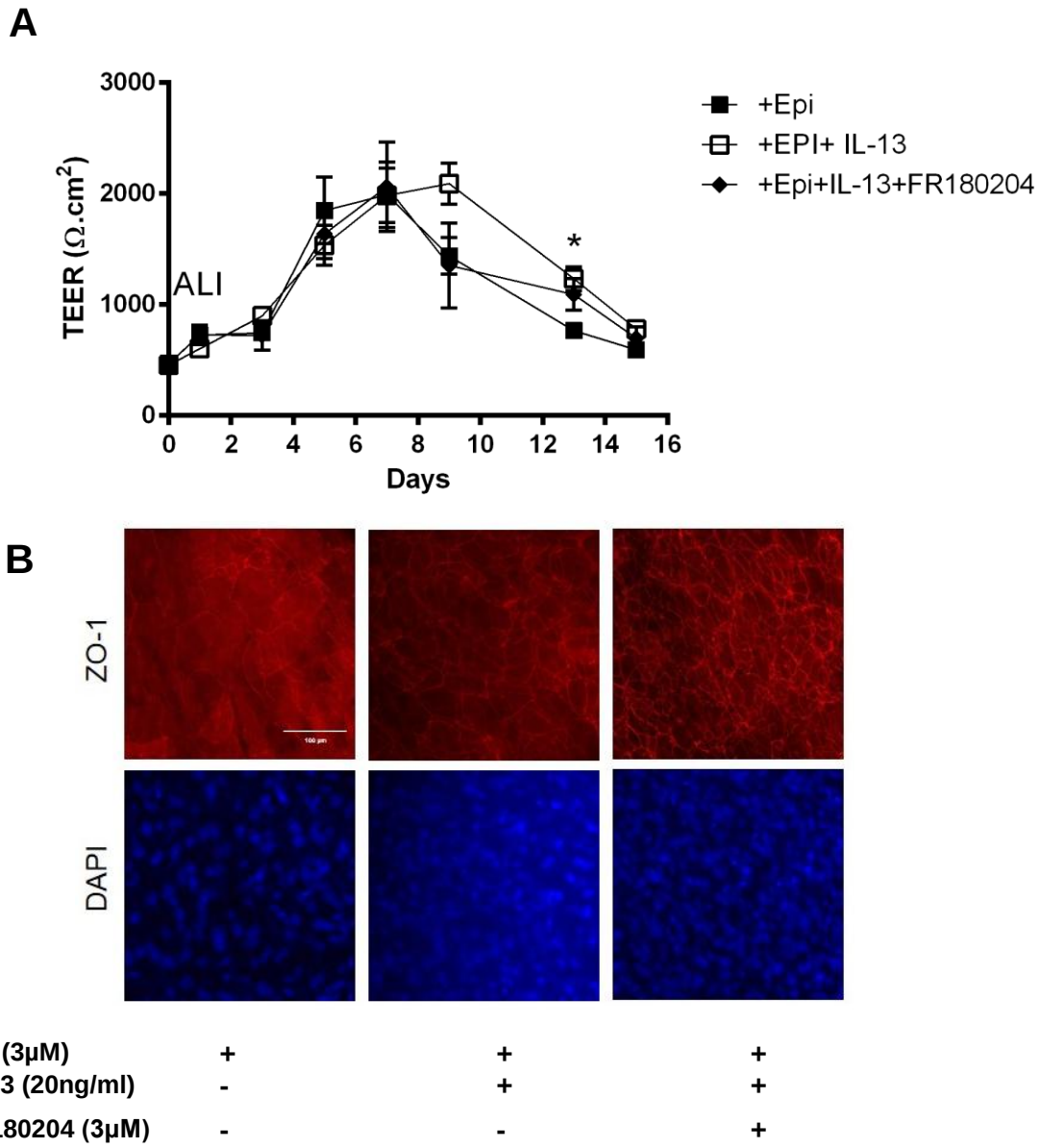
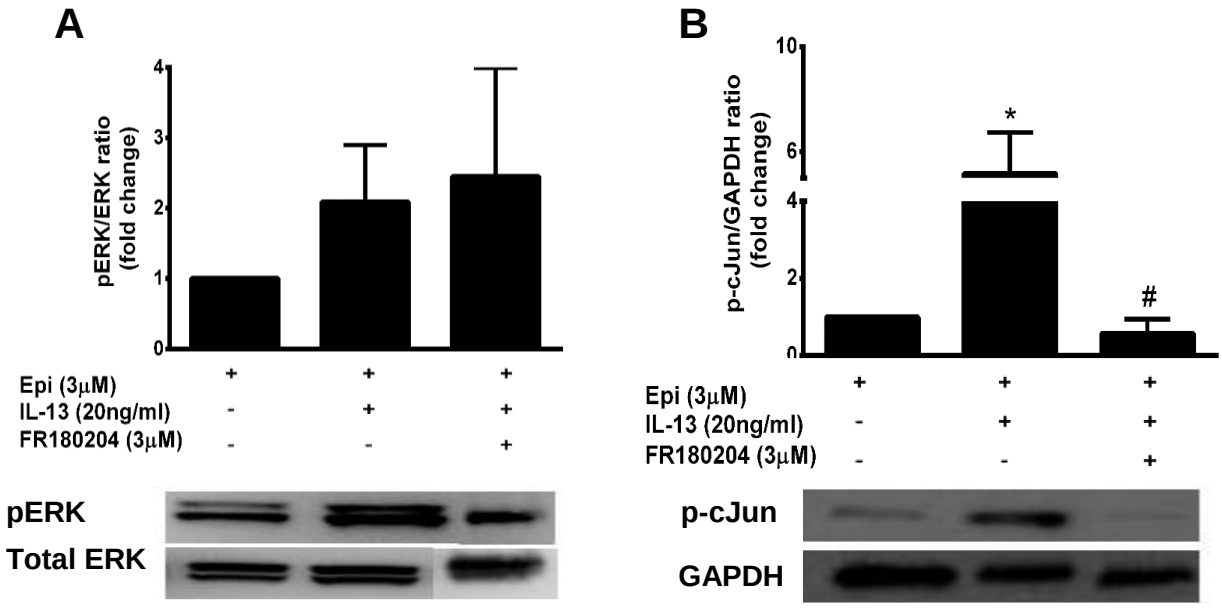



**Figure 28.** ***Effect of ERK1/2 inhibitor on the integrity of NHBE cells monolayer in response to IL-13:*** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM FR180204 (ERK1/2 inhibitor) for 14 days. **A)** Trans-epithelial electrical resistance (TEER) was measured one day before ALI and throughout ALI period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 μm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Data are presented as means ± SEM from three donors. * indicates $p<0.05$ significant difference between +epi and +epi+IL-13.



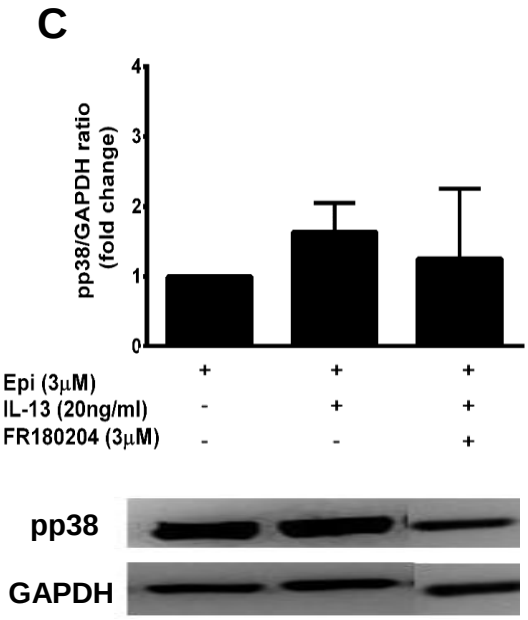




**Figure 29.** ***Effects of ERK1/2 inhibitor on activation of MAPKs in NHBE cells in response to IL-13:*** At ALI, NHBE cells were treated with 20 ng/ml IL-13 in combination with 3 μM FR180204 (ERK1/2 inhibitor) for 14 days in the presence of 3 μM epinephrine. Total proteins were harvested and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation. **B)** cJun phosphorylation. **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. *and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

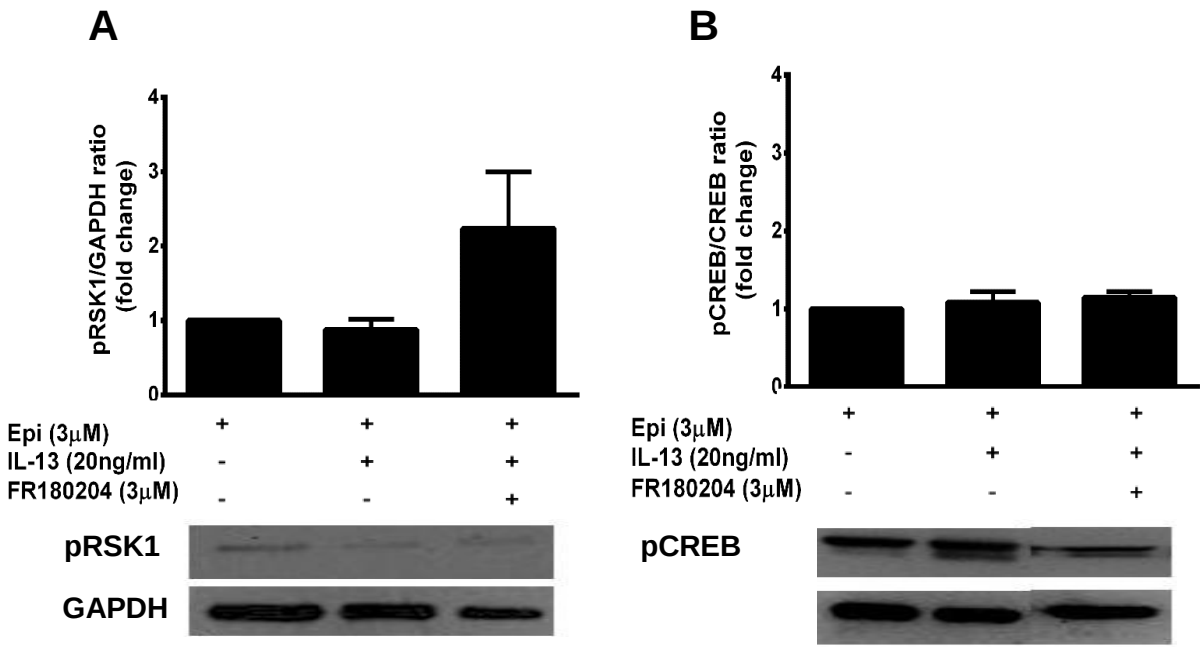



**Figure 30.** ***Effects of ERK1/2 inhibitor on activation of RSK-1 and CREB in NHBE cells in response to IL-13:*** At ALI, NHBE cells were treated with 20 ng/ml IL-13 in combination with 3 μM FR180204 (ERK1/2 inhibitor) for 14 days in the presence of 3 μM epinephrine. Total proteins were harvested, and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** RSK-1 phosphorylation in NHBE cells. **B)** CREB phosphorylation. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

### 4.3.2 Role of JNK signaling in mucin production in response to IL-13 in NHBE cells

Since IL-13 increased cJun phosphorylation, we studied the effect of JNK on mucin parameters induced by IL-13 only in the presence of epinephrine. We treated the cells with 3 μM SP600125, a JNK inhibitor, for 14 days in combination with IL-13 in the presence of epinephrine. SP600125 blocked the increase in MUC5AC expression by IL-13 (15.18 ± 3.76 fold increase by IL-13 vs 0.77 ± 0.39 fold by SP600125, $p<0.05$) (Figure 31A). Moreover, mucin 5AC and mucin glycoproteins were also inhibited significantly with SP600125 as compared to the levels induced by IL-13 (Figure 31B and Figure 32). 3 μM SP600125 did not reduce the integrity of the cultured epithelial monolayer below the control cells, as evaluated by measuring TEERs and ZO-1 protein localization (Figure 33A and B).

We then examined the effect of inhibiting JNK activity on several downstream signaling molecules that might mediate the effect of IL-13 on mucin production in NHBE cells. We found that SP600125 was associated with reduction in cJun phosphorylation in response to IL-13 (Figure 34A). However, SP600125 did not affect the levels of ERK1/2, p38 or CREB phosphorylation when combined with IL-13 as compared to IL-13 treated cells (Figure 34 B, C and Figure 35).

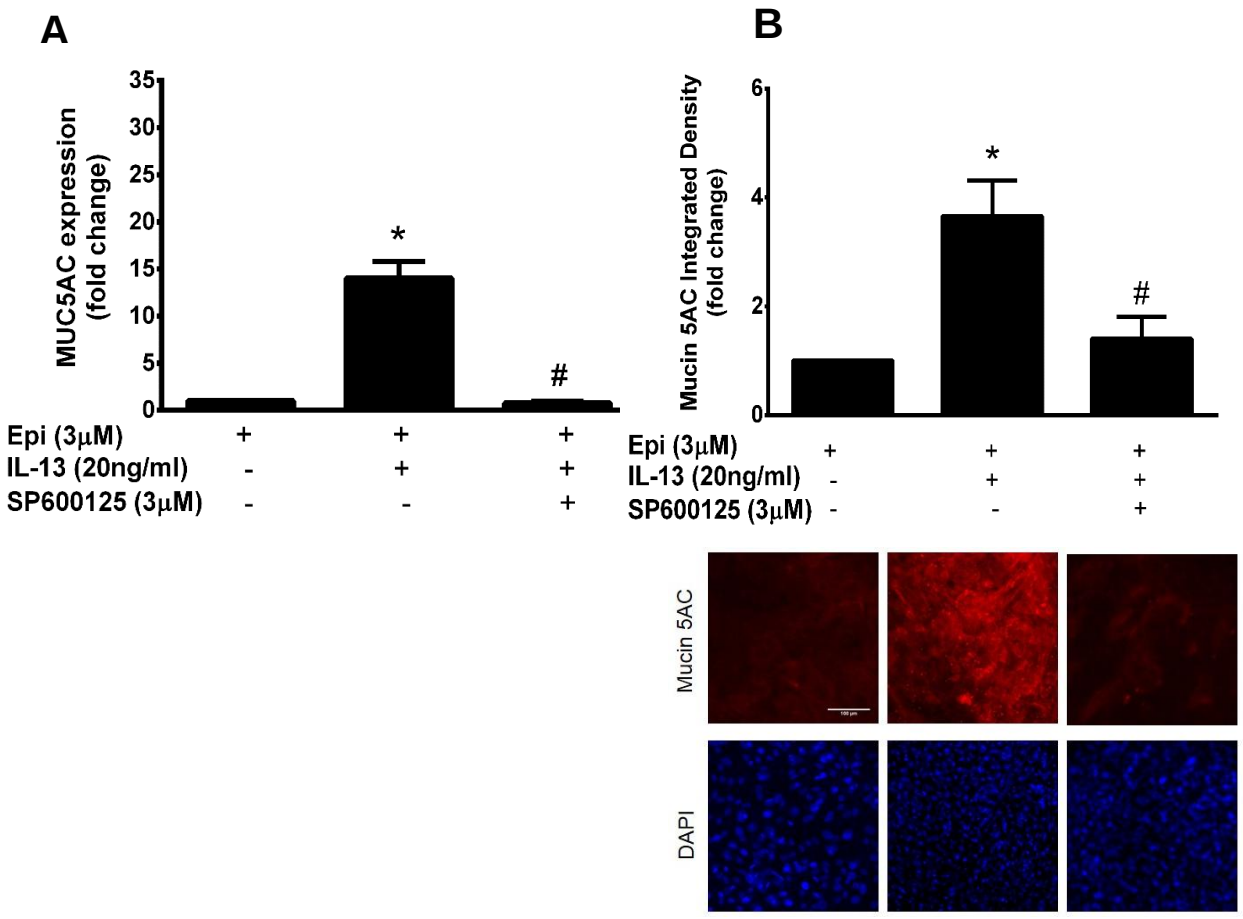



**Figure 31.** ***Role of JNK signaling in mucin 5AC production in response to IL-13 in NHBE cells*****:** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM SP600125 (JNK inhibitor) for 14 days. **A)** MUC5AC transcripts were measured by qRT-PCR, and the data presented as fold change compared to cells grown in the presence of epinephrine only. **B)** The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi+IL-13 treated cells respectively.

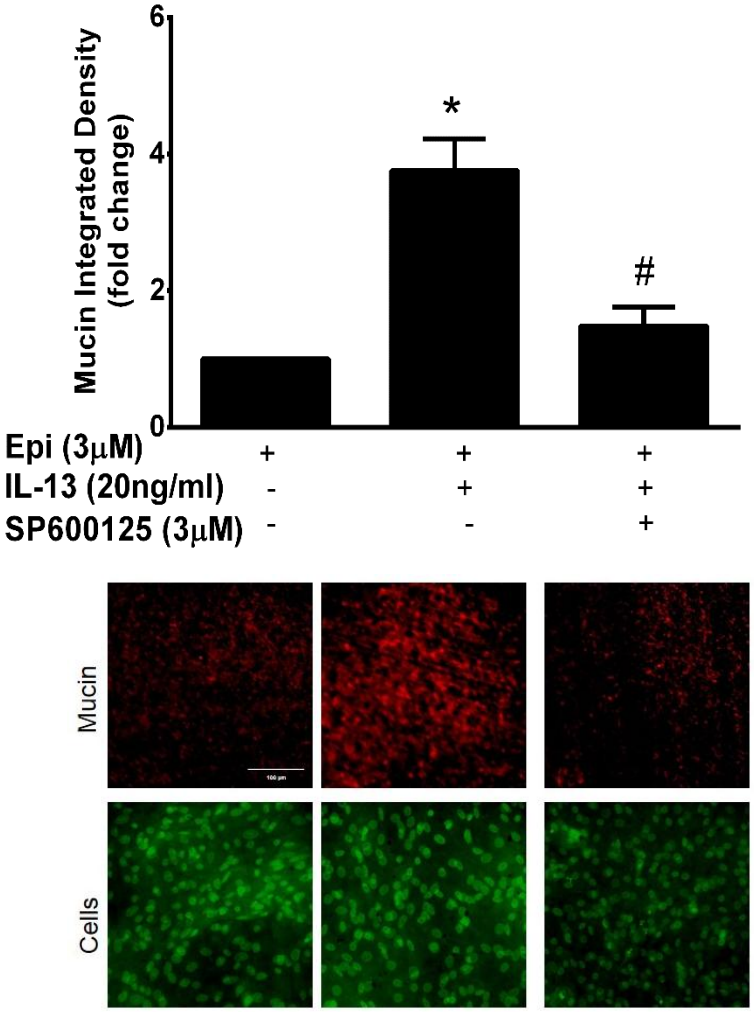



**Figure 32.** ***Role of JNK signaling in mucin production in response to IL-13 in NHBE cells*****:** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM SP600125 (JNK inhibitor) for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

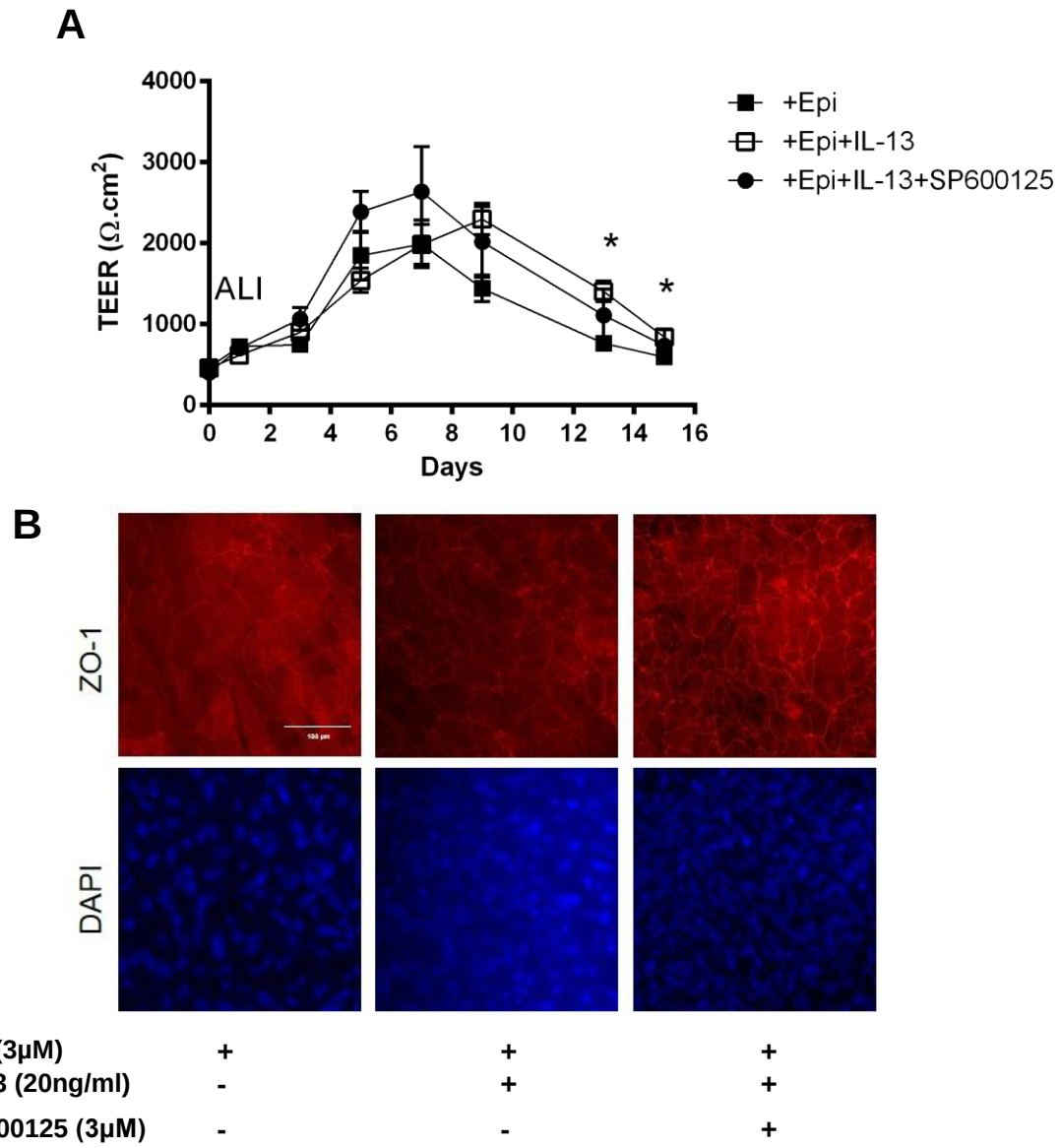
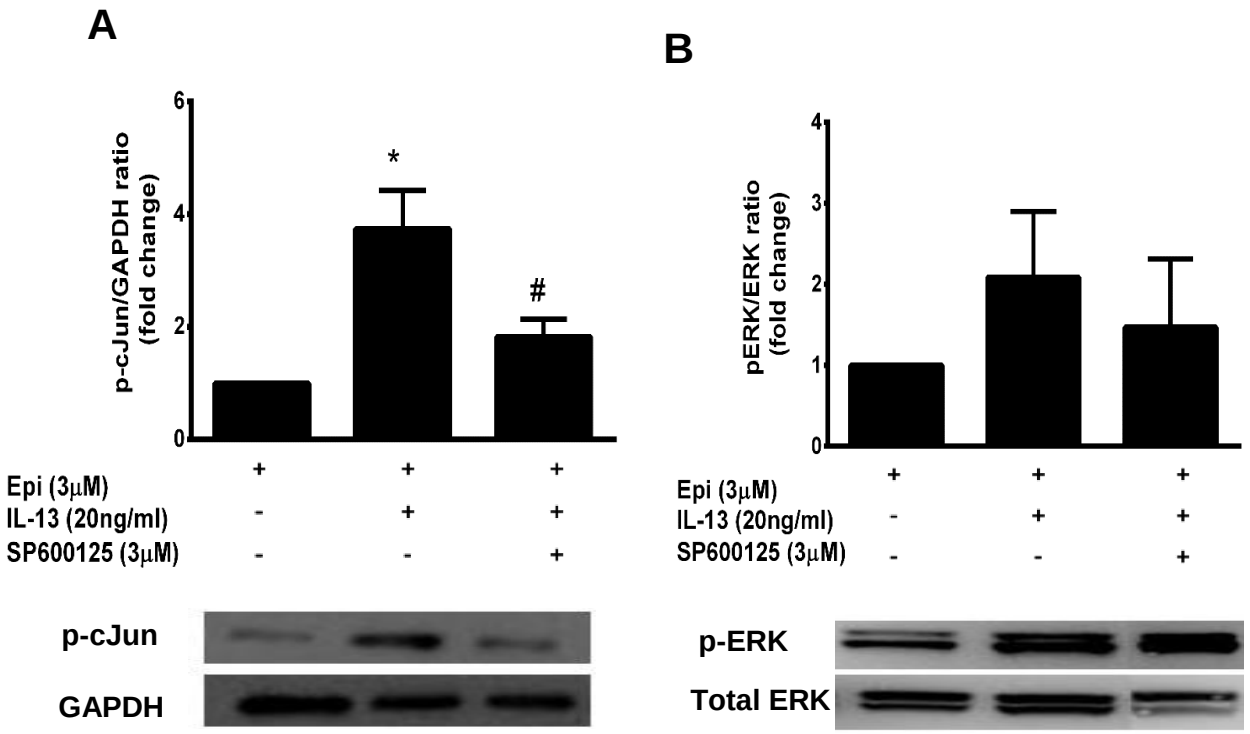



**Figure 33.** ***Effect of JNK inhibitor on the integrity of NHBE cells monolayer in response to IL-13:*** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM SP600125 (JNK inhibitor) for 14 days. **A)** Trans-epithelial electrical resistance (TEER) was measured one day before ALI and throughout ALI period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 μm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Data are presented as means ± SEM from three donors. * indicates $p<0.05$ significant difference between +epi and +epi+IL-13 treated cells.



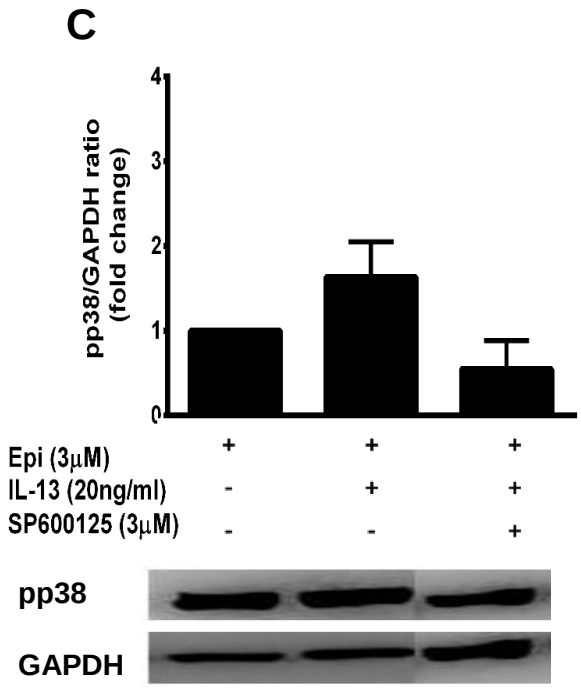




**Figure 34.** ***Effects of JNK inhibitor on activation of MAPKs in NHBE cells in response to IL-13:*** At ALI, NHBE cells were treated with 20 ng/ml IL-13 in combination with 3 μM SP600125 (JNK inhibitor) for 14 days in the presence of 3 μM epinephrine. Total proteins were harvested, and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** cJun phosphorylation in NHBE cells. **B)** ERK1/2 phosphorylation and **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

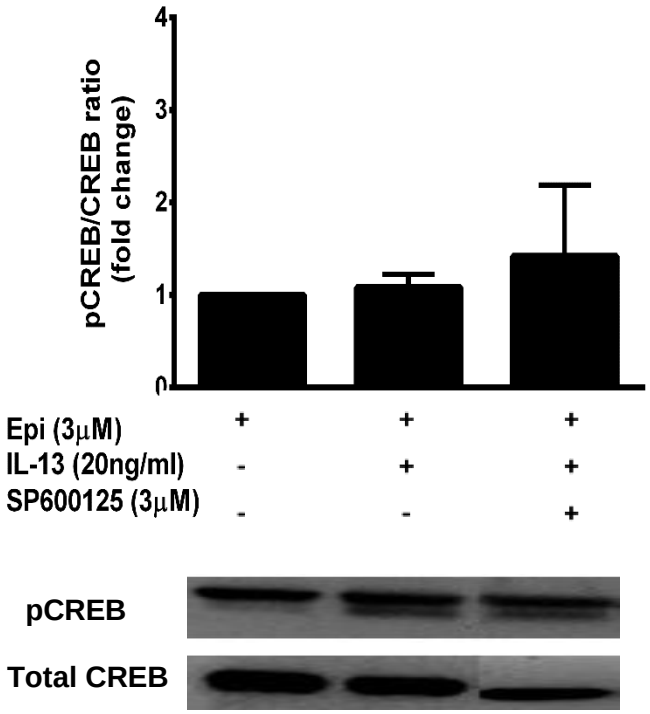



**Figure 35.** ***Effect of JNK inhibitor on activation of CREB in NHBE cells in response to IL-13:*** At ALI, NHBE cells were treated with 20 ng/ml IL-13 in combination with 3 μM SP600125 (JNK inhibitor) for 14 days in the presence of 3 μM epinephrine. Total proteins were harvested. Immunoblots were performed using antibodies to phosphorylated CREB proteins. The signal densities of the phosphorylated proteins were normalized to total CREB protein density and the relative intensities were reported as the degree of activation of the protein. The data presented as fold change compared to the corresponding control cells. CREB phosphorylation. Data are presented as means ± SEM from three donors.

### 4.3.3 Role of p38 signaling in mucin production in response to IL-13 in NHBE cells

Next, we examined the involvement of the third member of the traditional MAPK family, p38. IL-13 did not increase p38 phosphorylation either in the presence or absence of p38 (Figure 24C). Subsequently, we studied if the basal level of phosphorylated p38 plays any role in modulating the mucin parameters induced by IL-13 in the presence of epinephrine. We treated the cells with 3 μM SB203580, a p38 inhibitor, in combination with IL-13 and in the presence of epinephrine for 14 days. SB203580 significantly attenuated the IL-13 induced increase in MUC5AC expression (15.18 ± 3.76 fold increase by IL-13 vs 0.80 ±0. 65 fold by SB203580, $p<0.05$) (Figure 36A). Intracellular mucin 5AC and mucin glycoproteins were also inhibited by SB203580 (Figure 36B and 37).

SB203580 did not reduce the TEER readings below the control cells, instead it increased these values as compared to control or IL-13 treated cells (Figure 38A). Morphologically, the localization of ZO-1 at the cell peripheries did not change (Figure 38B).

Inhibiting p38 did not affect the phosphorylation of other MAPKs; p38, ERK1/2 or of cJun, a specific substrate of JNK (Figure 39A, B and C). Moreover, inhibiting p38 activity by SB203580 did not affect the activation of MAP kinase activated protein kinase-2 (MAPKAP kinase-2), a downstream substrate of p38, or of the CREB transcription factor (Figure 40A and B).

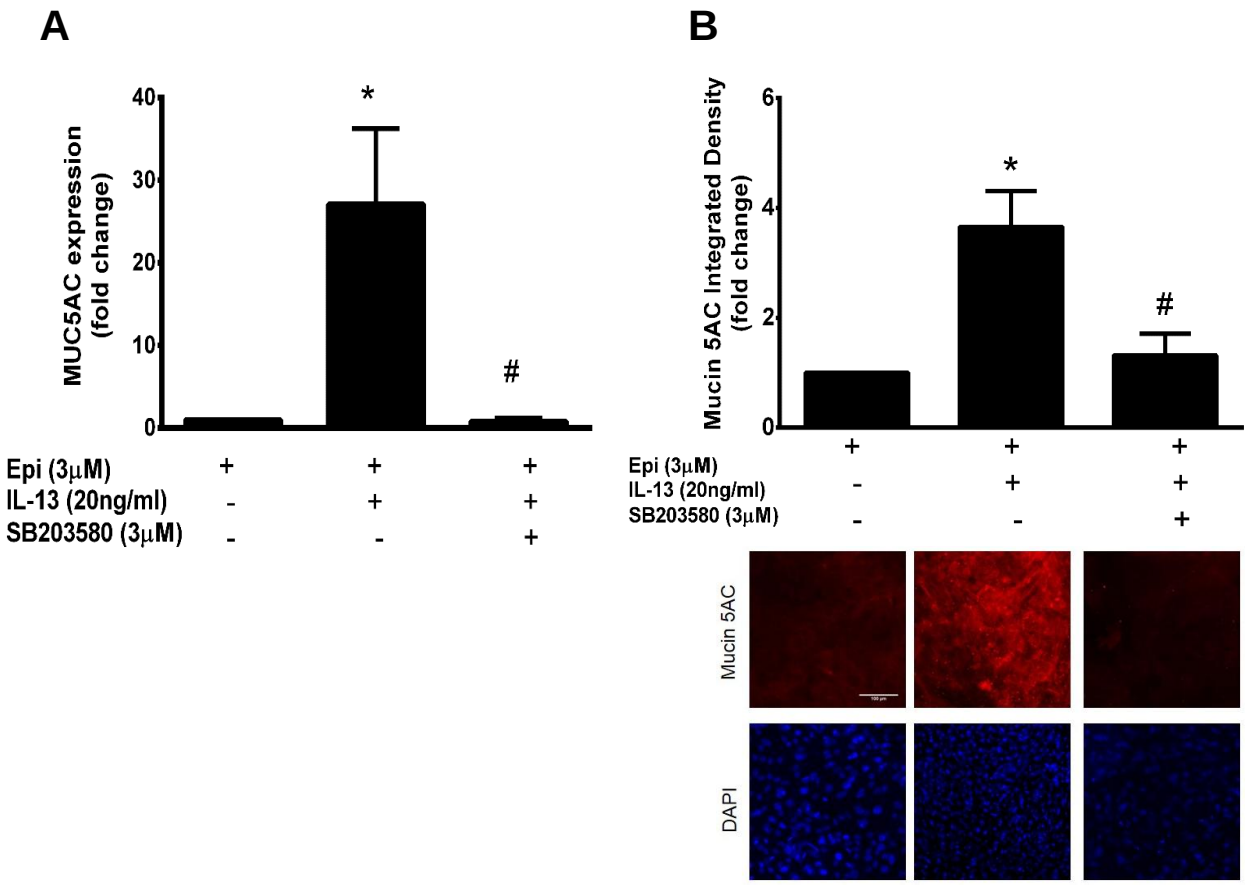



**Figure 36.** ***Role of p38 signaling in mucin 5AC production in response to IL-13 in NHBE cells*****:** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM SB203580 (p38 inhibitor) for 14 days. **A)** MUC5AC transcripts were measured by qRT-PCR, and the data presented as fold change compared to cells grown in the presence of epinephrine only. **B)** The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi+IL-13 treated cells respectively.

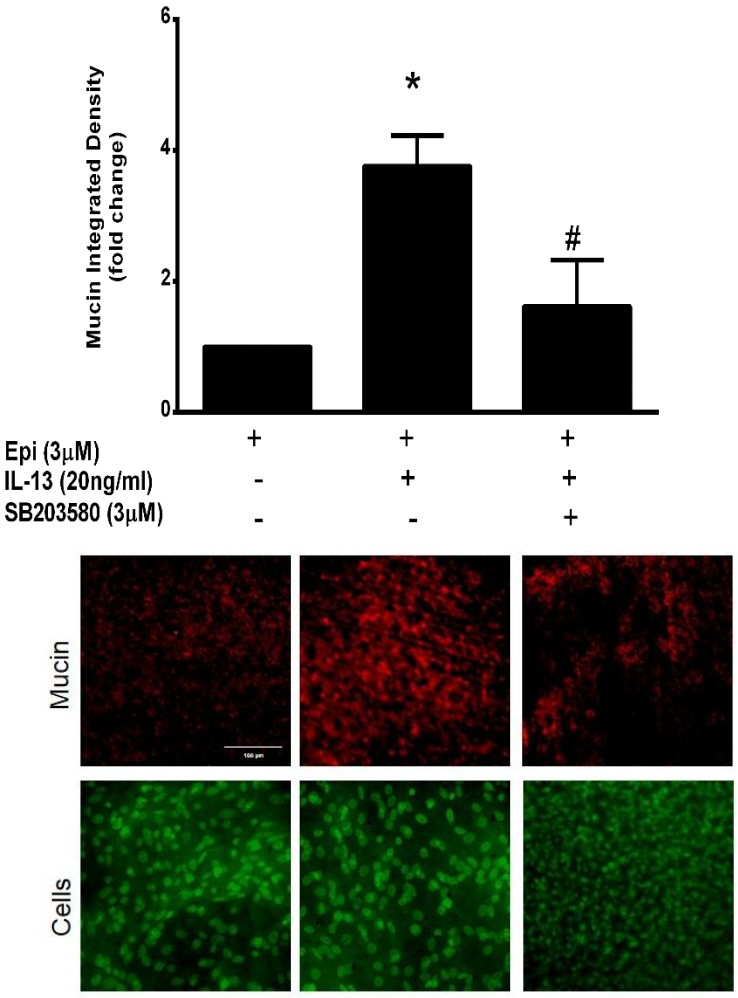



**Figure 37.** ***Role of p38 signaling in mucin production in response to IL-13 in NHBE cells*****:** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM SB203580 (p38 inhibitor) for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

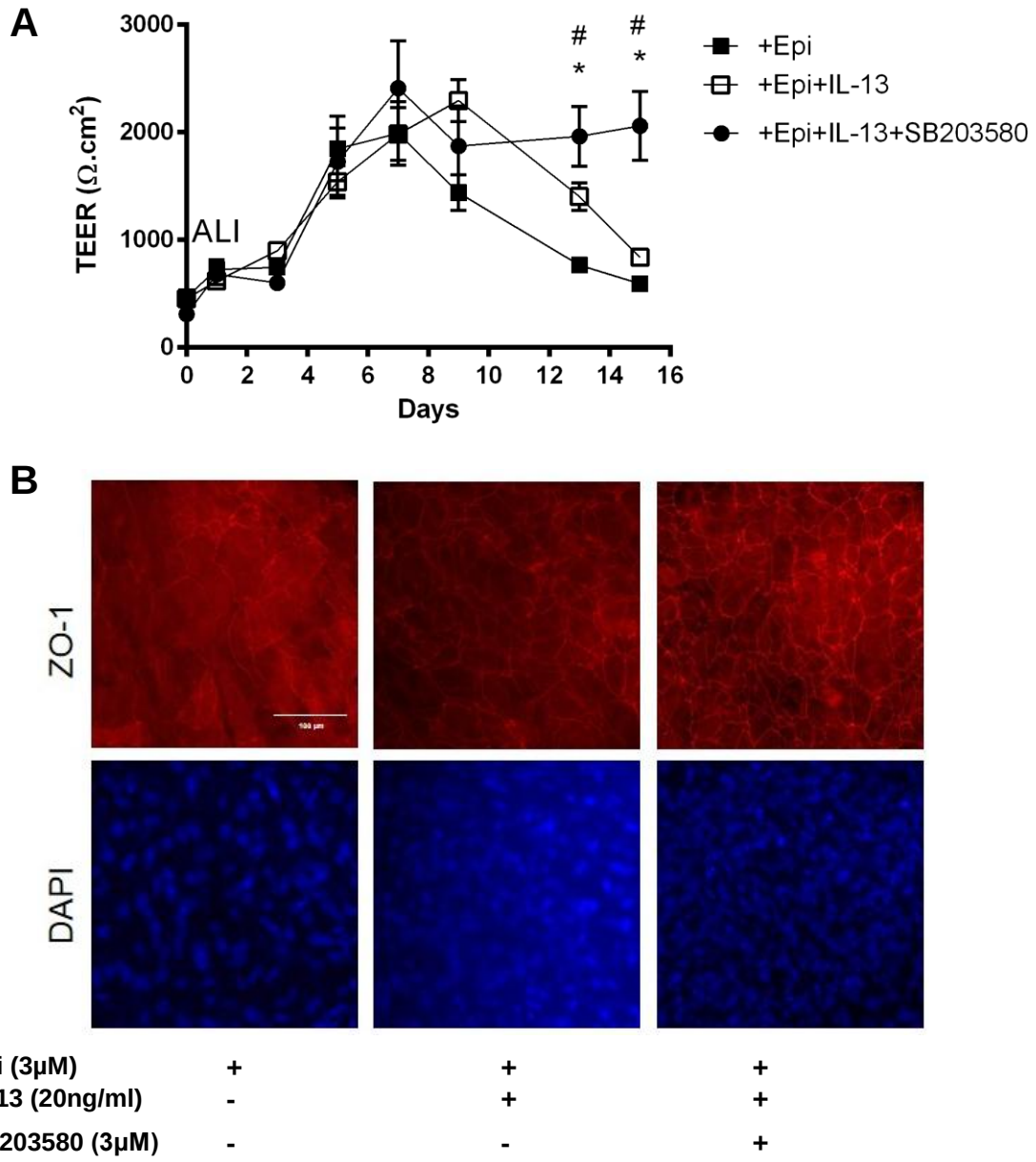
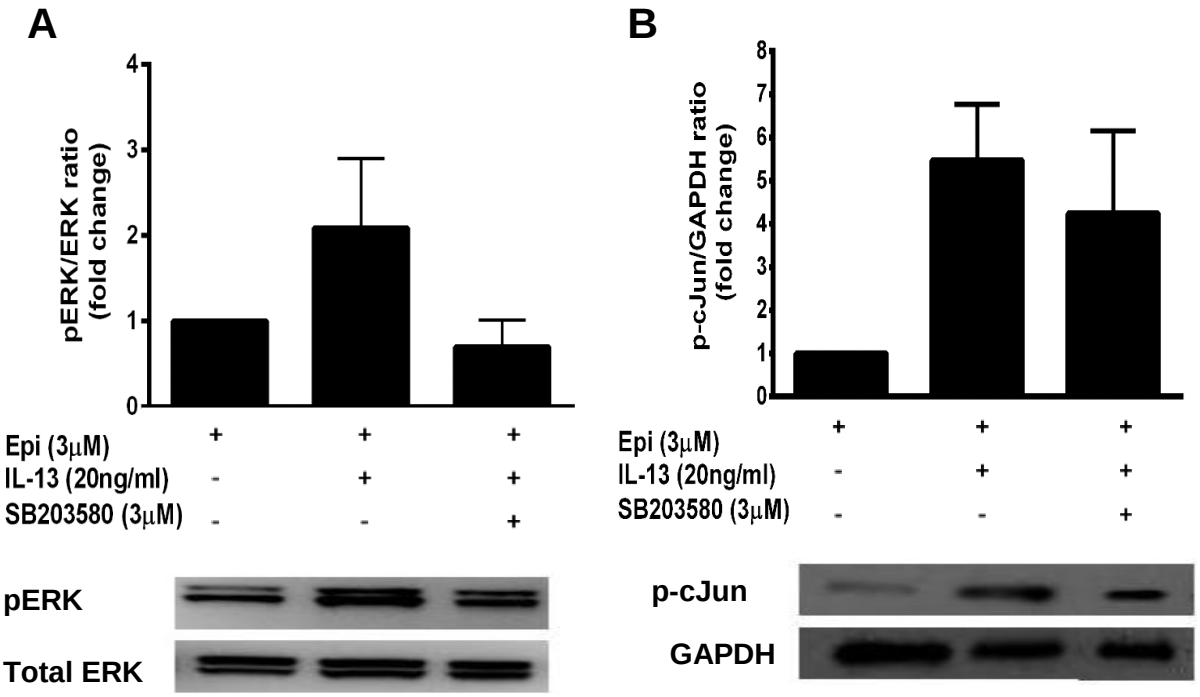



**Figure 38.** ***Effect of p38 inhibitor on the integrity of NHBE cells monolayer in response to IL-13:*** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM SB203580 (p38 inhibitor) for 14 days. **A)** Trans-epithelial electrical resistance (TEER) was measured one day before ALI and throughout the ALI period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 μm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Data are presented as means ± SEM from three donors. *, @ indicates $p<0.05$ significant difference between +epi and +epi+IL-13 and between +epi and +epi+IL-13+SB203580 respectively.



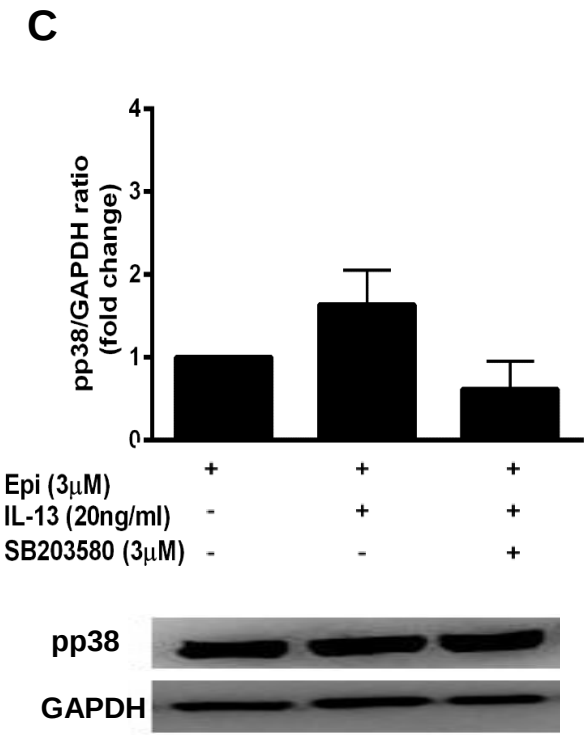




**Figure 39.** ***Effects of p38 inhibitor on activation of MAPKs in NHBE cells in response to IL-13:*** At ALI, NHBE cells were treated with 20 ng/ml IL-13 in combination with 3 μM SB203580 (p38 inhibitor) for 14 days in the presence of 3 μM epinephrine. Total proteins were harvested, and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation in NHBE cells. **B)** cJun phosphorylation and **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

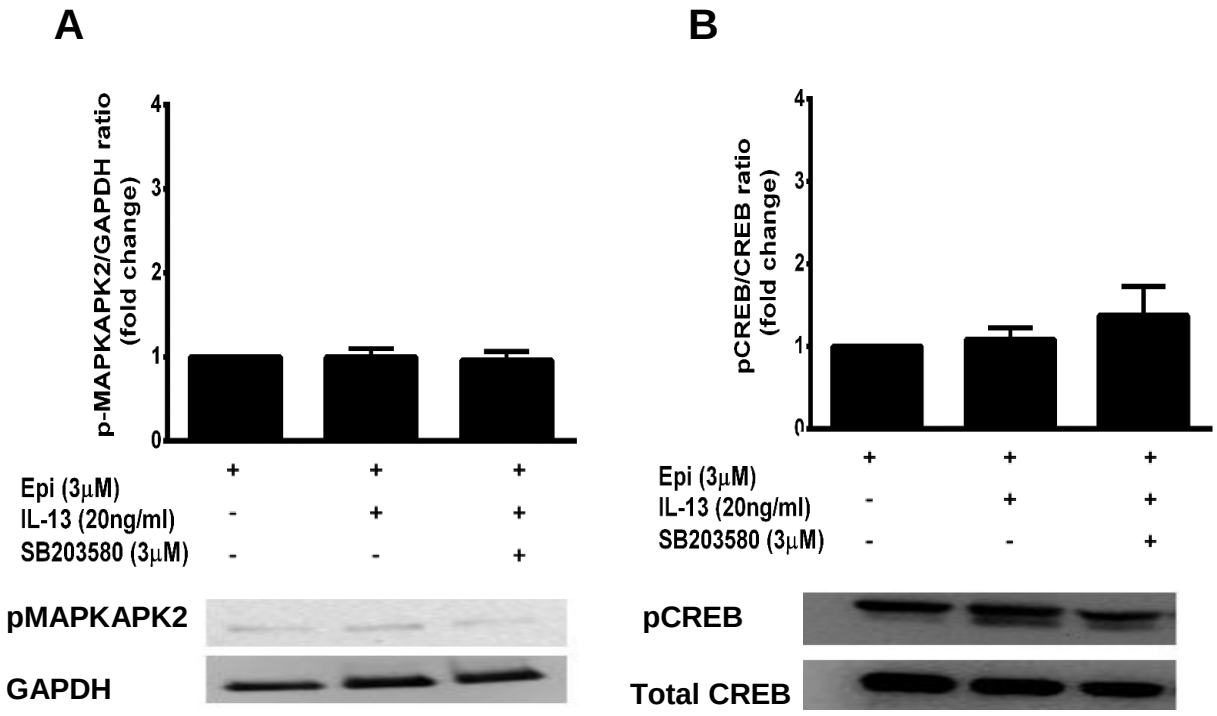



**Figure 40.** ***Effects of p38 inhibitor on activation of MAPKAPK2 and CREB in NHBE cells in response to IL-13:*** At ALI, NHBE cells were treated with 20 ng/ml IL-13 in combination with 3 μM SB203580 (p38 inhibitor) for 14 days in the presence of 3 μM epinephrine. Total proteins were harvested, and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** MAPKAPK2 phosphorylation in NHBE cells. **B)** CREB phosphorylation. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

## 4.4 Role of PKA signaling in mucin production in response to IL-13 in NHBE cells

$\beta_2$AR can signal through the canonical Gs-AC pathway and β-arrestin signaling cascades. The canonical Gs pathway leads to increase intracellular cAMP and PKA activation. Here we studied the involvement of PKA and cAMP separately and their effect on IL-13 induced mucin production in NHBE cells.

First, we treated the cells with 3μM H89, a PKA inhibitor, in combination with IL-13 and in the presence of epinephrine. H89 reduced MUC5AC expression significantly in response to IL-13 (15.02± 8.42 fold by IL-13 vs 2.09± 2.28 fold by H89, $p<0.05$) (Figure 41A). Since PKA activates ERK1/2 (Schmitt and Stork 2000; Gerits, Kostenko et al. 2008), we measured ERK1/2 phosphorylation in response to IL-13 and IL-13+H89. H89 did not reduce ERK1/2 phosphorylation as compared to IL-13 induced levels (Figure 41B).

Due to the several limitations of H89 that will be discussed later, we studied the involvement of PKA using a competitive cAMP analogue, Rp-cAMPS, in combination with IL-13 and epinephrine. Rp-cAMPS did not significantly reduce the levels of MUC5AC expression at 50 μM (5.97 ± 4.29 fold *vs* 12.50 ± 5.38 fold increase by IL-13, $p>0.05$) while at 100 μM, there was a significant reduction (2.35 ± 1.63 fold *vs* 12.50 ± 5.38 fold increase by IL-13, $p<0.05$) (Figure 42A). The intracellular mucin 5AC protein level was significantly reduced when the cells were treated with 100 μM Rp-cAMPS but not at 50 μM, while mucin

glycoproteins levels were reduced at both concentrations (Figure 42B and Figure 43).

The two concentrations of Rp-cAMPS that we used in our studies did not induce any cytotoxicity to the monolayer, as evaluated by TEER measurement and ZO-1 localization (Figure 44 A and B).

To understand the mechanism by which Rp-cAMPS reduced mucin production in NHBE cells, we examined the activity of several PKA downstream signaling molecules. Rp-cAMPS at 100 μM increased ERK1/2 phosphorylation as compared to control cells or IL-13 treated cells (Figure 44A). However, cJun, p38, CREB and STAT6 phosphorylation state by IL-13 was not affected by Rp-cAMPS (Figure 45B, C and Figure 46A, B).

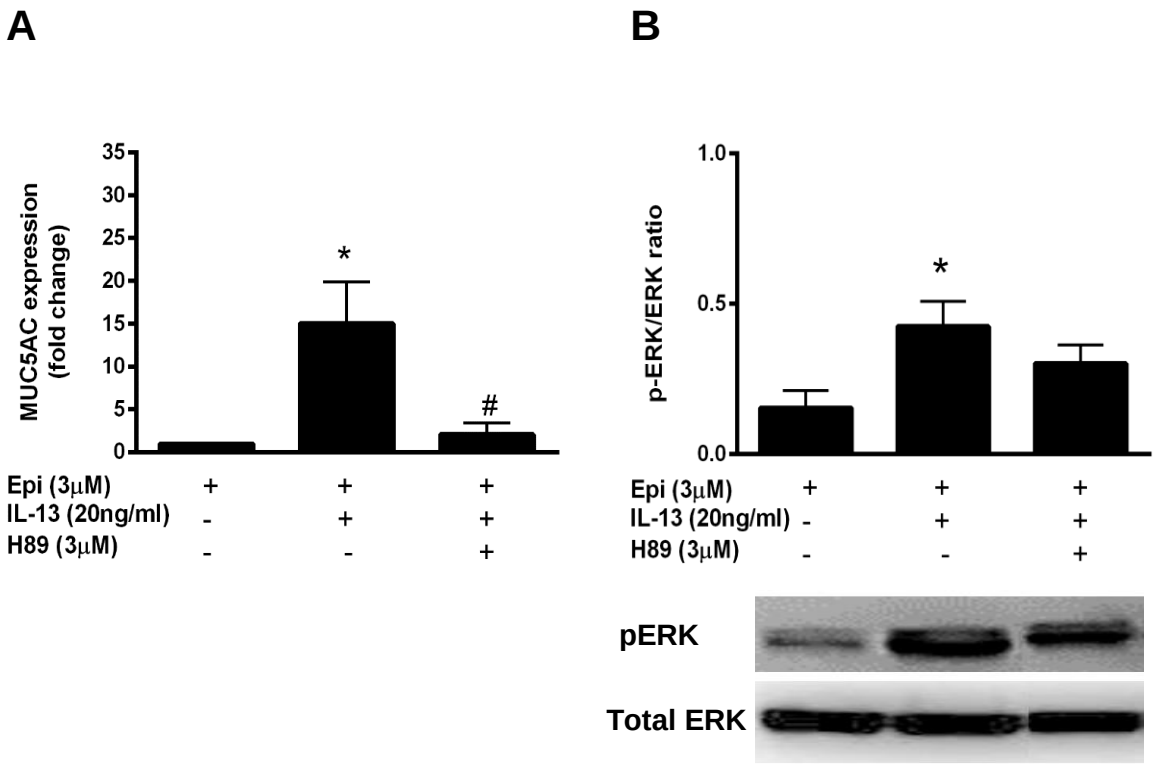



**Figure 41.** ***Role of H89 in MUC5AC expression in response to IL-13 in NHBE cells:*** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 3 μM H89 (PKA inhibitor) for 14 days. **A)** MUC5AC transcripts were measured by qRT-PCR, and the data presented as fold change compared to cells grown in the presence of epinephrine only. **B)** Immunoblots were performed using antibodies to the indicated phosphorylated ERK1/2. The signal densities of the phosphorylated proteins were normalized to total ERK1/2 protein density and the relative intensities were reported as the degree of activation of the protein. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi+IL-13 treated cells respectively.

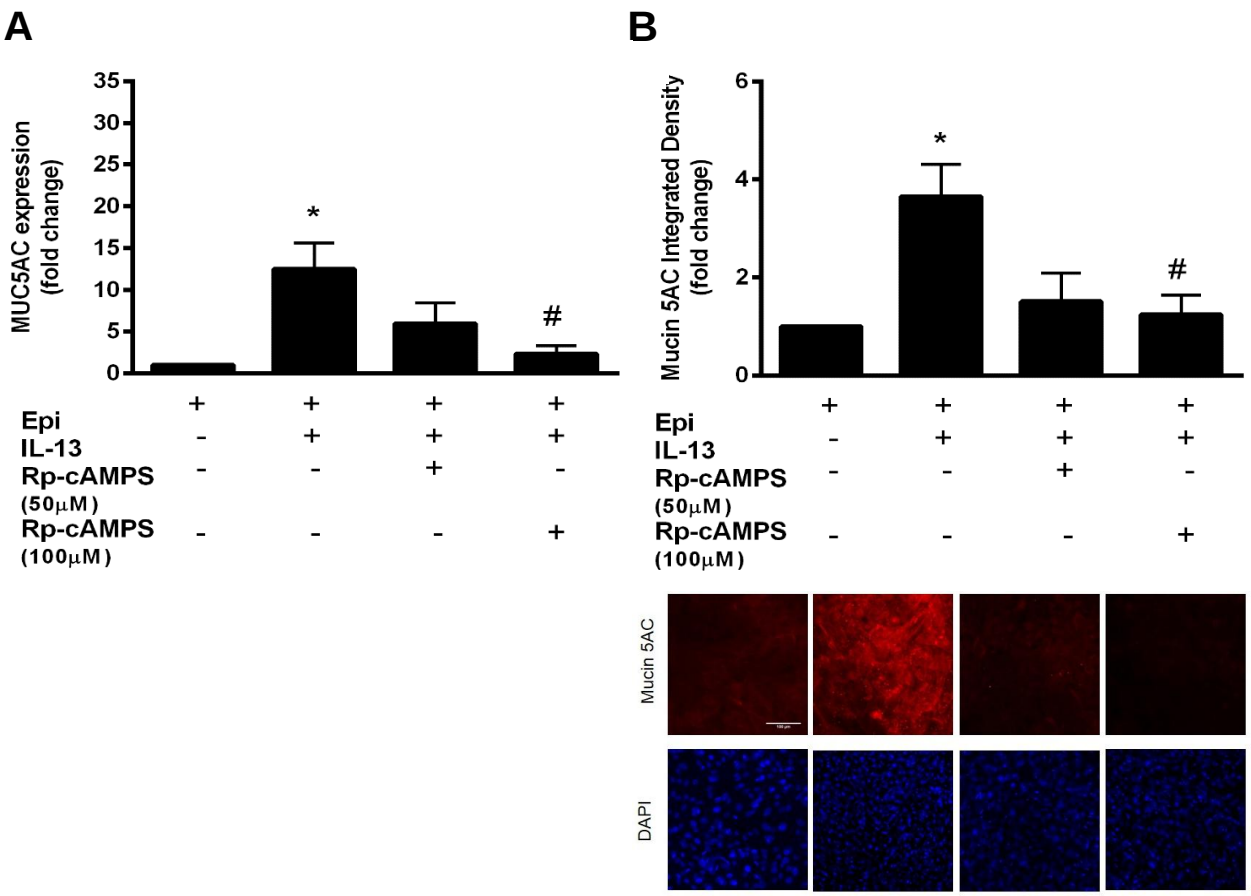



**Figure 42.** ***Role of PKA signaling in mucin 5AC production in response to IL-13 in NHBE cells*****:** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with either 50 μM or 100 μM Rp-cAMPS (cAMP dependent protein kinase inhibitor) for 14 days. **A)** MUC5AC transcripts were measured by qRT-PCR, and the data presented as fold change compared to cells grown in the presence of epinephrine only. **B)** The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are (scale bar = 100 μm). Data are presented as means ± SEM from three donors. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi+IL-13 treated cells respectively.

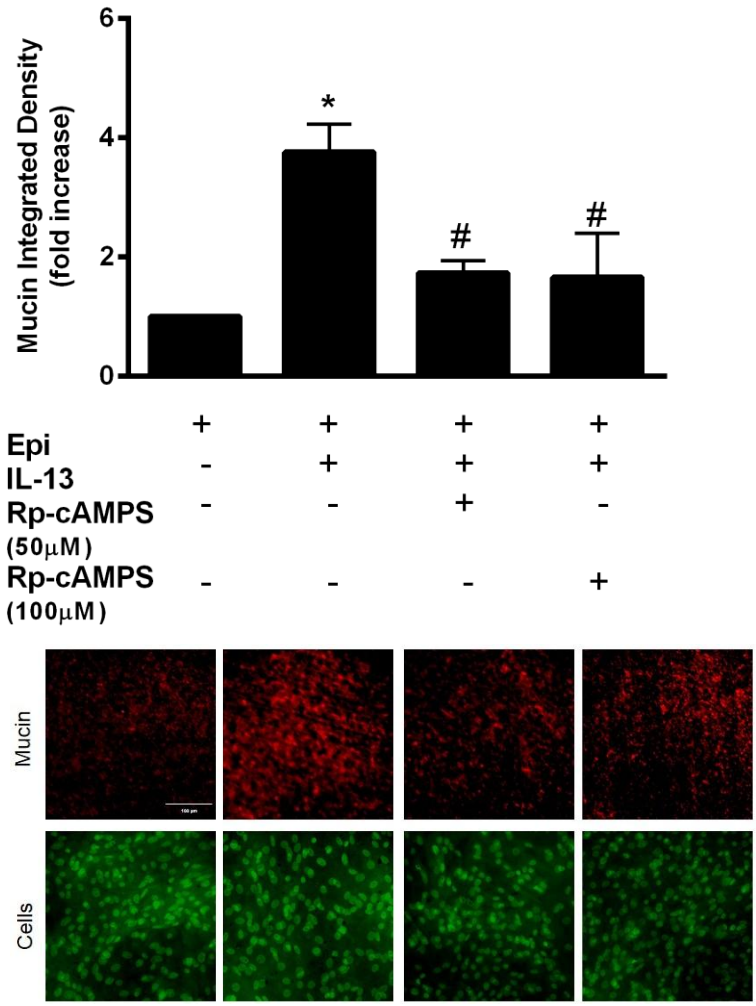



**Figure 43.** ***Role of PKA signaling in mucin production in response to IL-13 in NHBE cells*****:** The cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with either 50 μM or 100 μM Rp-cAMPS (cAMP dependent protein kinase inhibitor) for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

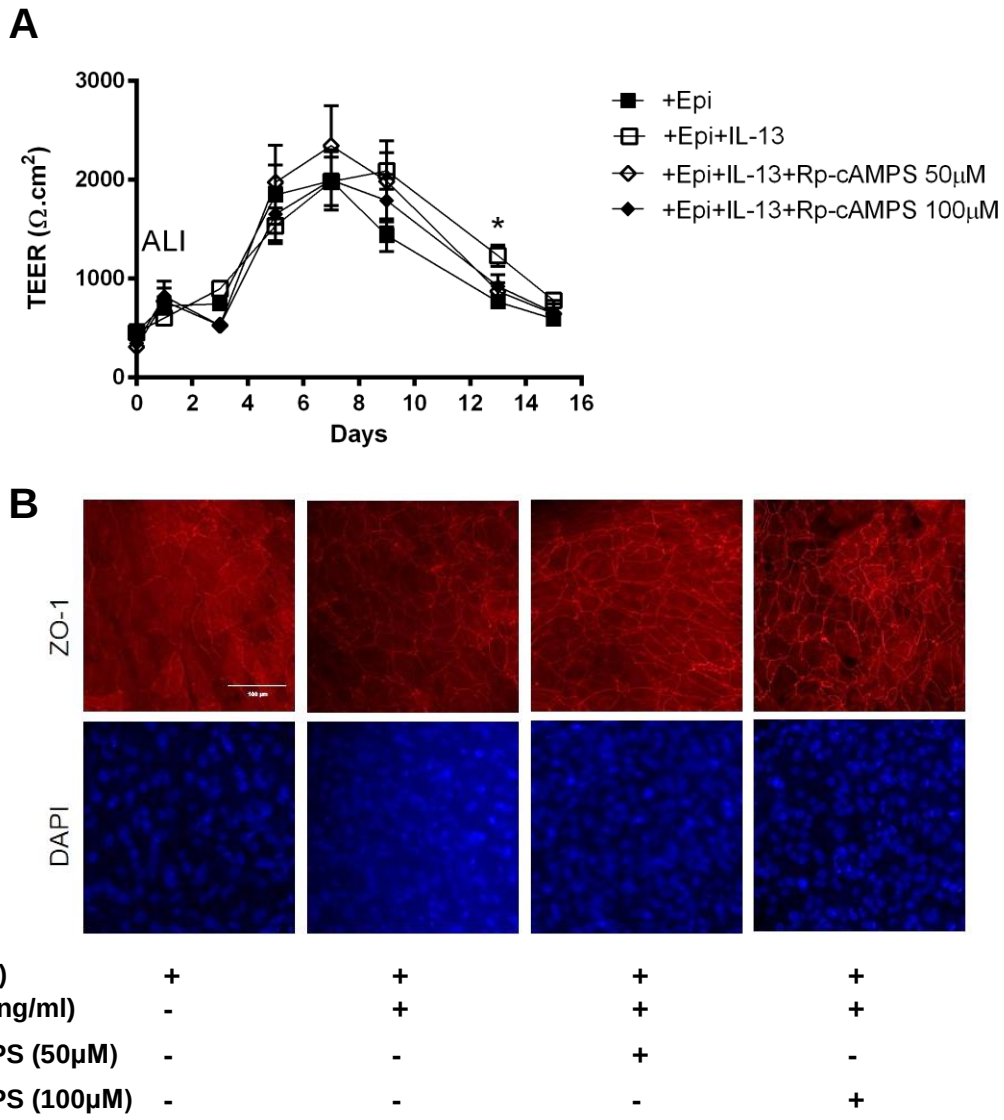
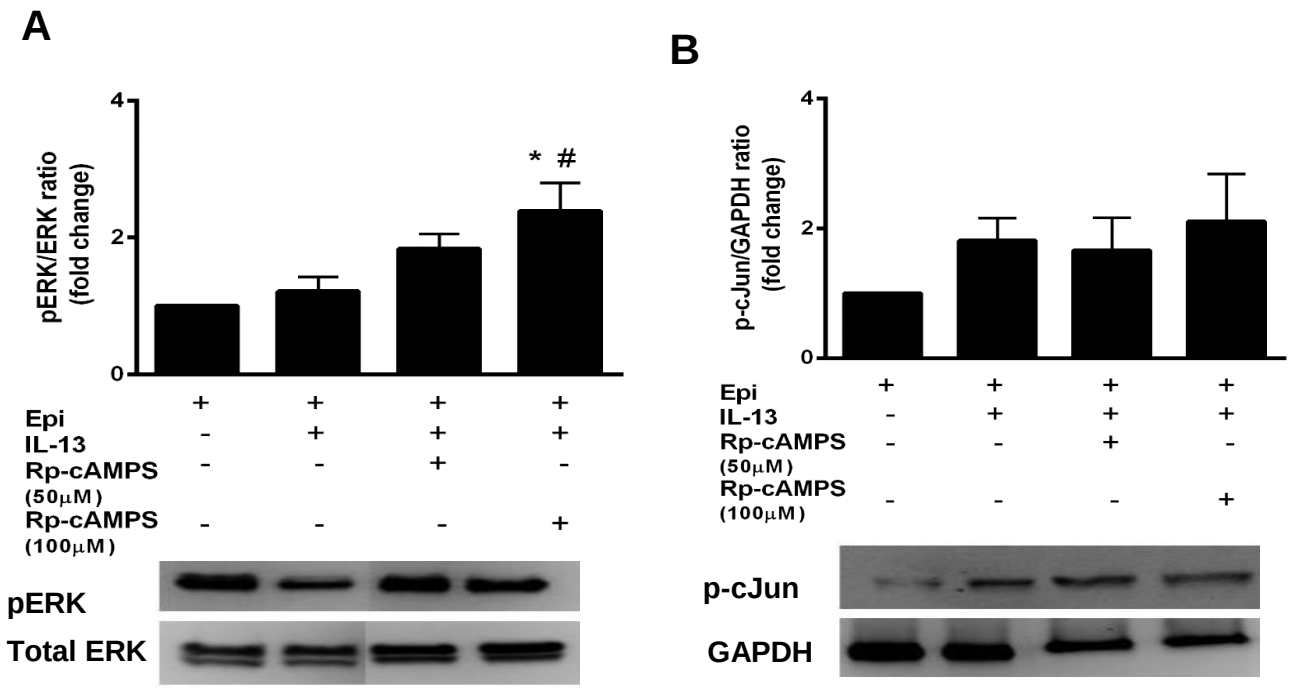



**Figure 44.** ***Effect of PKA inhibitor on the integrity of NHBE cells monolayer in response to IL-13:*** NHBE cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with either 50 μM or 100 μM Rp-cAMPS (cAMP dependent protein kinase inhibitor) for 14 days. **A)** Trans-epithelial electrical resistance (TEER) was measured one day before ALI and throughout ALI period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 μm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Data are presented as means ± SEM from three donors. * indicates $p<0.05$ significant difference between +epi and +epi+IL-13.



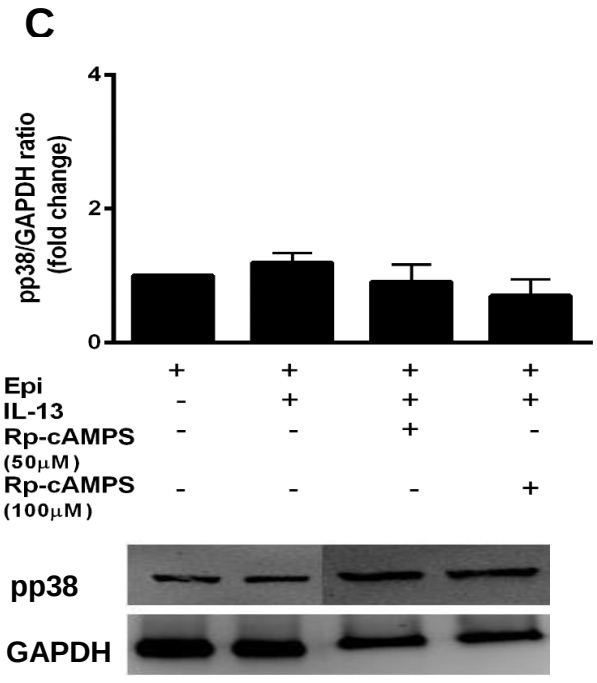




**Figure 45.** ***Effects of PKA inhibitor on activation of MAPKs in NHBE cells in response to IL-13:*** NHBE cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with either 50 μM or 100 μM Rp-cAMPS (cAMP dependent protein kinase inhibitor) for 14 days. Total proteins were harvested, and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation in NHBE cells. **B)** cJun phosphorylation and **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

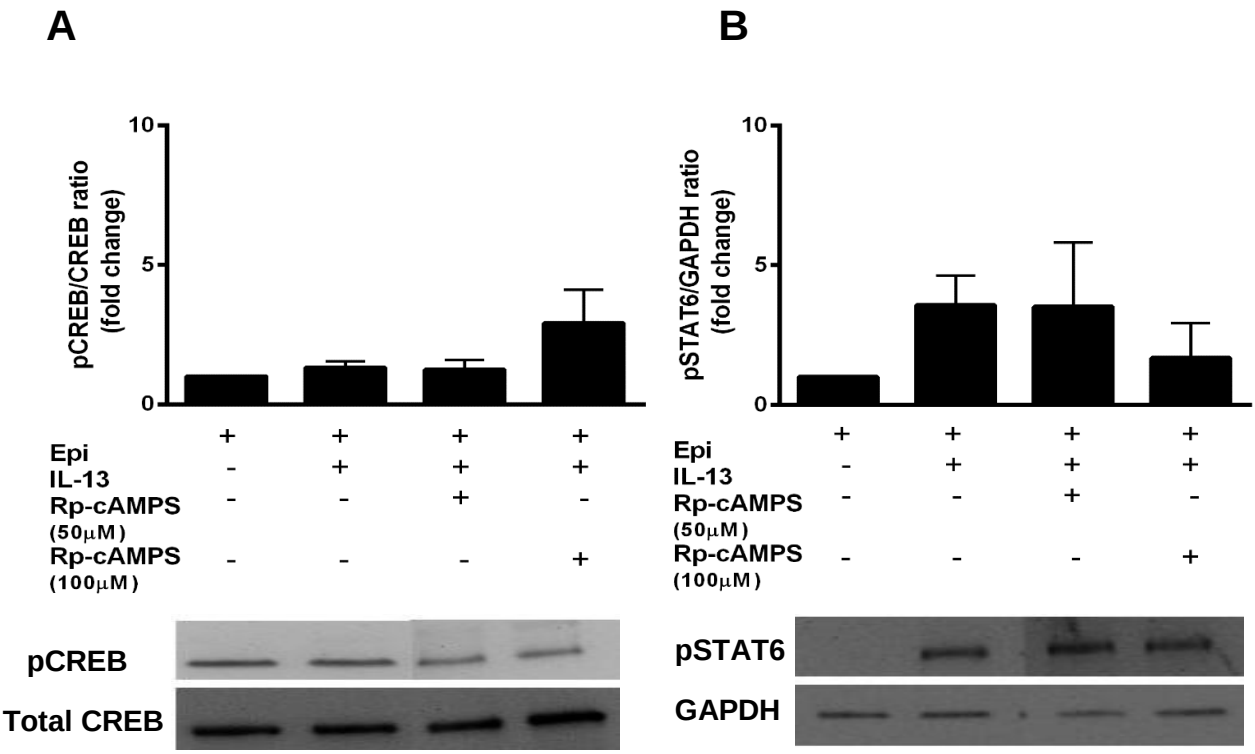



**Figure 46.** ***Effects of PKA inhibitor on activation of CREB and STAT6 in NHBE cells in response to IL-13:*** At ALI, NHBE cells were treated with 20 ng/ml IL-13 in combination with either 50 μM or 100 μM Rp-cAMPS (cAMP dependent protein kinase inhibitor) for 14 days in the presence of 3 μM epinephrine. Total proteins were harvested, and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** CREB phosphorylation in NHBE cells. **B)** STAT6 phosphorylation. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi +IL-13 treated cells respectively.

### 4.5 Effect of cAMP elevating agents in mucin production in response to IL-13 in NHBE cells

#### 4.5.1 Effect of forskolin+IBMX in mucin production in response to IL-13 in NHBE cells

We evaluated the effect of elevating intracellular cAMP Levels on mucin parameters in response to IL-13. For this purpose, we treated cells with 10 μM forskolin combined with 100 μM 3-isobutyl-l-methylxan-thine (IBMX), in the absence of epinephrine for 14 days. This treatment caused a dramatic increase in MUC5AC expression (75.73 ± 66.59 fold *vs* 0.56 ± 0.40 fold by IL-13, $p<0.05$) (Figure 47A). Then we tested if the forskolin+IBMX combination potentiates the effect of IL-13 on mucin production in the *presence* epinephrine. MUC5AC expression was increased in response to IL-13 in the presence of epinephrine +forskolin+IBMX combination (120.29 ± 85.5 fold *vs* 12.29 ± 7.55 fold increase by IL-13, $p<0.05$) (Figure 47B). The forskolin+IBMX combination also increased intracellular mucin 5AC and mucin glycoproteins accumulation in response to IL-13 either in the presence or absence of epinephrine (Figure 48A, B and Figure 49A, B).

The effect of forskolin+IBMX combination did not alter the barrier function and the integrity of NHBE monolayer, as evaluated by TEERs measurements and ZO-1 apical localization (data not shown) (Figure 50A and B).

We next measured intracellular cAMP levels to evaluate the effectiveness of forskolin+IBMX combination in raising cAMP. The forskolin+IBMX combination resulted in an increased accumulation of intracellular cAMP under all conditions (Figure 51A and B). We then studied the involvement of different signaling molecules that could be activated by increasing intracellular cAMP levels and thereby mediate the increase in mucin production in response to IL-13. ERK1/2 phosphorylation in response to IL-13 did not change with forskolin+IBMX combination, either in the presence or absence of epinephrine (Figure 52A and Figure 53A). p38 and cJun activation were also not affected by forskolin+IBMX combination (Figure 52B, C and Figure 53B,C), while CREB activation was significantly increased by forskolin+IBMX and IL-13 treatment but only in the presence of epinephrine (Figure 54A,B). This increase was significant as compared to cells grown in the presence of epinephrine. Moreover, the phosphorylation of STAT6, an effector of IL-13 signaling cascade, was also increased by IL-13 in the presence of forskolin+IBMX and epinephrine (Figure 54C).

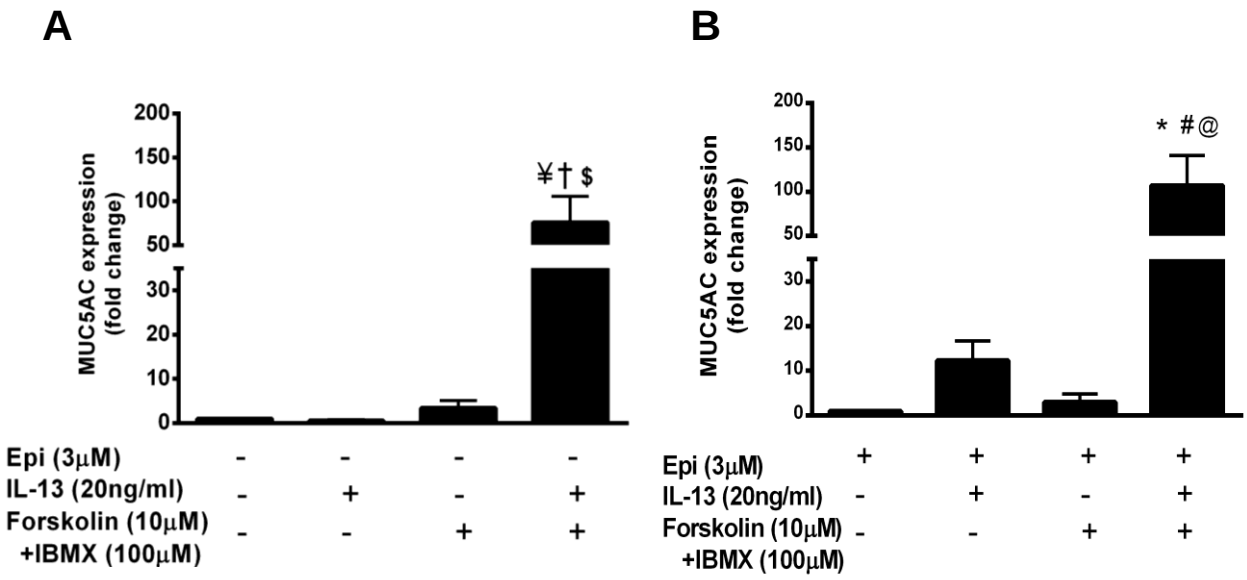



**Figure 47.** ***cAMP potentiates MUC5AC expression in response to IL-13 in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. MUC5AC transcripts levels were measured by qRT-PCR and the data presented as fold change compared to corresponding control cells grown in the absence of IL-13, IBMX and forskolin. Data are presented as means ± SEM from three donors. †, ¥,$ and *, #, @ indicate $p<0.05$ significance as compared to -epi, -epi+ IL-13,-epi+forskolin+IBMX, +epi and +epi+IL-13, +epi+forskolin+IBMX treated cells respectively.

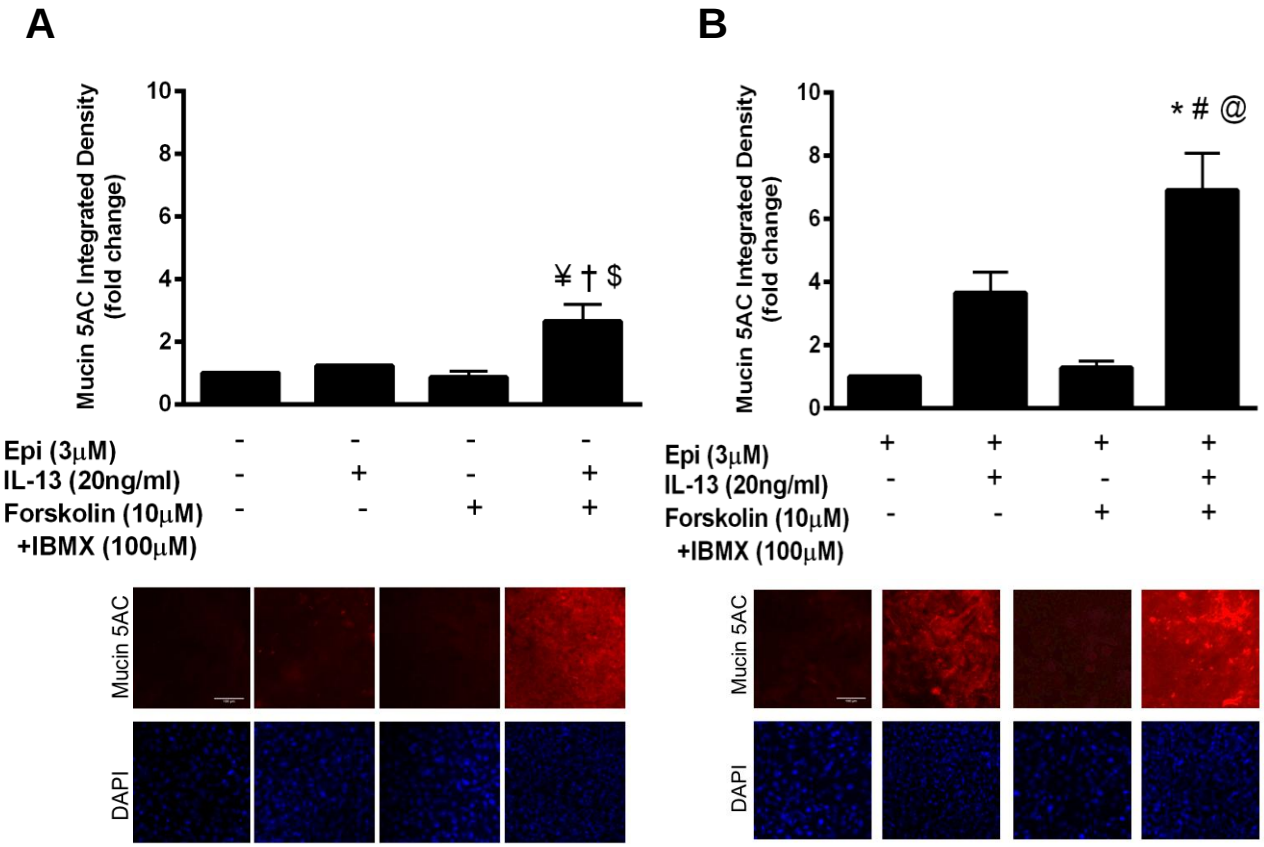



**Figure 48.** ***cAMP potentiates mucin 5AC production in response to IL-13 in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. †, ¥,$ and *, #, @ indicate $p<0.05$ significance as compared to -epi, -epi+ IL-13,-epi+forskolin+IBMX, +epi and +epi+IL-13, +epi+forskolin+IBMX treated cells respectively.

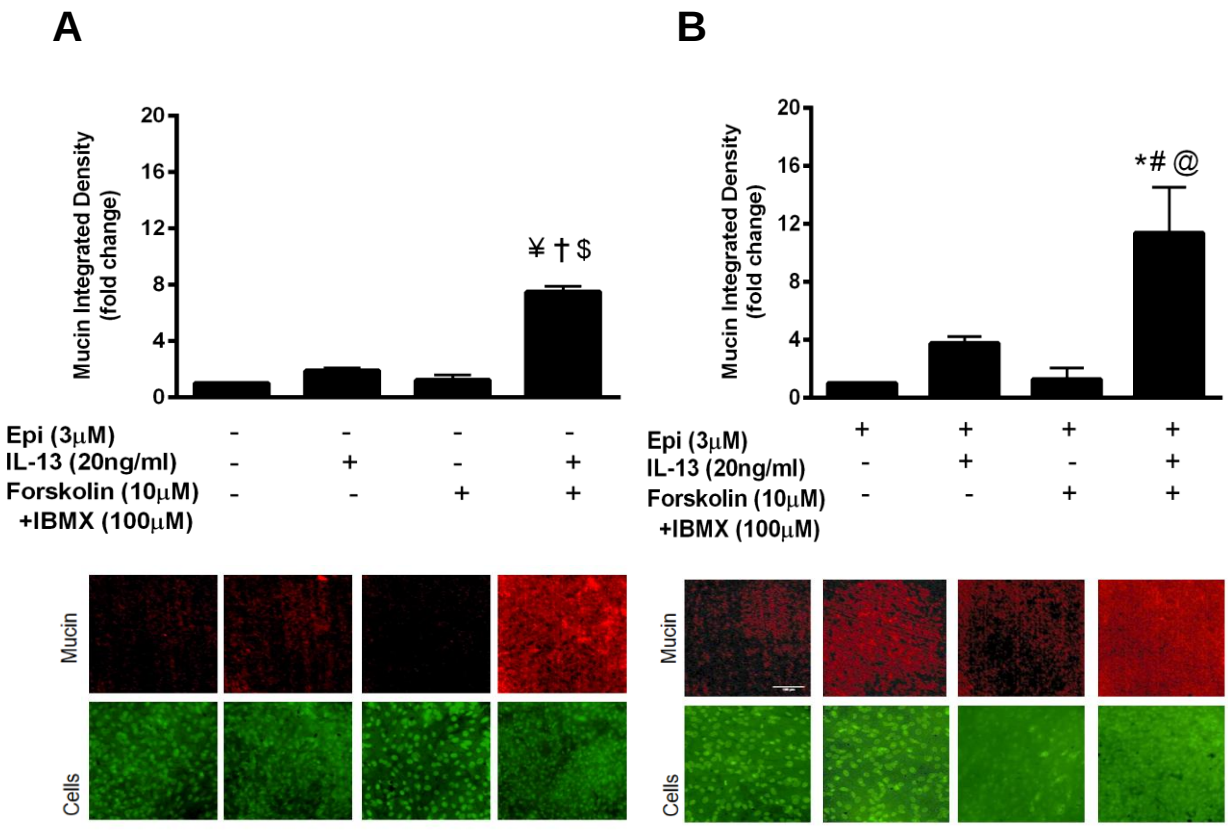



**Figure 49.** ***cAMP potentiates mucin production in response to IL-13 in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. †, ¥,$ and *, #, @ indicate $p<0.05$ significance as compared to -epi, -epi+ IL-13,-epi+forskolin+IBMX, +epi and +epi+IL-13, +epi+forskolin+IBMX treated cells respectively.

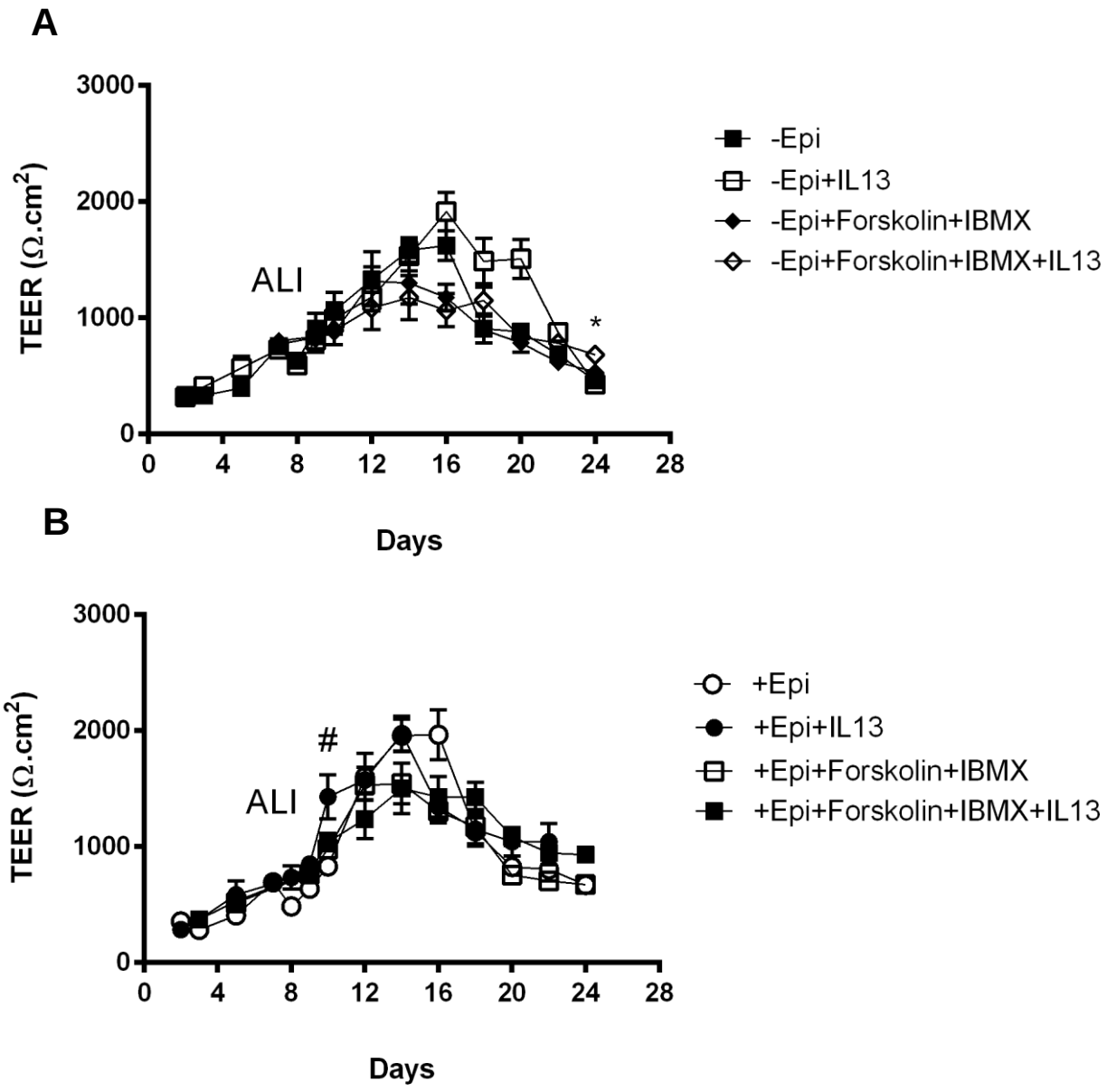



**Figure 50.** ***Effect of cAMP elevating agents on the integrity of NHBE cell monolayers****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. Trans-epithelial electrical resistance (TEER) was measured throughout the culture period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance between -epi and -epi+forskolin+IBMX+IL-13, and between +epi and +epi+IL-13 treated cells respectively.

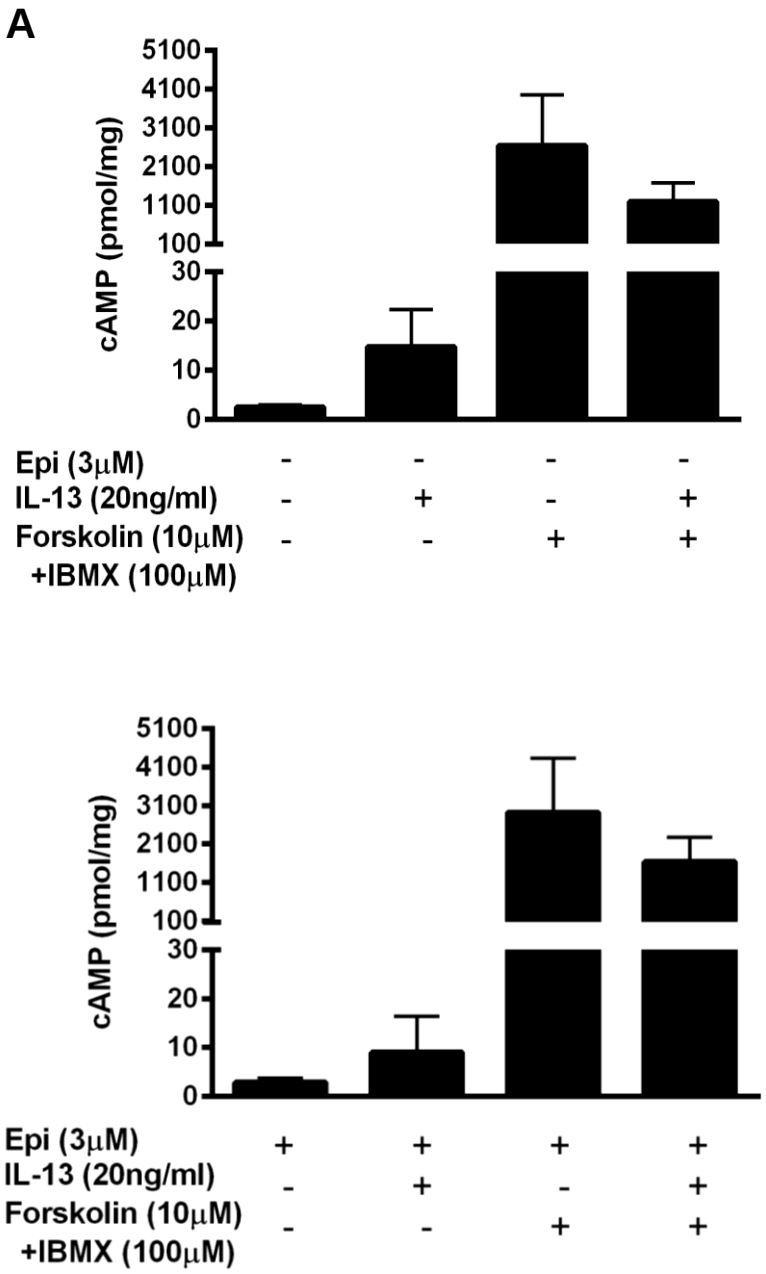
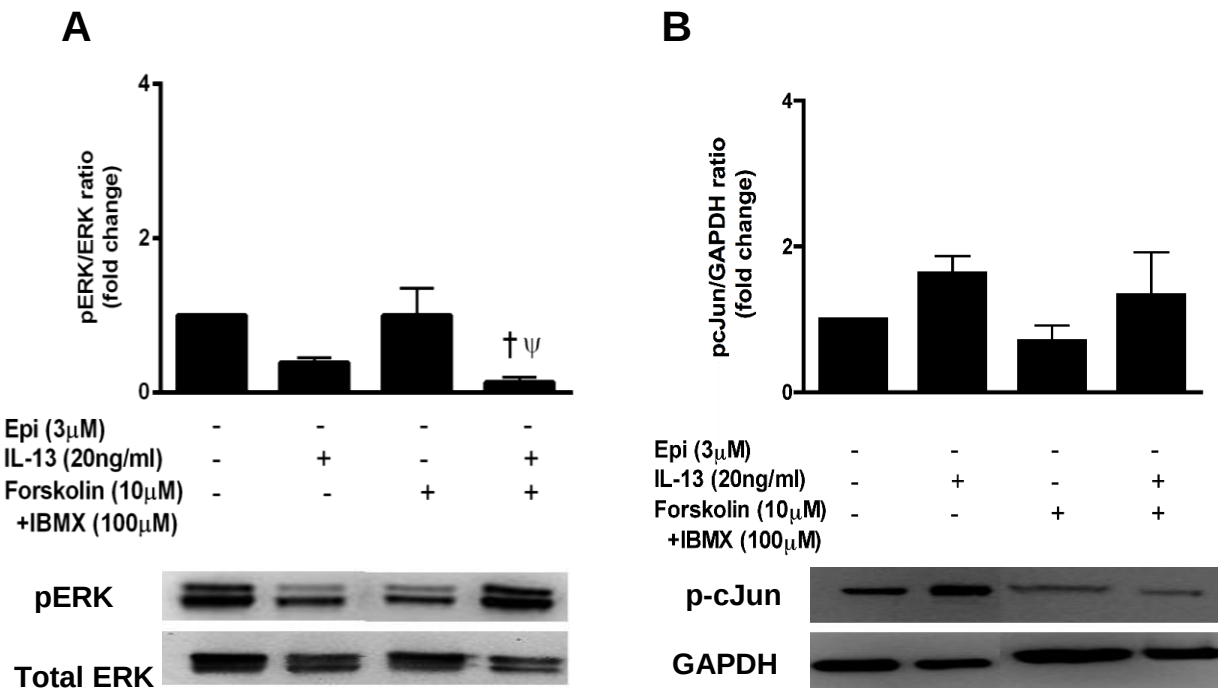



**Figure 51.** ***Effect of forskolin+IBMX combination on intracellular cAMP levels in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. Intracellular cAMP was measured in pmol and presented as pmol/mg after normalizing to total protein concentration. Data are presented as means ± SEM from three donors.



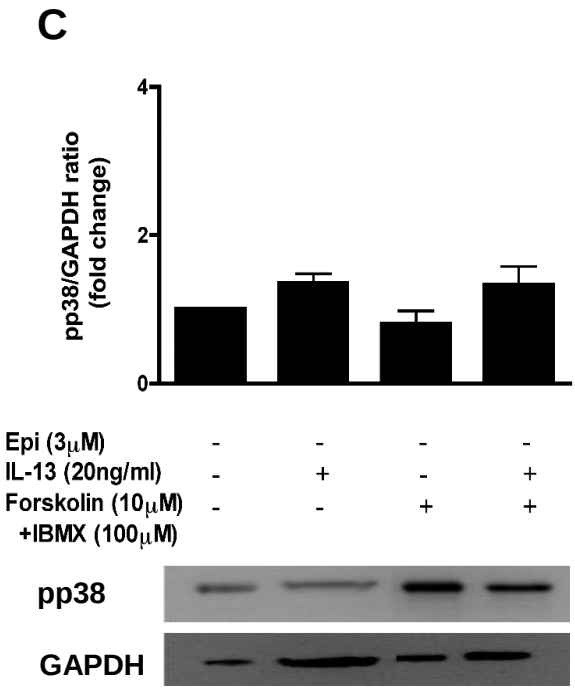
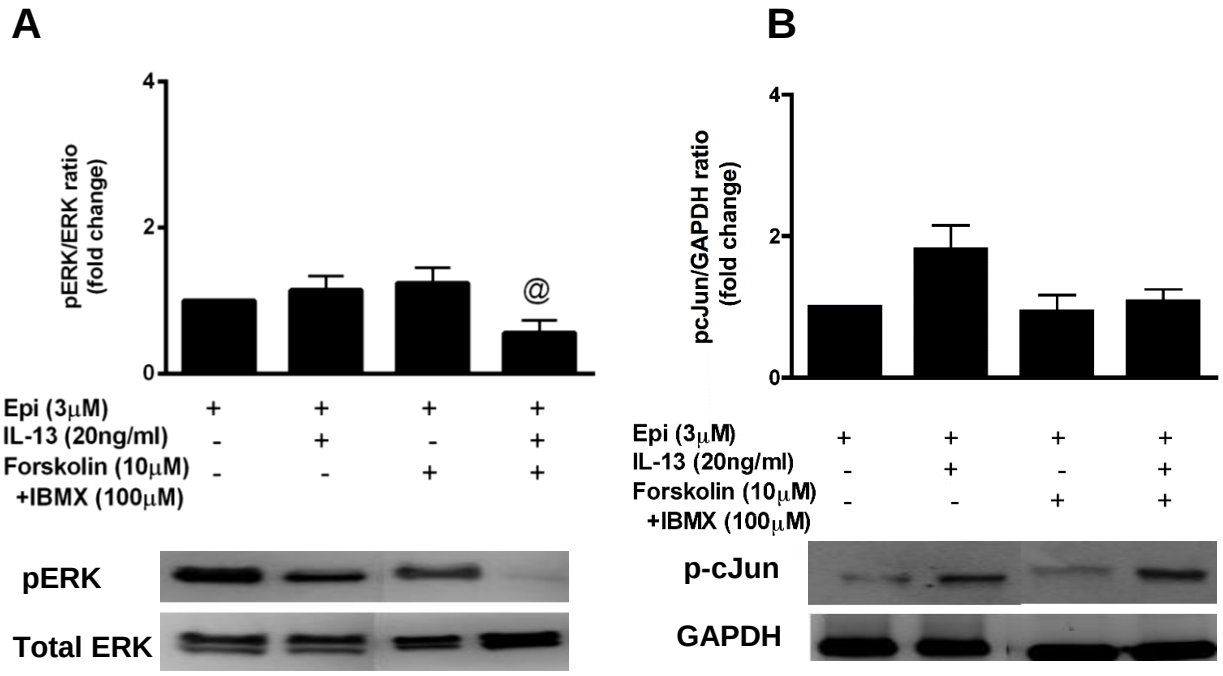




**Figure 52.** ***Effects of elevated intracellular cAMP on MAPK activation in NHBE cells in response to IL-13 in the absence of epinephrine:*** Cells were grown in the absence of epinephrine 72 hours before ALI**,** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. Total proteins were harvested and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation in NHBE cells. **B)** cJun phosphorylation and **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. † and ψ indicate $p<0.05$ significance as compared to -epi and -epi+forskolin+IBMX respectively.



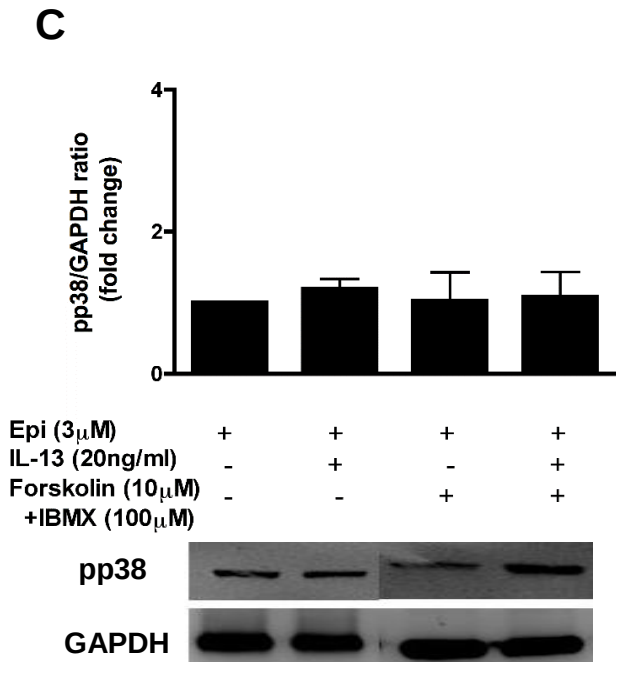
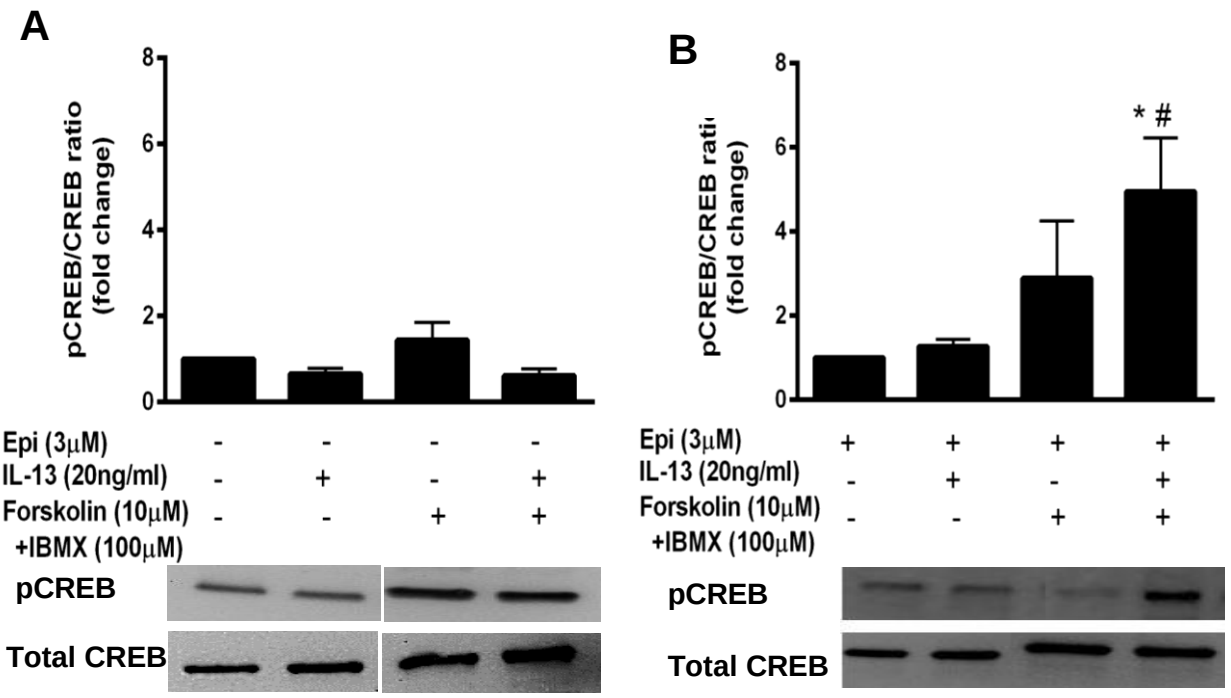




**Figure 53.** ***Effects of elevated intracellular cAMP on MAPK activation in NHBE cells in response to IL-13 in the presence of epinephrine:*** Cells were grown in the presence of epinephrine**,** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. Total proteins were harvested and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation in NHBE cells. **B)** cJun phosphorylation and **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. @ indicate $p<0.05$ significance as compared to +epi+forskolin+IBMX treated cells respectively.



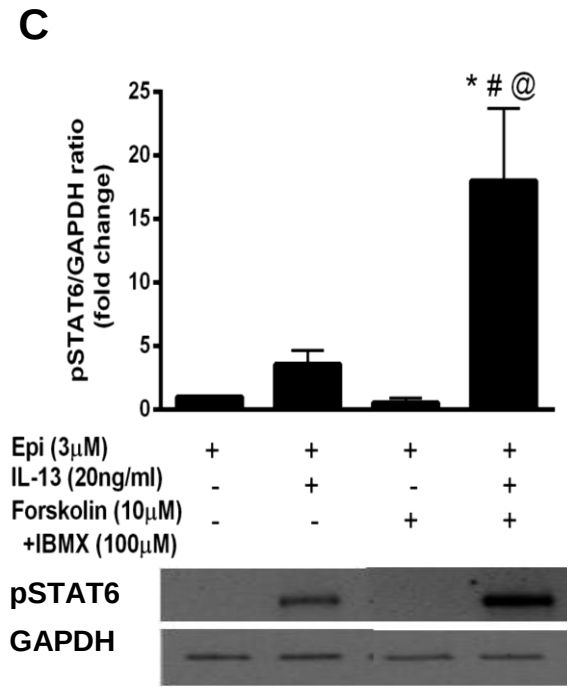




**Figure 54.** ***Effects of elevated intracellular cAMP on CREB, STAT6 activation in NHBE cells in response to IL-13:*** Cells were grown in the absence of epinephrine 72 hours before ALI **(A)**, or in presence of epinephrine **(B, C),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. Total proteins were harvested and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of CREB phosphorylation in NHBE cells **(A, B)** and STAT6 phosphorylation **(C)** were measured and normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. Data are presented as means ± SEM from three donors. *,# and @ indicate $p<0.05$ significance as compared to +epi, +epi+IL-13 and +epi+forskolin+IBMX treated cells respectively.

### 4.5.2 Role of a specific PDE inhibitor in mucin production in response to IL-13 in NHBE cells

Besides studying the global intracellular cAMP elevation induced by forskolin+IBMX combination, we used roflumilast, a specific PDE4 inhibitor, to study the involvement of compartmentalized cAMP signaling. Subcellular compartmentalization of cAMP is responsible for spatially and temporally controlling the generation and degradation of cAMP (Arora, Sinha et al. 2013) and consequently phosphorylation of specific downstream substrates (Di Benedetto, Zoccarato et al. 2008). We treated NHBE cells with 100 nM roflumilast in combination with IL-13 in the presence or absence of epinephrine. In the absence of epinephrine, roflumilast did not increase MUC5AC expression (1.57 ± 1.61 fold *vs* 0.72 ± 0.47 fold by IL-13, $p>0.05$) (Figure 55A). Intracellular accumulation of mucin 5AC and mucin glycoproteins were also not increased by roflumilast under the same conditions (Figure 56A and Figure 57A). Also, roflumilast did not alter the levels of MUC5AC transcripts that were induced by IL-13 and in the presence of epinephrine (3.48 ± 3.57 fold *vs* 12.29 ± 7.55 fold increase by IL-13, $p>0.05$) (Figure 55B). Intracellular accumulation of mucin 5AC and mucin glycoproteins were reduced in roflumilast+ IL-13 treated cells as compared to IL-13 treated cells (Figure 56B and Figure 57B). Roflumilast did not affect the integrity of epithelial cells monolayer, as evaluated by TEER measurements (Figure 58A and B).

Intracellular cAMP levels were not elevated in roflumilast+IL-13 treated cells as compared to cells grown in the absence or presence of epinephrine (Figure 59A and B).

We examined the activation state of several downstream signaling molecules to identify the involved signaling molecules in mediating the effect of roflumilast. Roflumilast did not alter the levels of activated ERK1/2, p38, cJun and CREB in response to IL-13, either in the presence or absence of epinephrine (Figure 60A-C, Figure 61A-C, Figure 62A and B). Furthermore, STAT6 phosphorylation was also not altered by roflumilast in the presence of epinephrine (Figure 62C).

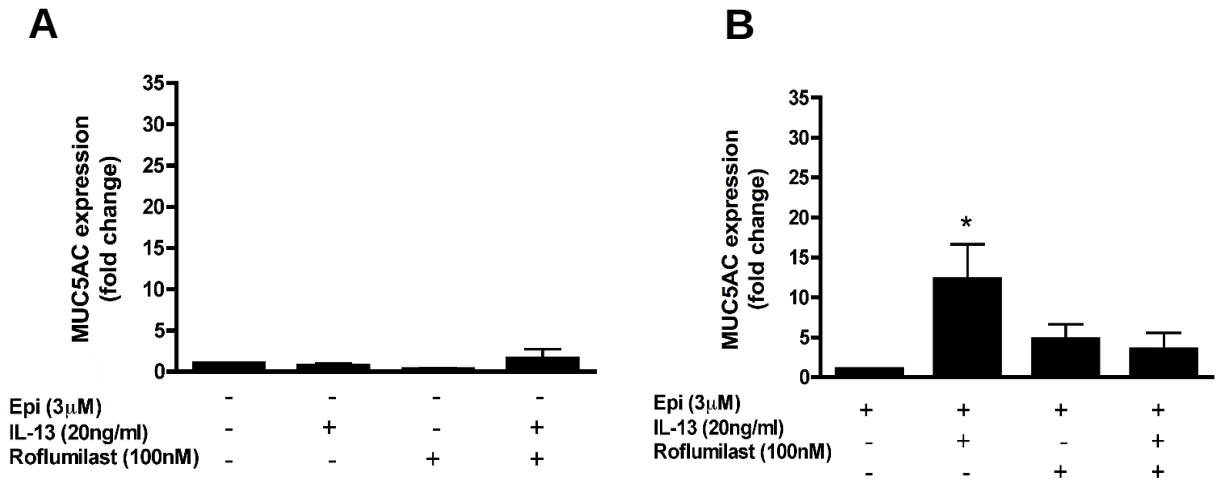



**Figure 55.** ***Effect of roflumilast on MUC5AC transcripts in response to IL-13 in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM roflumilast for 14 days. MUC5AC transcripts levels were measured by qRT-PCR and the data presented as fold change compared to corresponding control cells grown in the absence of IL-13 and roflumilast. Data are presented as means ± SEM from three donors.* indicates $p<0.05$ significance as compared to +epi treated cells.

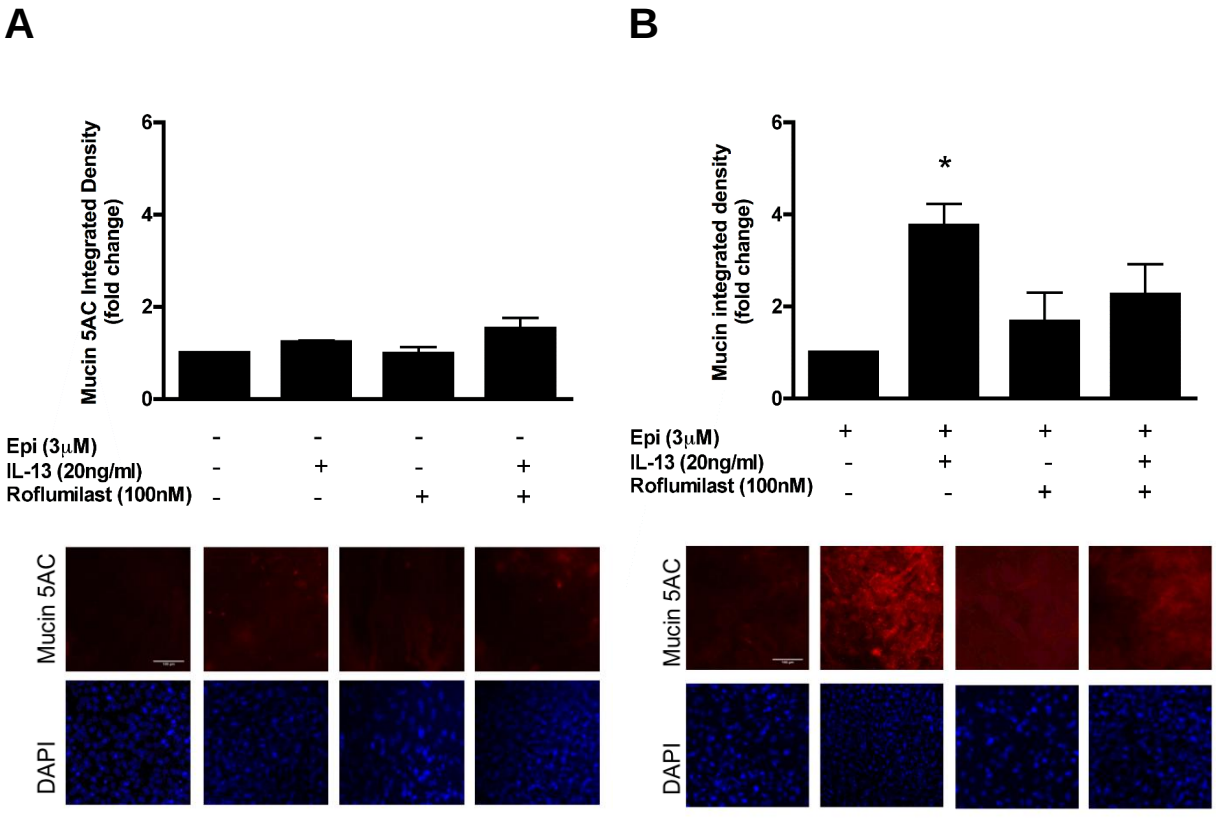



**Figure 56.** ***Effect of roflumilast on mucin 5AC production in response to IL-13 in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM roflumilast for 14 days. The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. * indicates $p<0.05$ significance as compared to +epi treated cells.

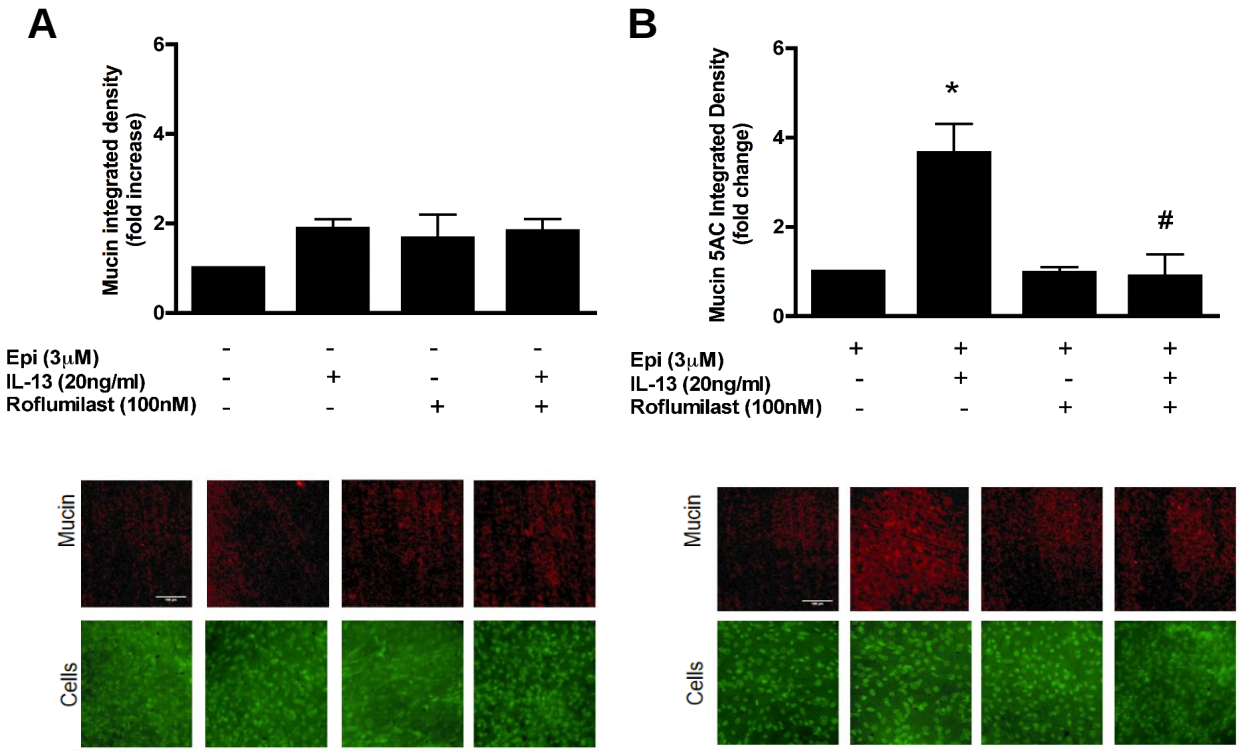



**Figure 57.** ***Effect of roflumilast on mucin production in response to IL-13 in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM roflumilast for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. Data are presented as means ± SEM from three donors. * and # indicate $p<0.05$ significance as compared to +epi and +epi+IL-13 treated cells.

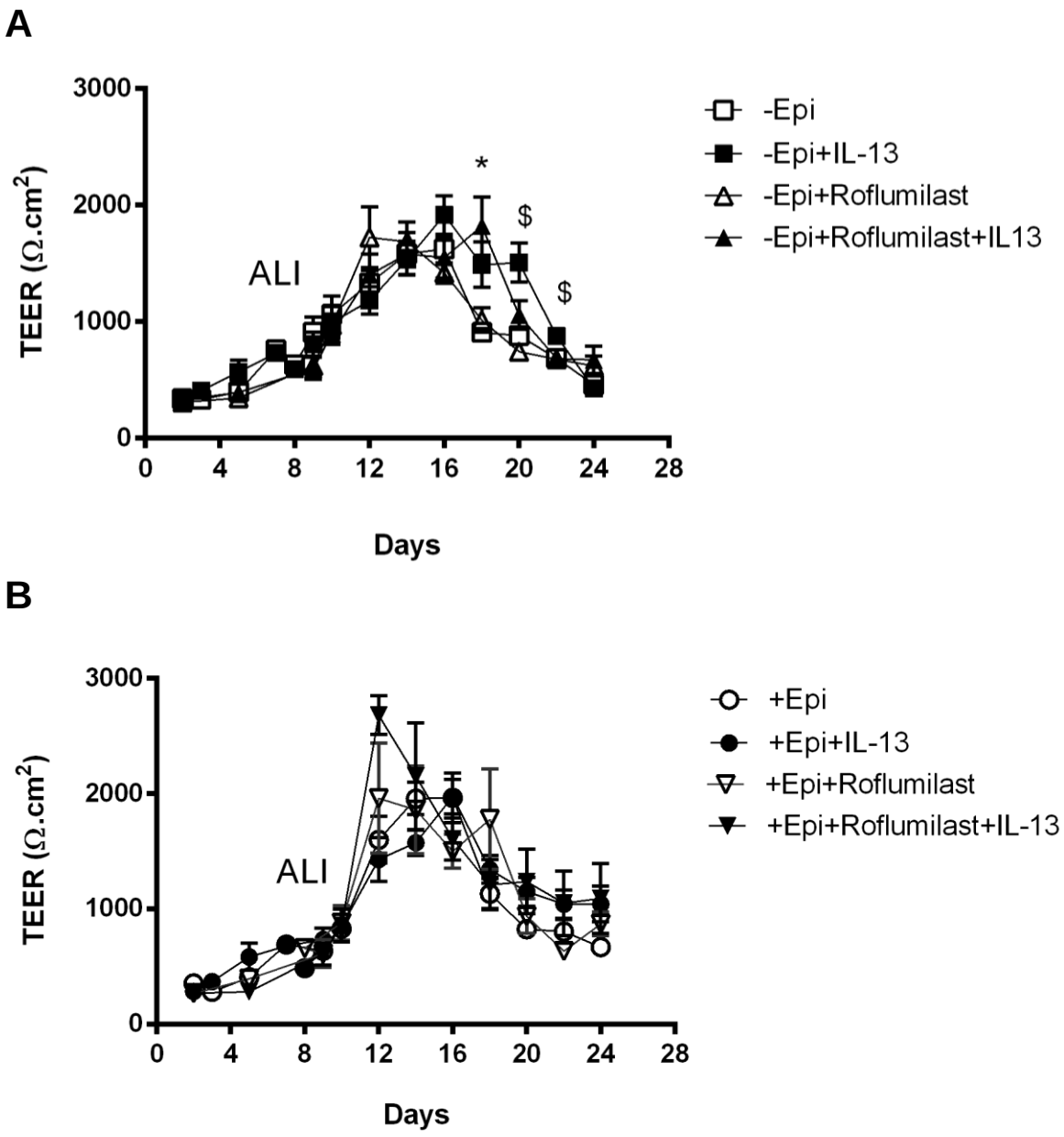



**Figure 58.** ***Effect of roflumilast on the integrity of NHBE cell monolayers****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM roflumilast for 14 days. Trans-epithelial electrical resistance (TEER) was measured throughout the culture period. Data are presented as mean ohm.cm$^2$ of insert surface ± SEM from three donors. * and $ indicate $p<0.05$ significance between -epi and -epi+roflumilast+IL-13, and between -epi and -epi+IL-13 treated cells respectively.

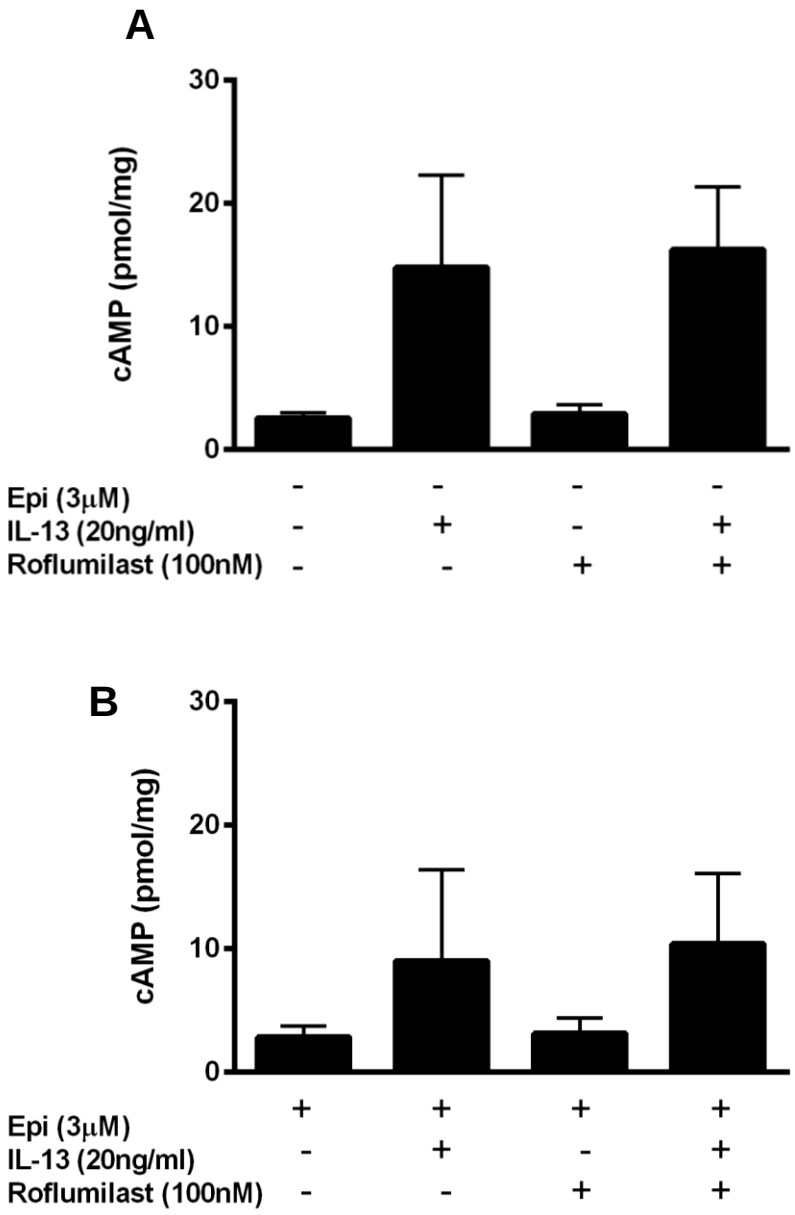
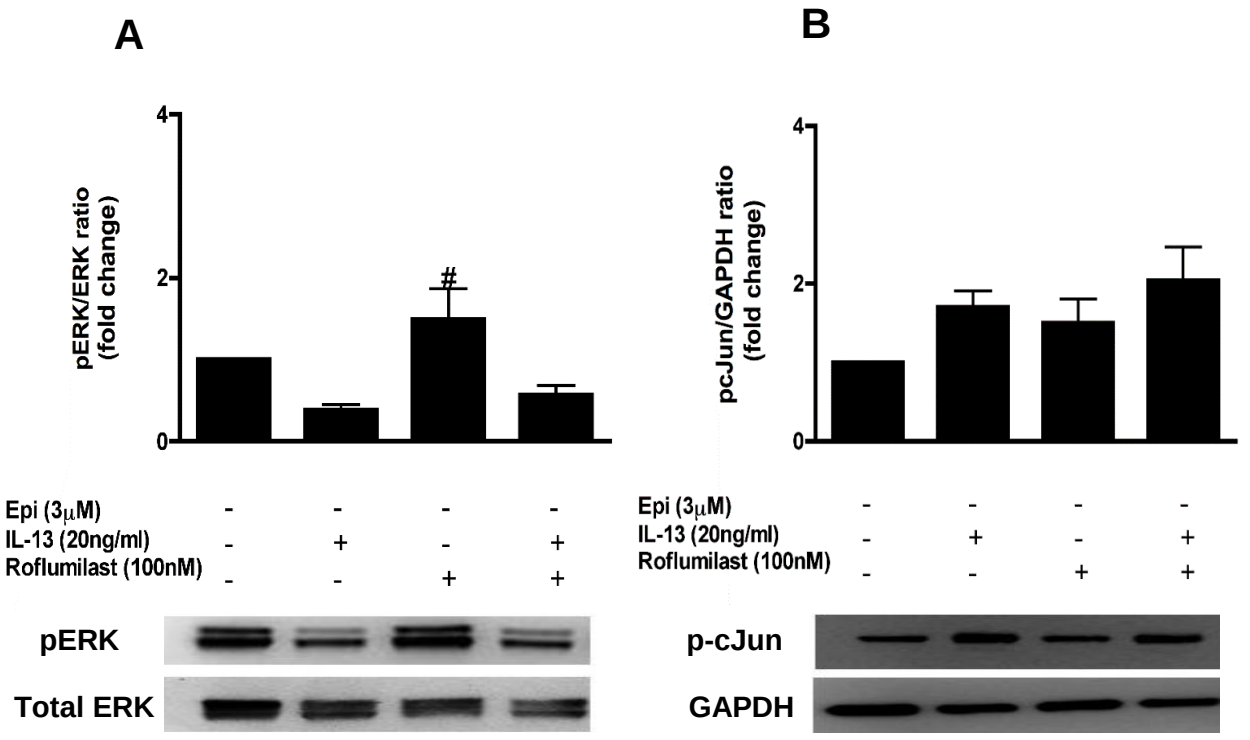



**Figure 59.** ***Effect of roflumilast on intracellular cAMP levels in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM roflumilast for 14 days. Intracellular cAMP was measured in pmol and presented as pmol/mg after normalizing to total protein concentration. Data are presented as means ± SEM from three donors.



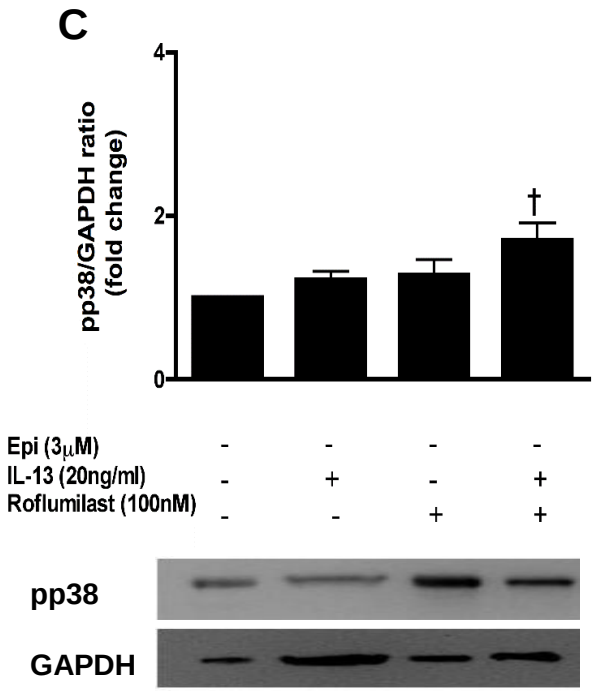
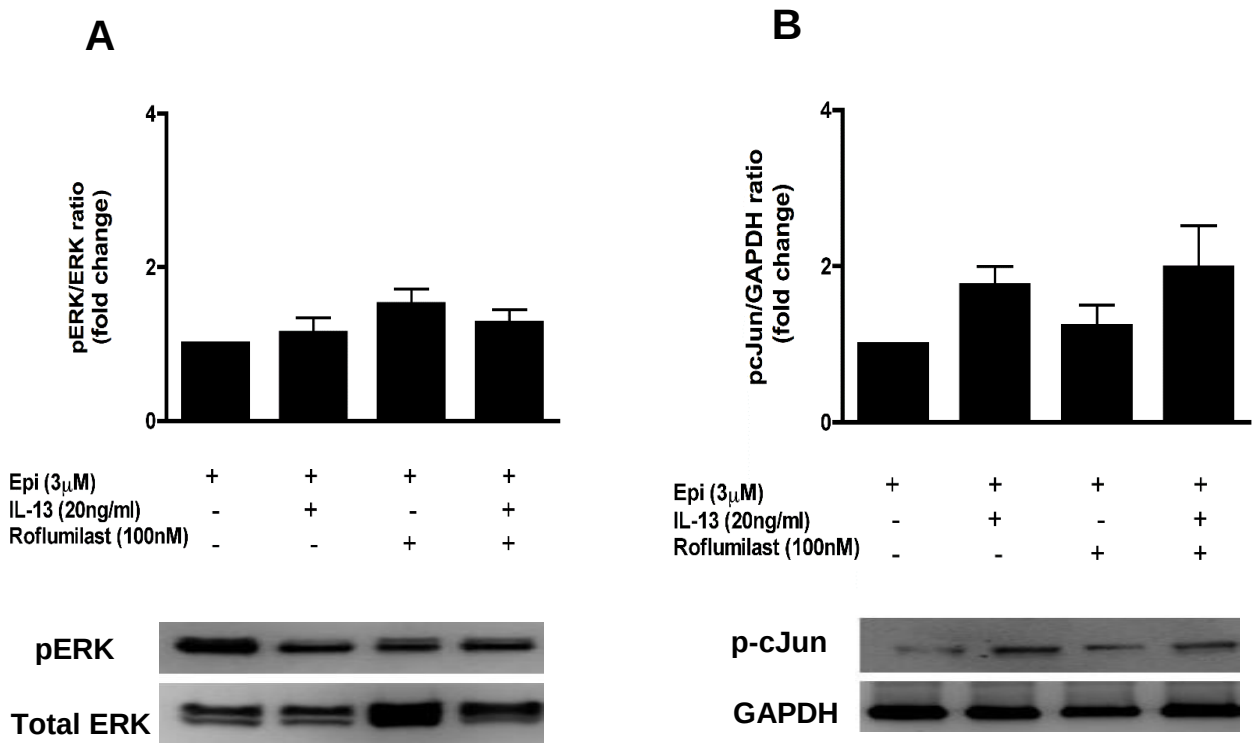




**Figure 60.** ***Effects of roflumilast on activation of MAPKs in NHBE cells in response to IL-13 in the absence of epinephrine:*** Cells were grown in the absence of epinephrine 72 hours before ALI**,** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM roflumilast for 14 days. Total proteins were harvested and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation in NHBE cells. **B)** cJun phosphorylation and **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. # and † indicate $p<0.05$ significance as compared to -epi+IL-13 and -epi respectively.



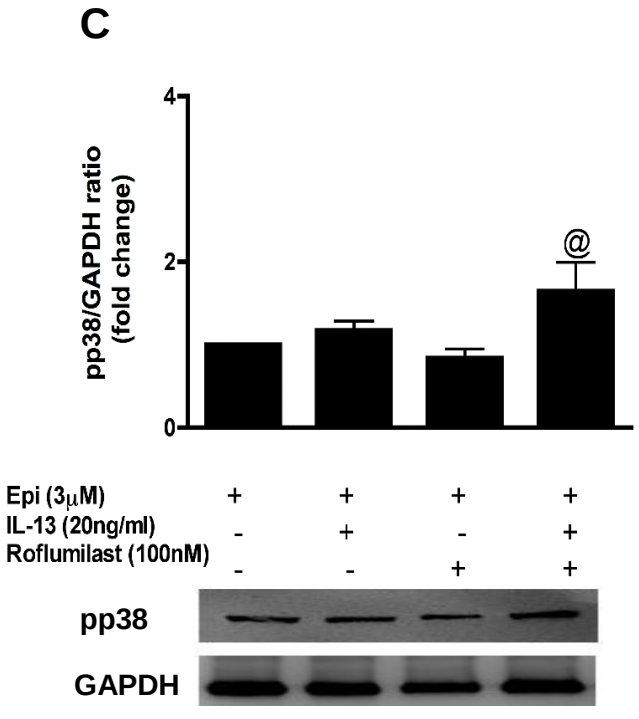
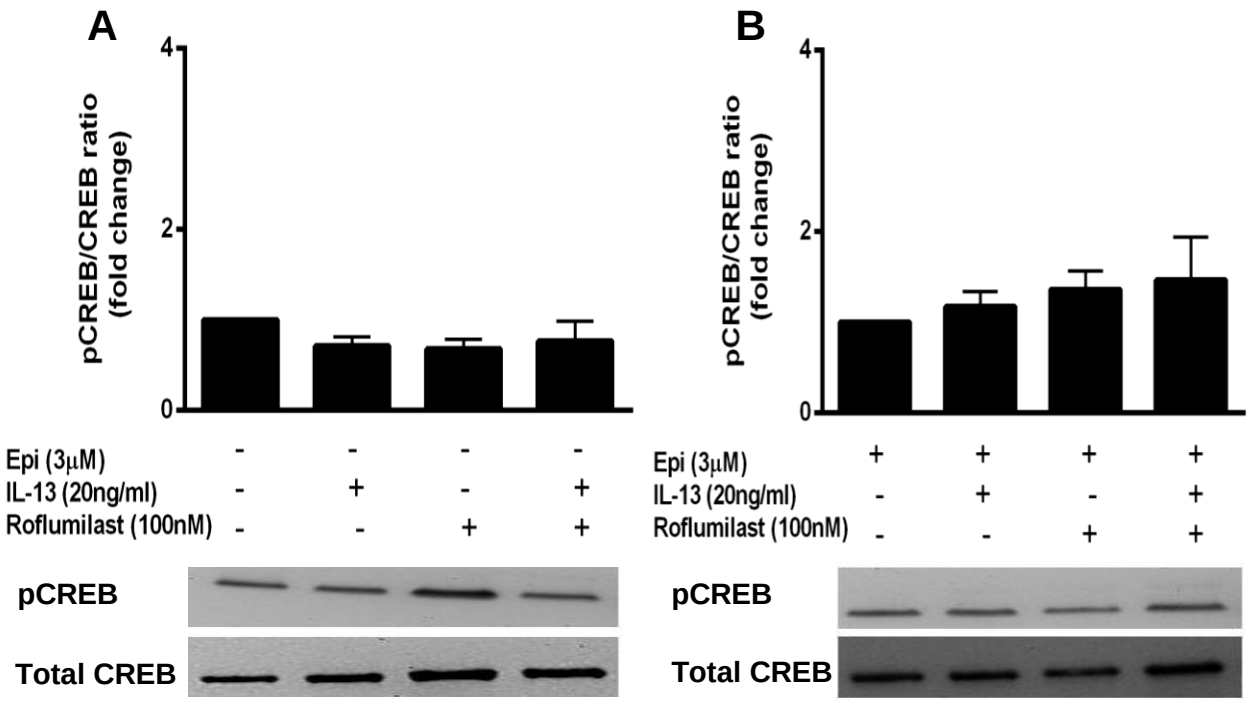




**Figure 61.** ***Effects of roflumilast on activation of MAPKs in NHBE cells in response to IL-13 in the presence of epinephrine:*** Cells were grown in the presence of epinephrine then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM roflumilast for 14 days. Total proteins were harvested and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation in NHBE cells. **B)** cJun phosphorylation and **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. @ indicate $p<0.05$ significance as compared to +epi+roflumilast treated cells respectively.



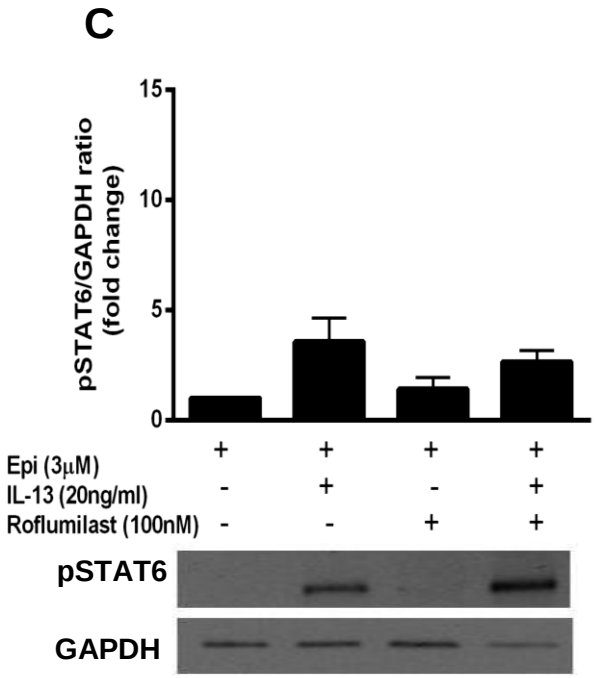




**Figure 62.** ***Effects of roflumilast on CREB and STAT6 activation in NHBE cells in response to IL-13:*** Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B and C),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM roflumilast for 14 days. Total proteins were harvested and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A and B)** CREB phosphorylation in NHBE cells. **C)** STAT6 phosphorylation. Data are presented as means ± SEM from three donors.

## 4.6 Role of β-arrestin signaling in mucin production in response to IL-13 in airway epithelial cells

### 4.6.1 Effect of carvedilol on mucin production in response to IL-13 in NHBE cells

After evaluating the involvement of $\beta_2$-AR canonical Gs/adenylyl cyclase pathway, we assessed the role of β-arrestin signaling on mucin parameters and signaling cascades induced by IL-13. For this purpose, we treated NHBE cells with 100 nM carvedilol, a β-blocker that blocks the Gs/ adenylyl cyclase signaling while activating the β-arrestin pathway, for 14 days in the presence and absence of epinephrine. MUC5AC expression was not changed with carvedilol in the absence of epinephrine (2.20 ± 1.76 fold *vs* 0.72 ± 0.47 fold by IL-13, $p>0.05$) (Figure 63A). Also, in the presence of epinephrine, carvedilol did not alter the levels of MUC5AC expression induced by IL-13 (5.04 ± 8.50 fold *vs* 13.6 ± 5.72 fold by IL-13, $p>0.05$) (Figure 63B). Similar trends were also observed at the level of intracellular mucin 5AC accumulation and mucin glycoproteins either in the absence or presence of epinephrine (Figure 64A, B and Figure 65A, B). Carvedilol did not alter the barrier function of cultured NHBE cells, as assessed by measuring the TEER and ZO-1 localization (Figure 66A, B and 67A, B).

The accumulation of intracellular cAMP induced by carvedilol did not differ from control or IL-13 treated cells either in the presence or absence of epinephrine (Figure 68A, B). Next, we examined the levels of activated signaling molecules

downstream of β-arrestin signaling cascade. Carvedilol reduced ERK1/2 phosphorylation when combined with IL-13 in the absence of epinephrine as compared to control cells, whereas it did not affect ERK1/2 phosphorylation in the presence of epinephrine (Figure 69A and Figure 70A). On the other hand, carvedilol + IL-13 treatment increased cJun phosphorylation as compared to control cells only in the pesence of epinephrine (Figure 70B). p38 and CREB phosphorylation states were not changed by carvedilol either in the presence or absence of epinephrine (Figure 69C, Figure 70C and Figure 71A,B).

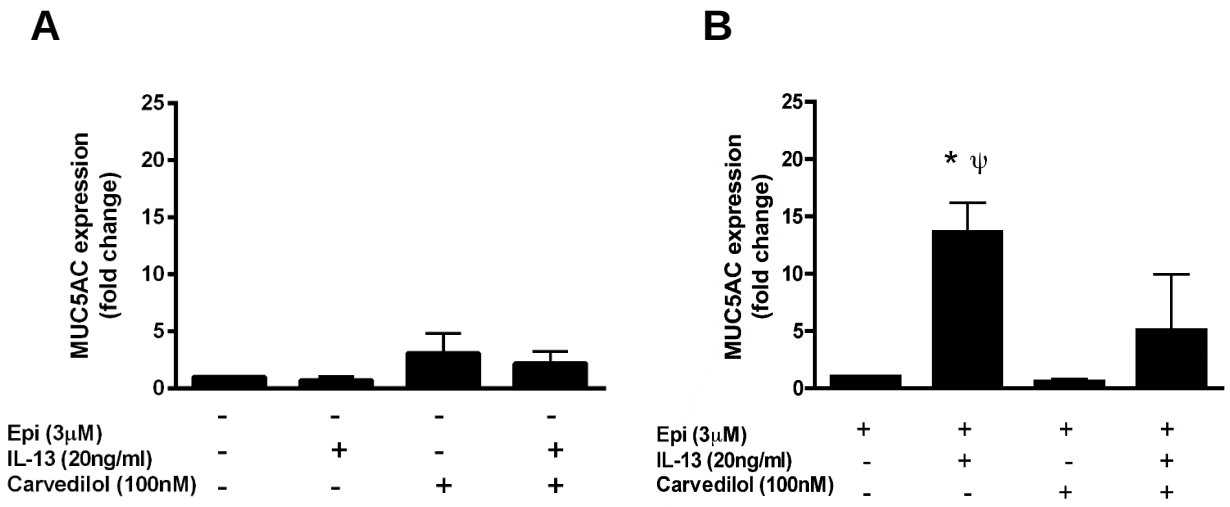



**Figure 63.** ***Effect of carvedilol on MUC5AC transcripts in response to IL-13 in NHBE cells:*** Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM carvedilol for 14 days. MUC5AC transcripts levels were measured by qRT-PCR and the data presented as fold change compared to corresponding control cells grown in the absence of IL-13 and carvedilol. Data are presented as means ± SEM from three donors.* and ψ indicate $p<0.05$ significance as compared to +epi and +epi+carvedilol treated cells respectively.

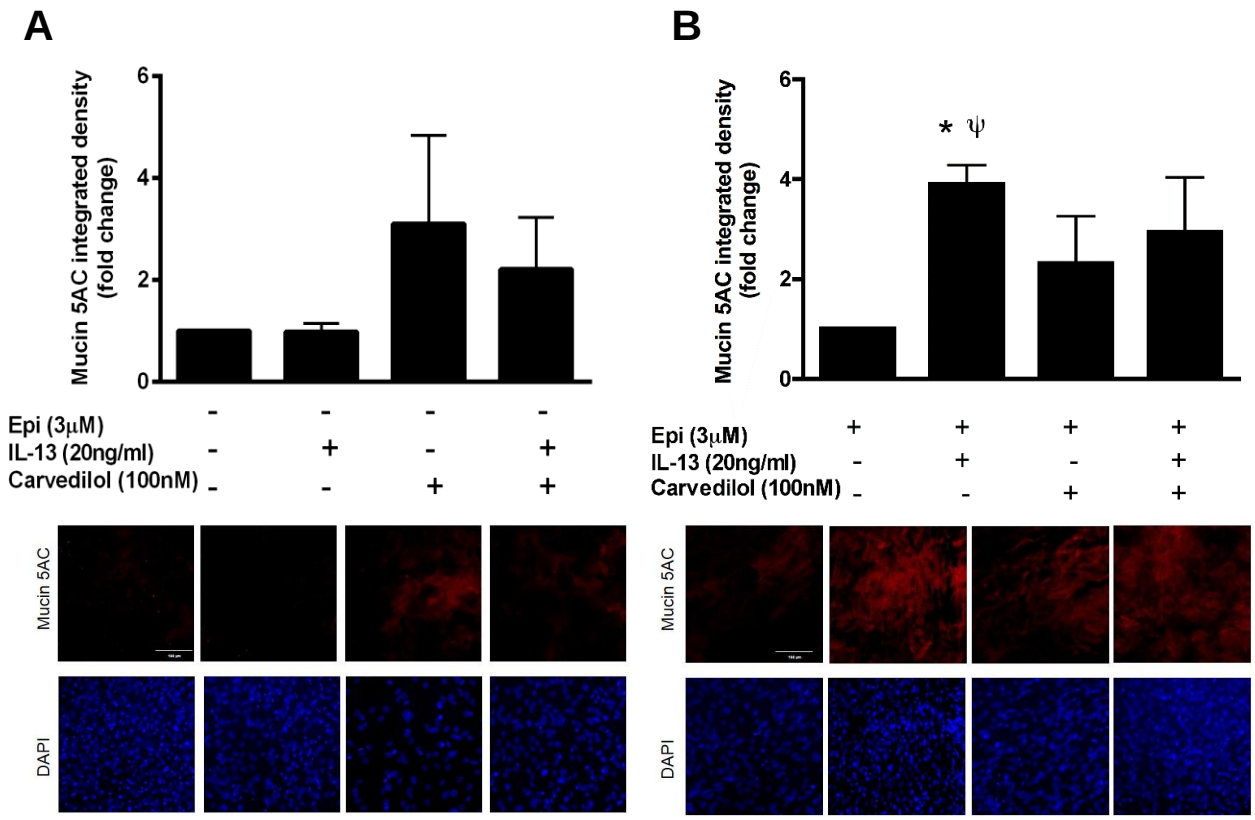



**Figure 64.** ***Effect of carvedilol on mucin 5AC production in response to IL-13 in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM carvedilol for 14 days. The Transwell® membranes were incubated with a rabbit antibody against human mucin 5AC (red) and DAPI to counterstain the nuclei (blue). The ratio of integrated fluorescence density of each group to the integrated mucin 5AC density of the corresponding control group was calculated and expressed as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three donors. .* and ψ indicate $p<0.05$ significance as compared to +epi and +epi+carvedilol treated cells respectively.

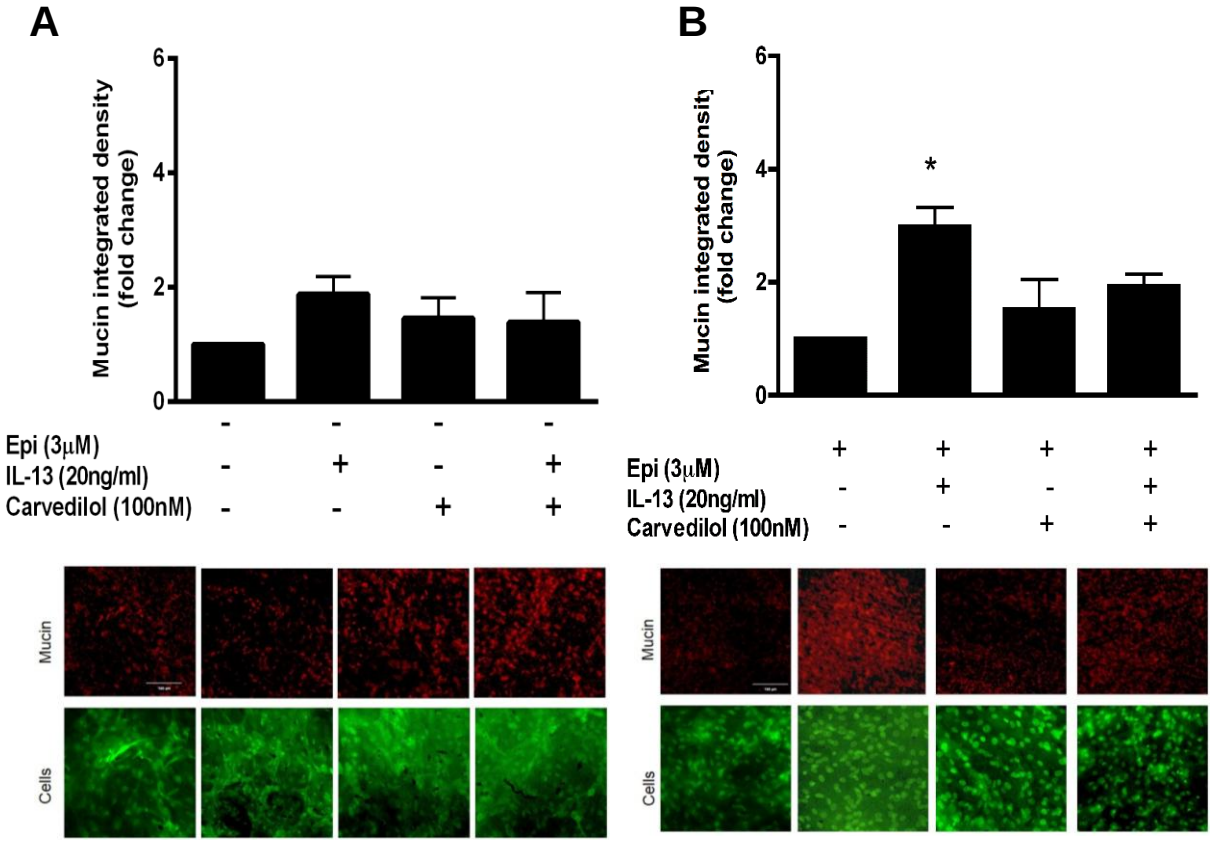



**Figure 65.** ***Effect of carvedilol on mucin production in response to IL-13 in NHBE cells:*** Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM carvedilol for 14 days. PAFS staining of NHBE cells to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three donors. Data are presented as means ± SEM from three donors. * indicates $p<0.05$ significance as compared to +epi treated cells.

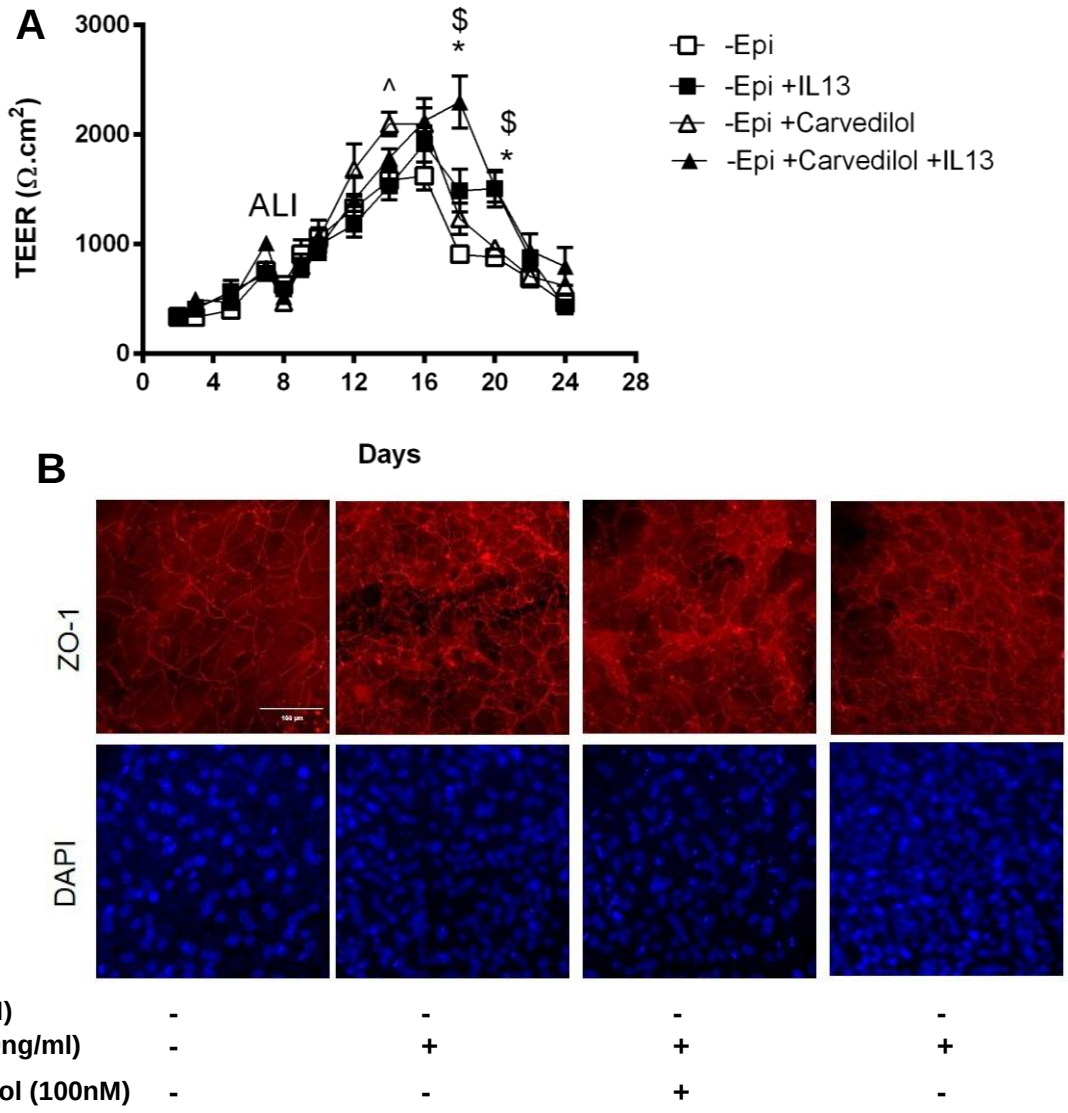
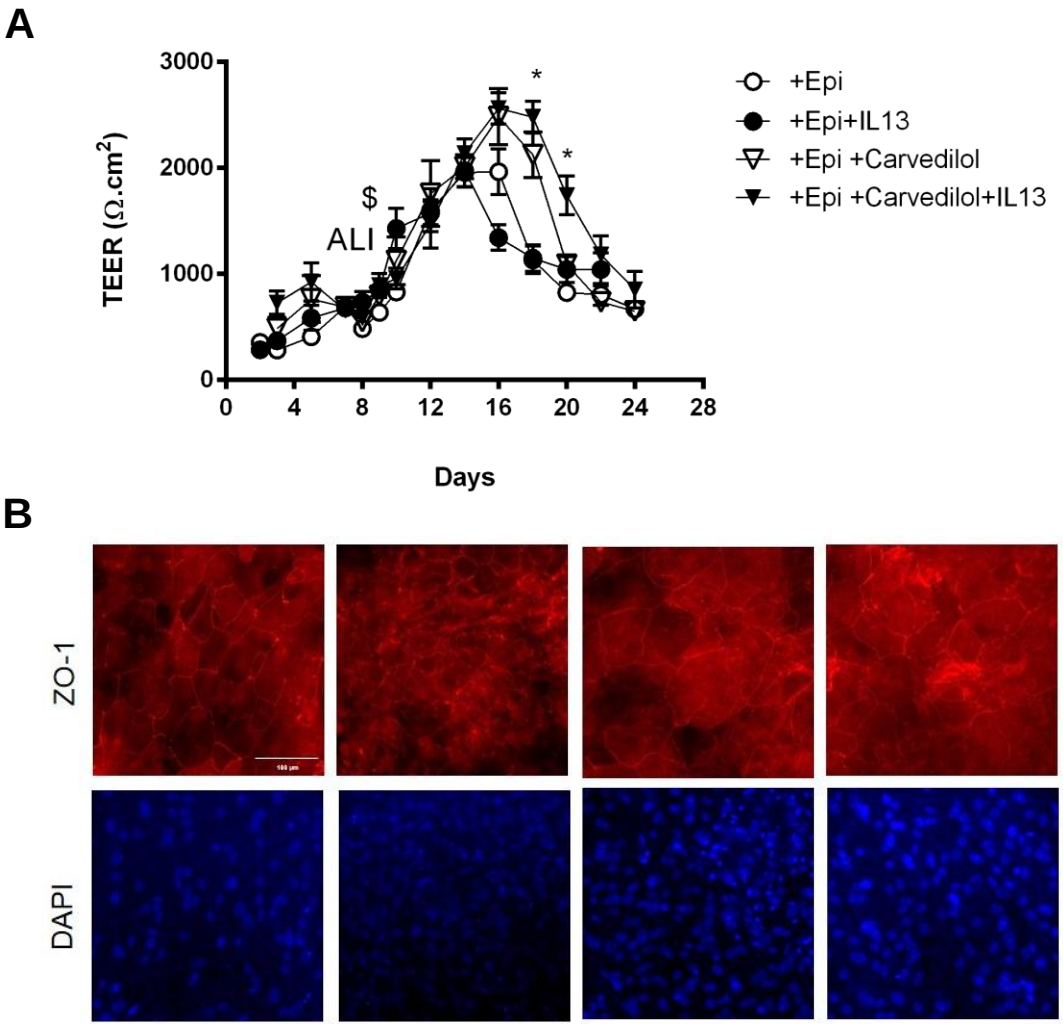



**Figure 66.** ***Effect of carvedilol on the integrity of NHBE cells monolayer in response to IL-13 in the absence of epinephrine:*** NHBE cells were grown in the absence of epinephrine 72 hours before ALI, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 100 nM carvedilol for 14 days. **A)** Trans-epithelial electrical resistance (TEER) was measured thought the culture period one day before ALI and throughout ALI period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 μm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Data are presented as means ± SEM from three donors. * and $ indicate $p<0.05$ significant difference between -epi and -epi+IL-13 and between -epi and -epi+carvedilol+IL-13 treated cells respectively.




| | | | | |
|---|---|---|---|---|
| **Epi (3μM)** | **+** | **+** | **+** | **+** |
| **IL-13 (20ng/ml)** | **-** | **+** | **+** | **+** |
| **Carvedilol (100nM)** | **-** | **-** | **+** | **-** |

**Figure 67.** ***Effect of carvedilol on the integrity of NHBE cells monolayer in response to IL-13 in the presence of epinephrine:*** NHBE cells were grown in the presence of 3 μM epinephrine, then at ALI, they were treated with 20 ng/ml IL-13 in combination with 100 nM carvedilol for 14 days. **A)** Trans-epithelial electrical resistance (TEER) was measured throughout the culture period. Data are presented as ohm.cm$^2$ of insert surface area. Data are presented as means ± SEM from three donors. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 μm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Data are presented as means ± SEM from three donors. * indicates $p<0.05$ significant difference between +epi and +epi+carvedilol+IL-13 treated cells respectively.

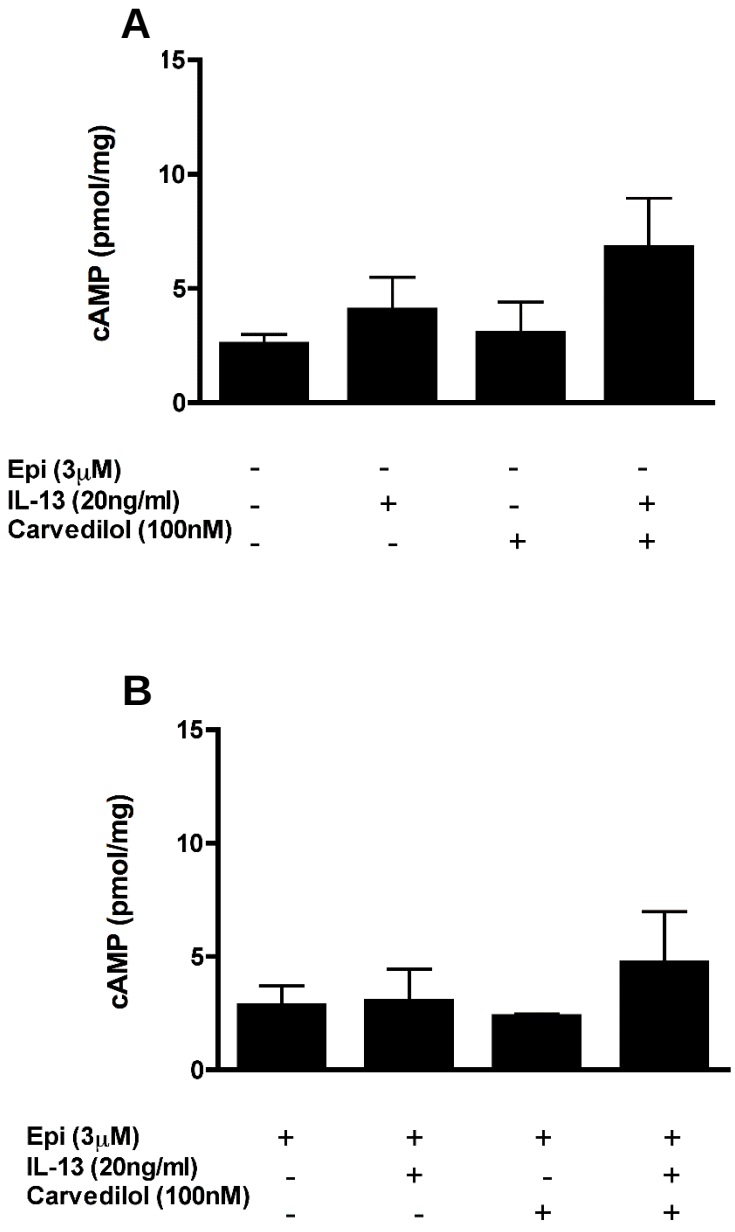
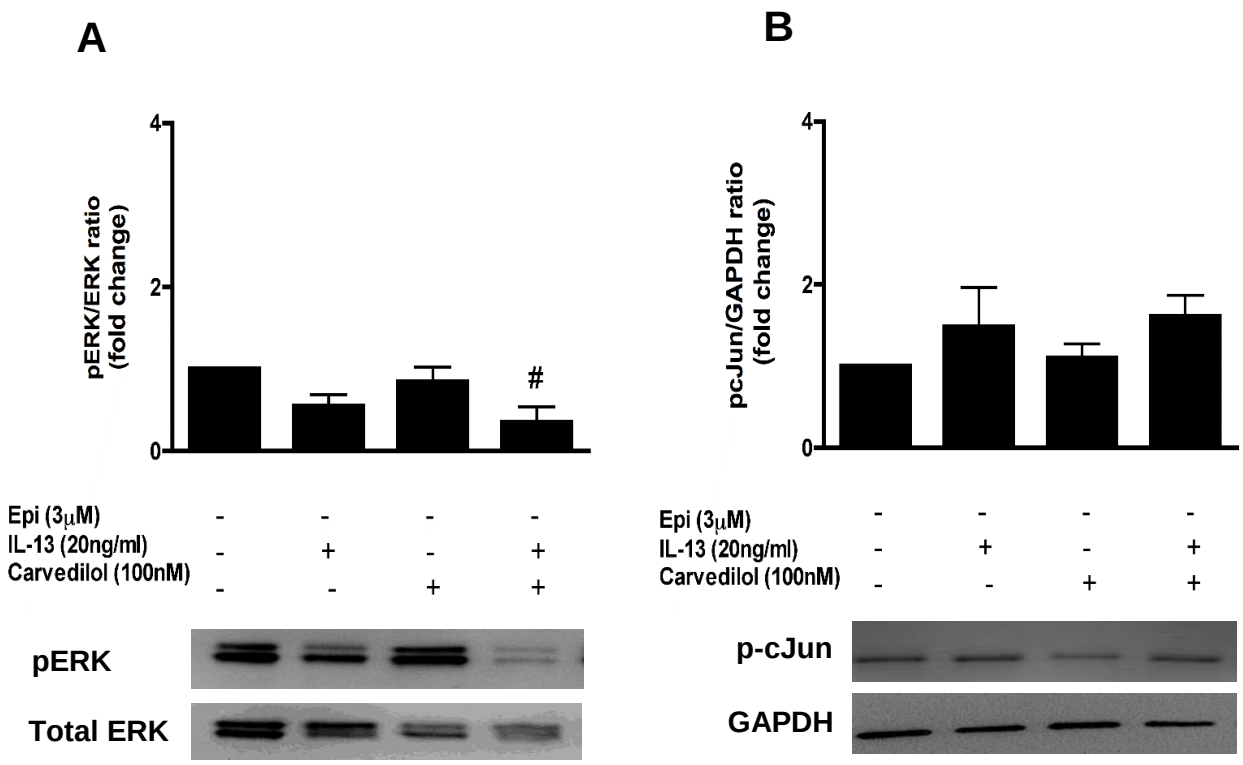



**Figure 68.** ***Effect of carvedilol on intracellular cAMP levels in NHBE cells****:* Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM carvedilol for 14 days. Intracellular cAMP was measured in pmol and presented as pmol/mg after normalizing to total protein concentration. Data are presented as means ± SEM from three donors.



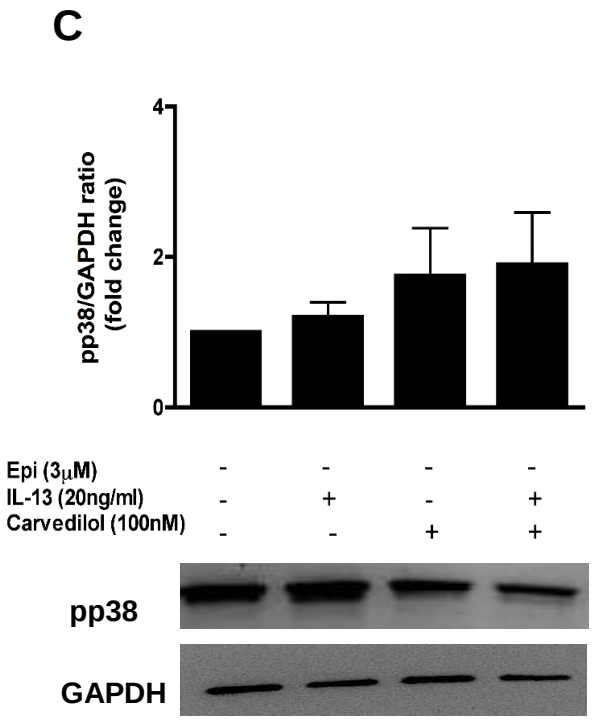
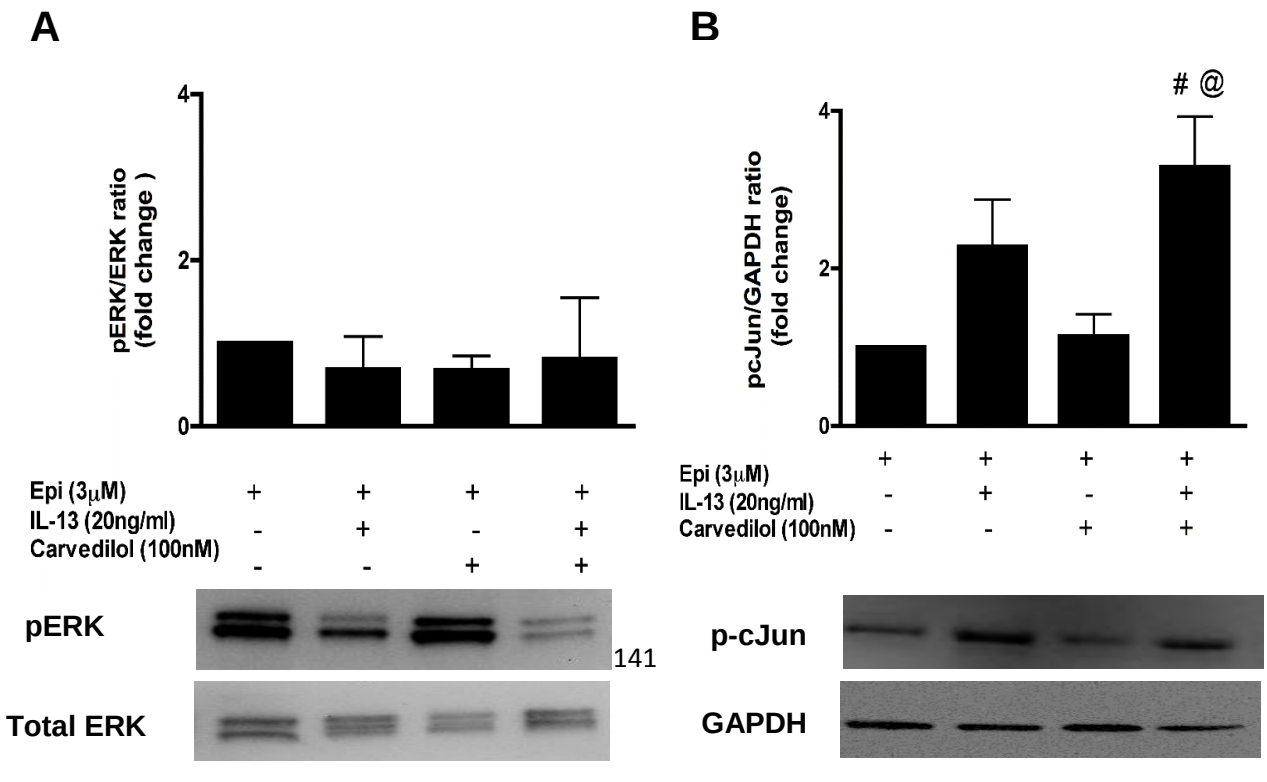
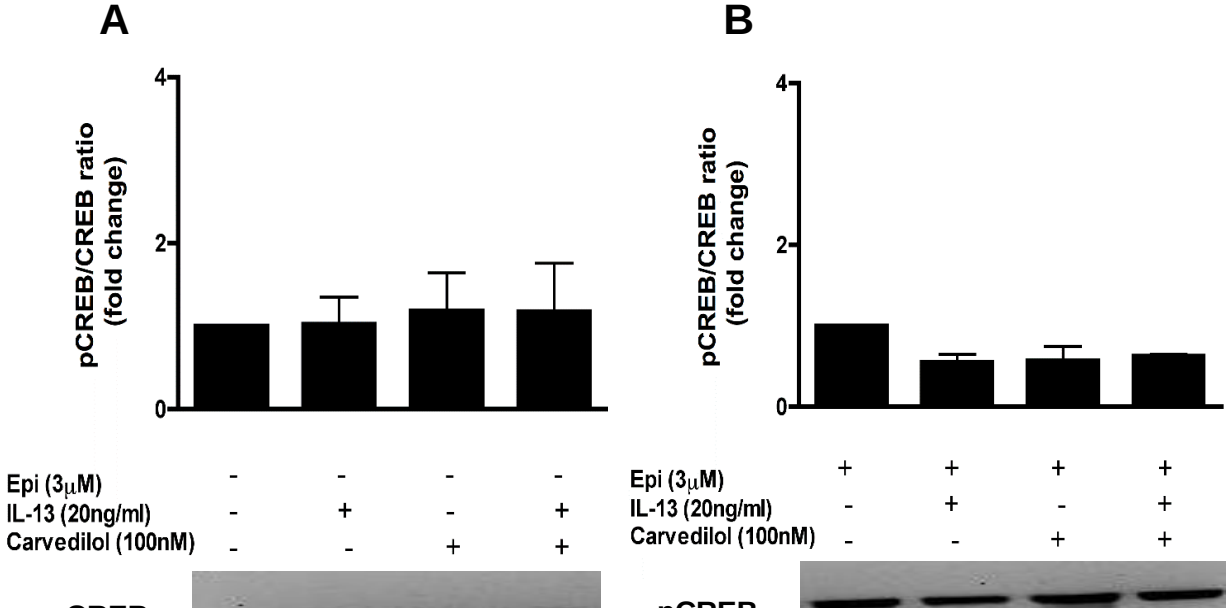




**Figure 69.** ***Effects of carvedilol on activation of MAPKs in NHBE cells in response to IL-13 in the absence of epinephrine:*** Cells were grown in the absence of epinephrine 72 hours before ALI**,** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 100 nM carvedilol for 14 days. Total proteins were harvested and immunoblots were performed using antibodies to phosphorylated proteins. The signal densities of the phosphorylated proteins were normalized to total protein or GAPDH protein density and the relative intensities were reported as the degree of phosphorylation of the protein. The data are presented as fold change compared to the corresponding control cells. **A)** ERK1/2 phosphorylation in NHBE cells. **B)** cJun phosphorylation and **C)** p38 phosphorylation. Data are presented as means ± SEM from three donors. # indicates $p<0.05$ significance as compared to -epi cells.


A
pERK/ERK ratio
(fold change )
4
2
0
Epi (3μM) + + + +
IL-13 (20ng/ml) - + - +
Carvedilol (100nM) - - + +
pERK
Total ERK
B
# @
pcJun/GAPDH ratio
(fold change)
4
2
0
Epi (3μM) + + + +
IL-13 (20ng/ml) - + - +
Carvedilol (100nM) - - + +
p-cJun
GAPDH


A
B
pCREB/CREB ratio (fold change)
4
2
0
Epi (3μM)
IL-13 (20ng/ml)
Carvedilol (100nM)
- - - -
- + - +
- - + +
+ + + +
- + - +
- - + +
pCREB
CREB

### 4.6.2 Effect of β-arrestin-2 on mucin production in response to IL-13 from isolated mouse tracheal epithelial cells (mTECs)

We next examined if the loss of β-arrestin-2 from airway epithelial cells affects the response to IL-13 by using a mouse with a knockout of β-arrestin-2. We isolated mTECs from β-arrestin-2 KO mice and WT mice and treated them with murine IL-13 for 14 days after establishing ALI for 14 days. The intracellular mucin 5ac content of mTECs from β-arrestin-2 KO was induced by IL-13 as compared to untreated cells from β-arrestin-2 KO. However, this increase was significantly lower as compared to IL-13 treated cells from WT mice (Figure 72).

In addition, we examined the total mucin glycoprotein accumulation in these treated cells. Intracellular mucin glycoprotein was induced by IL-13 in cells from WT mice, but not in cells from β-arrestin-2 KO mice (Figure 73).

Incubation of mTECs from β-arrestin-2 KO mice with IL-13 resulted in increased permeability of the monolayer, as evaluated by TEER measurement and ZO-1 localization after 14 days of IL-13 treatment (ALI day 28) (Figure74A, B). There was also a reduction in TEERs from IL-13 treated mTECs from WT mice, but it was not significant (Figure74A, B).

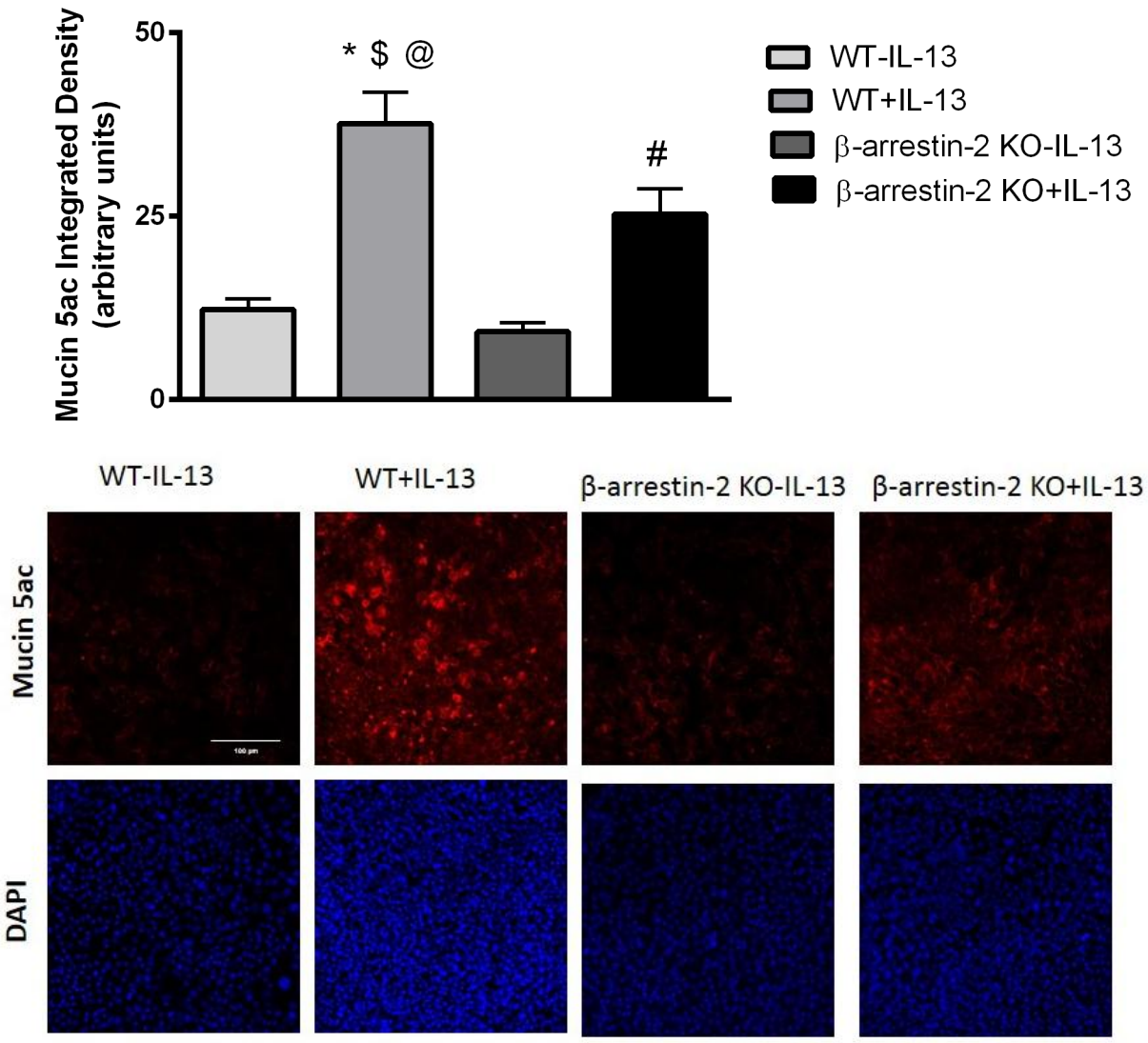



**Figure 72.** ***Role of β-arrestin-2 signaling in mucin 5ac production in response to IL-13 in cultured mTECs:*** Cells from WT and β-arrestin-2 KO mice were grown 14 days at ALI, then treated with or without 20 ng/ml IL-13 in the presence of epinephrine for another 14 days. The Transwell® membranes were incubated with mucin 5ac (red) antibody and DAPI to counterstain the nuclei (blue). The integrated fluorescence density of each group was calculated and expressed as arbitrary units. Representative images of immunofluorescence are shown (scale bar = 100 μm). Data are presented as means ± SEM from three inserts from N=3-4. *, $, @ and # indicate $p<0.05$ significance as compared to WT-IL-13, β-arrestin-2 KO-IL-13, β-arrestin-2 KO+IL-13 and β-arrestin-2 KO-IL-13 treated cells respectively.

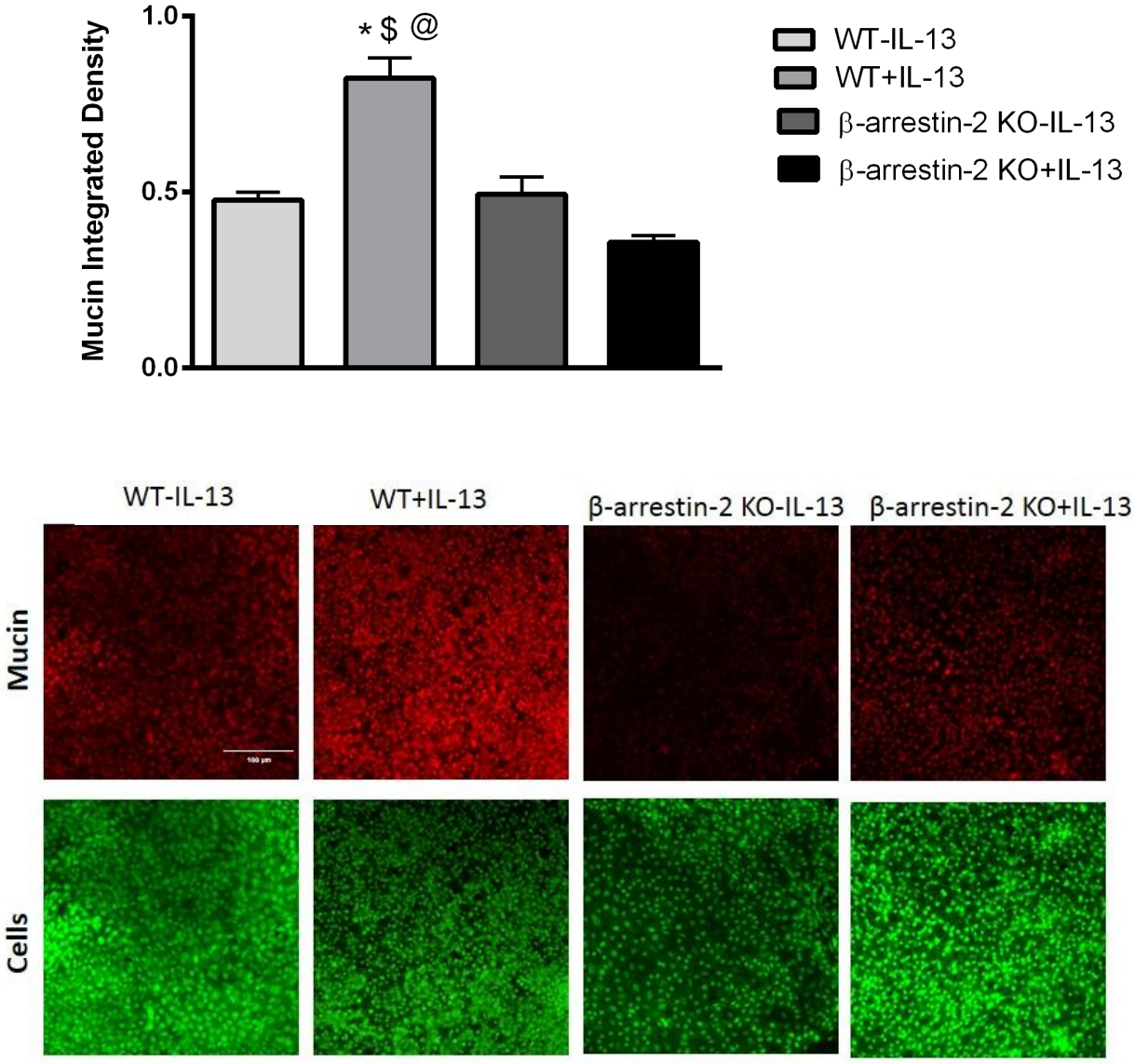



**Figure 73.** ***Role of β-arrestin-2 signaling in mucin production in response to IL-13 in cultured mTECs****:* Cells from WT and β-arrestin-2 KO mice were grown 14 days at ALI, then treated with or without 20 ng/ml IL-13 in the presence of epinephrine for another 14 days. PAFS staining of mTECs to quantify total intracellular mucin glycoproteins. Representative images are shown (scale bar = 100 μm). The ratio of mucin integrated density and nucleic acid/cytoplasm integrated density was calculated and the data presented as fold change compared to the corresponding control cells (in the absence of IL-13 treatment). Data are presented as means ± SEM from three inserts from N=3-4. *, $ and @ indicate $p<0.05$ significance as compared to WT-IL-13, β-arrestin-2 KO-IL-13 and β-arrestin-2 KO+IL-13 treated cells respectively.

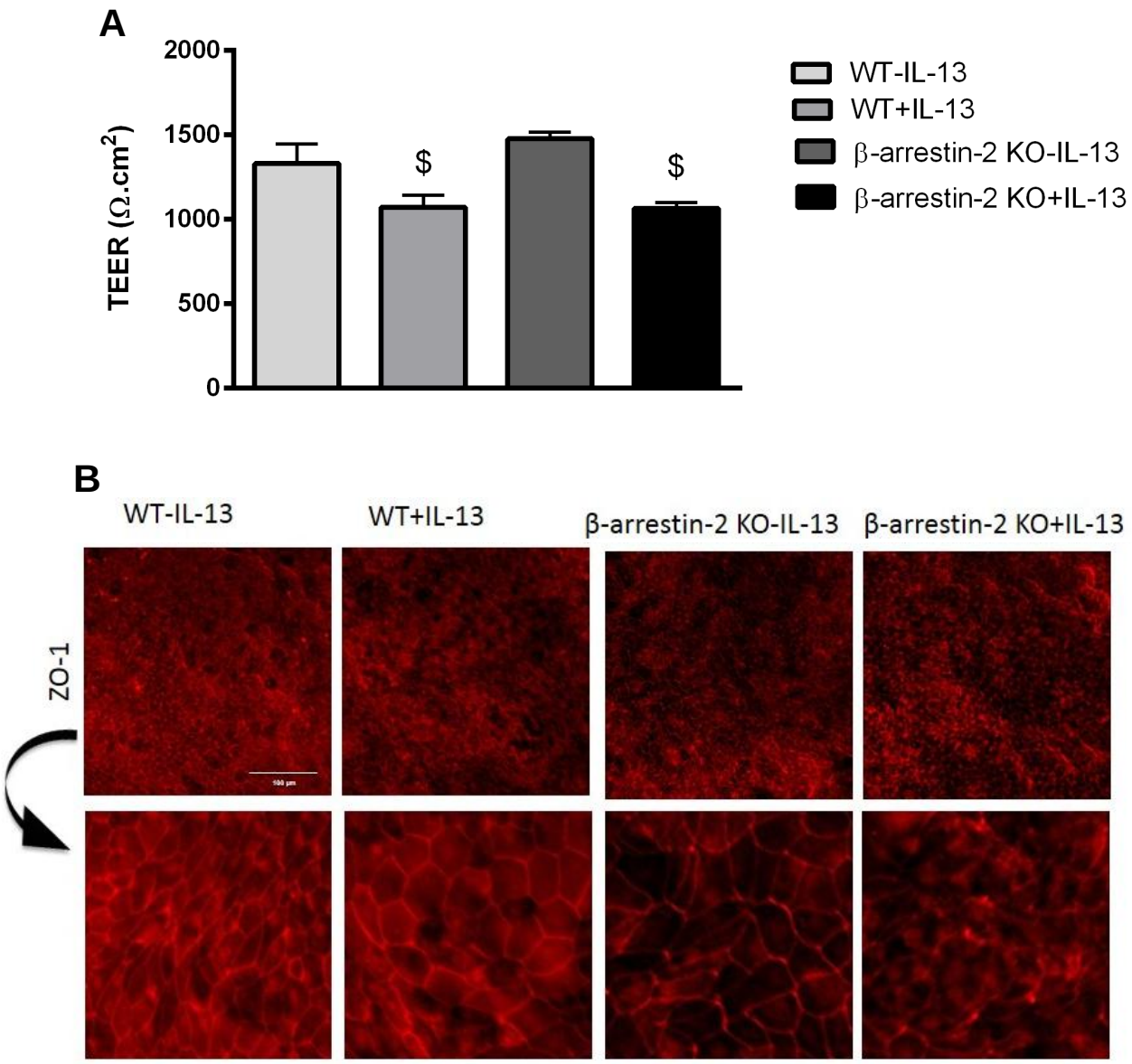



**Figure 74: *Effect of β-arrestin-2 signaling on the integrity of mTECS monolayer in response to IL-13:*** Cells were grown 14 days at ALI, then treated with or without 20 ng/ml IL-13 in the presence of epinephrine for another 14 days. **A)** Trans-epithelial electrical resistance (TEER) was measured on ALI day 28 (14 days after starting IL-13 treatment). Data are presented as mean ohm.cm$^2$ of insert surface area± SEM from three inserts from N=3-4. **B)** Representative images of immunofluorescence with a rabbit antibody against ZO-1 (red) (scale bar = 100 µm). The Transwell® membranes were incubated with DAPI to counterstain the nuclei (blue). Data are presented as means ± SEM from three donors. $ indicates $p<0.05$ significance as compared to β-arrestin-2 KO-IL-13 cells.

### 4.7 Effect of $\beta_2$-AR signaling in IL-13 modulation of mucociliary differentiation of NHBE cells.

We then assessed if $\beta_2$-AR signaling is involved in the transdifferentiation from ciliated cells to goblet cells induced by IL-13 and if this is the site of cross-talk between $\beta_2$-AR and IL-13 signaling pathways. For this purpose, we measured the expression level of FOXJ1, a marker for ciliated cells. Cells that were grown in the absence of epinephrine and treated with IL-13 showed a higher levels of FOXJ1, but this difference was not significant as compared to cells grown in the presence of epinephrine (Figure 75A). NHBE cells were treated with IL-13 and 1 μM ICI-118,551 (a highly preferential $\beta_2$-AR antagonist) or 3 μM CGP-20712A (a highly preferential $\beta_1$-AR antagonist) for 14 days in the presence of epinephrine. IL-13 did not alter FOXJ1 expression as compared to control cells. Moreover, CGP-20712A (0.60±0.27 fold *vs* 1.39±1.46 fold by IL-13, $p>0.05$) and ICI-118,551 (2.39±3.09 fold *vs* 1.39±1.46 fold by IL-13, $p>0.05$) did not affect the expression level in response to IL-13 (Figure 75B).

We next examined if the increase in mucin production in response to IL-13 in the presence of epinephrine is due to cell proliferation. We used DAPI staining of cell nuclei as a marker for cell number. IL-13 increased the cell number only in the presence of epinephrine (Figure 76A). Also, when treating the cells with CGP-20712A+IL-13, the cell number was significantly increased and ICI-118,551+ IL-13 significantly reduced the cell number as compared to CGP-

20712A+IL-13 (Figure 76B). In addition, elevated intracellular cAMP by forskolin+IBMX combination resulted in significant increase in cell number in response to IL-13 either in the absence or presence of epinephrine (Figure77A and B).

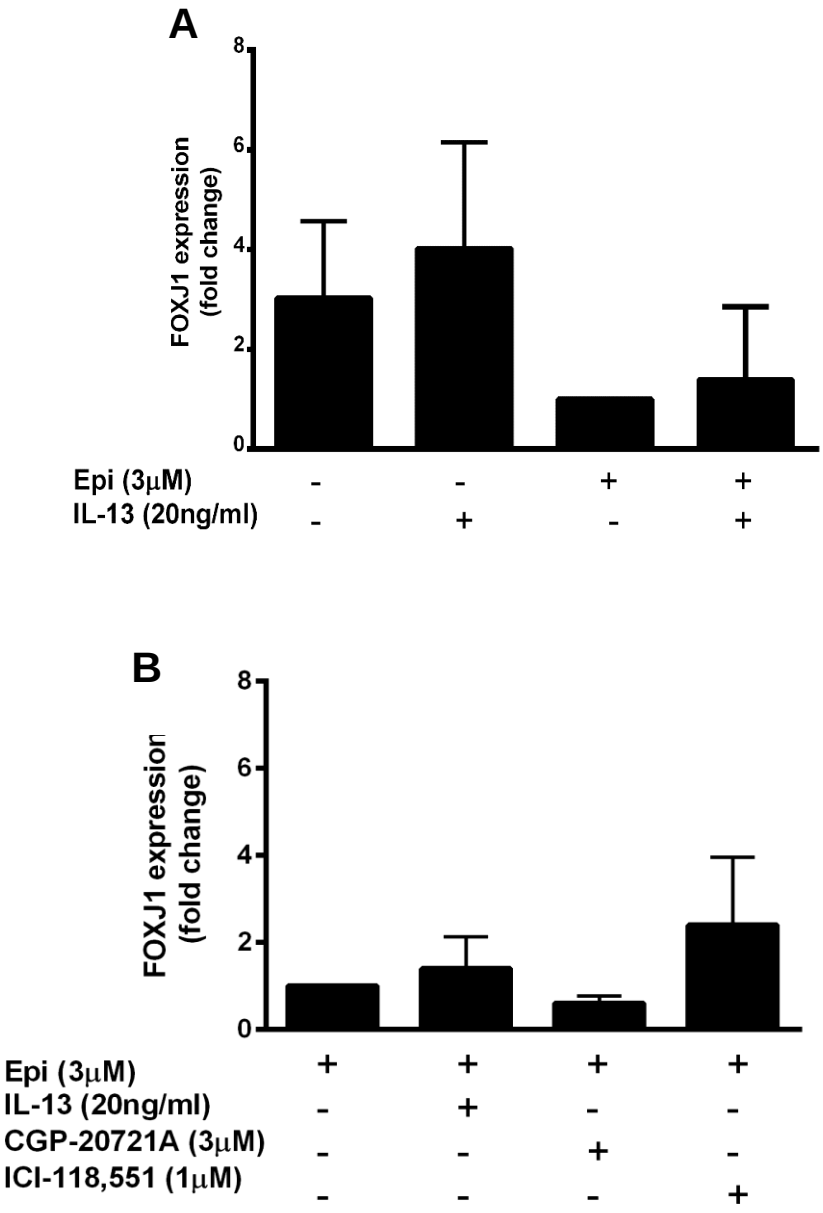



**Figure 75.** ***Role of $\beta_2$-AR signaling in FOXJ1 expression in response to IL-13 in NHBE cells:*** **A)** NHBE cells were grown in the absence of epinephrine 72 hours before ALI or in the presence of epinephrine, then at ALI, they were incubated with or without 20 ng/ml IL-13 for 14 days. FOXJ1 transcripts levels were measured by qRT-PCR and the data presented as fold change compared to cells grown in the presence of epinephrine. **B)** NHBE cells were grown in the presence of epinephrine, then at ALI, they were incubated with or without 20 ng/ml IL-13 with CGP-20721A or ICH-118,551 for 14 days. FOXJ1 was measured and the data presented as fold change compared to cells grown in the presence of epinephrine. Data are presented as

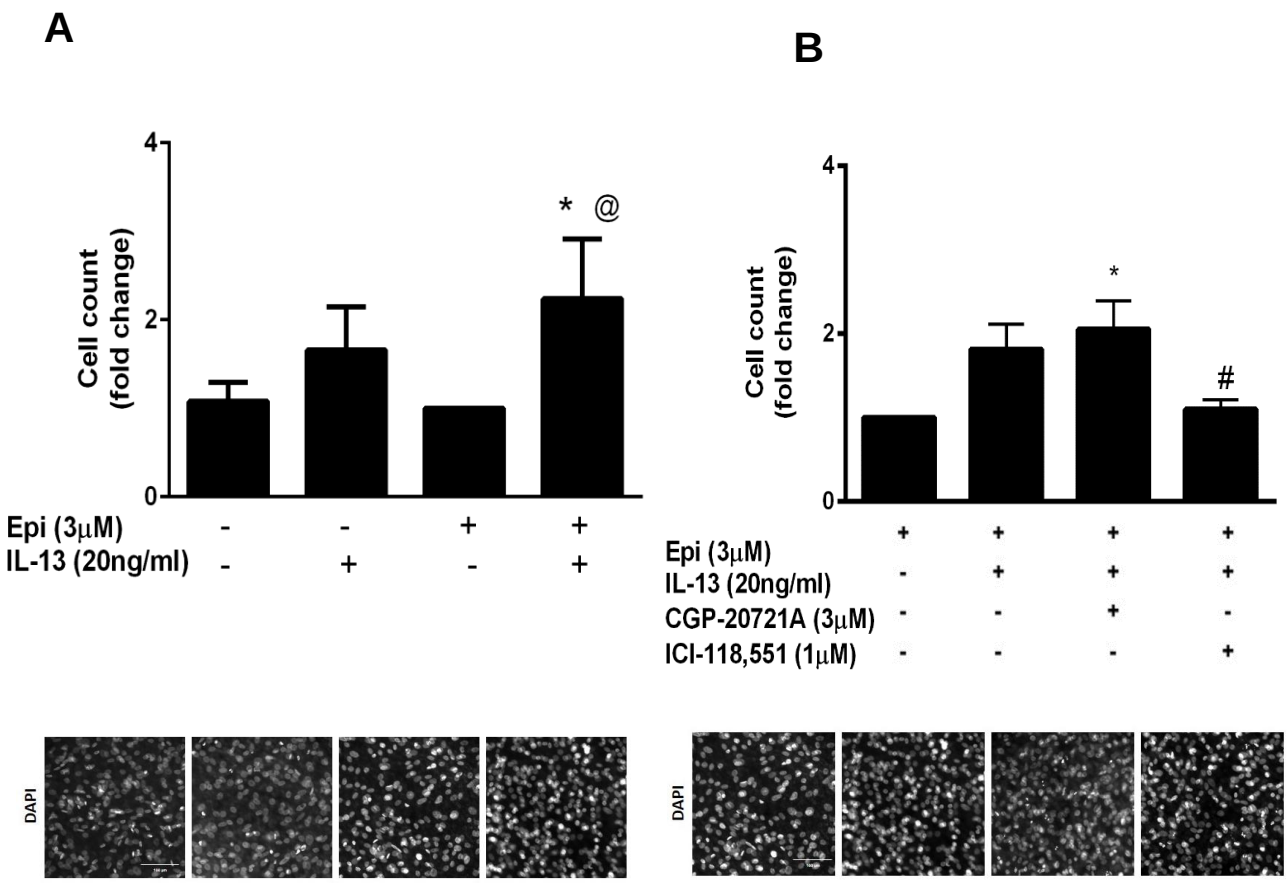



**Figure 76.** ***Role of $\beta_2$-AR signaling in cell proliferation in response to IL-13 in NHBE cells****:* **A)** NHBE cells were grown in the absence of epinephrine 72 hours before ALI or in the presence of epinephrine, then at ALI, they were incubated with or without 20 ng/ml IL-13 for 14 days. **B)** NHBE cells were grown in the presence of epinephrine, then at ALI, they were incubated with or without 20 ng/ml IL-13 with CGP-20721A or ICI-118,551 for 14 days. DAPI count was used as a marker of the cell number and the data presented as fold change compared to cells grown in the presence of epinephrine. Data are presented as means ± SEM from three donors. *, @ and # indicate $p<0.05$ significant difference between +epi and –epi and +epi+IL-13+ CGP-20721A treated cells respectively.

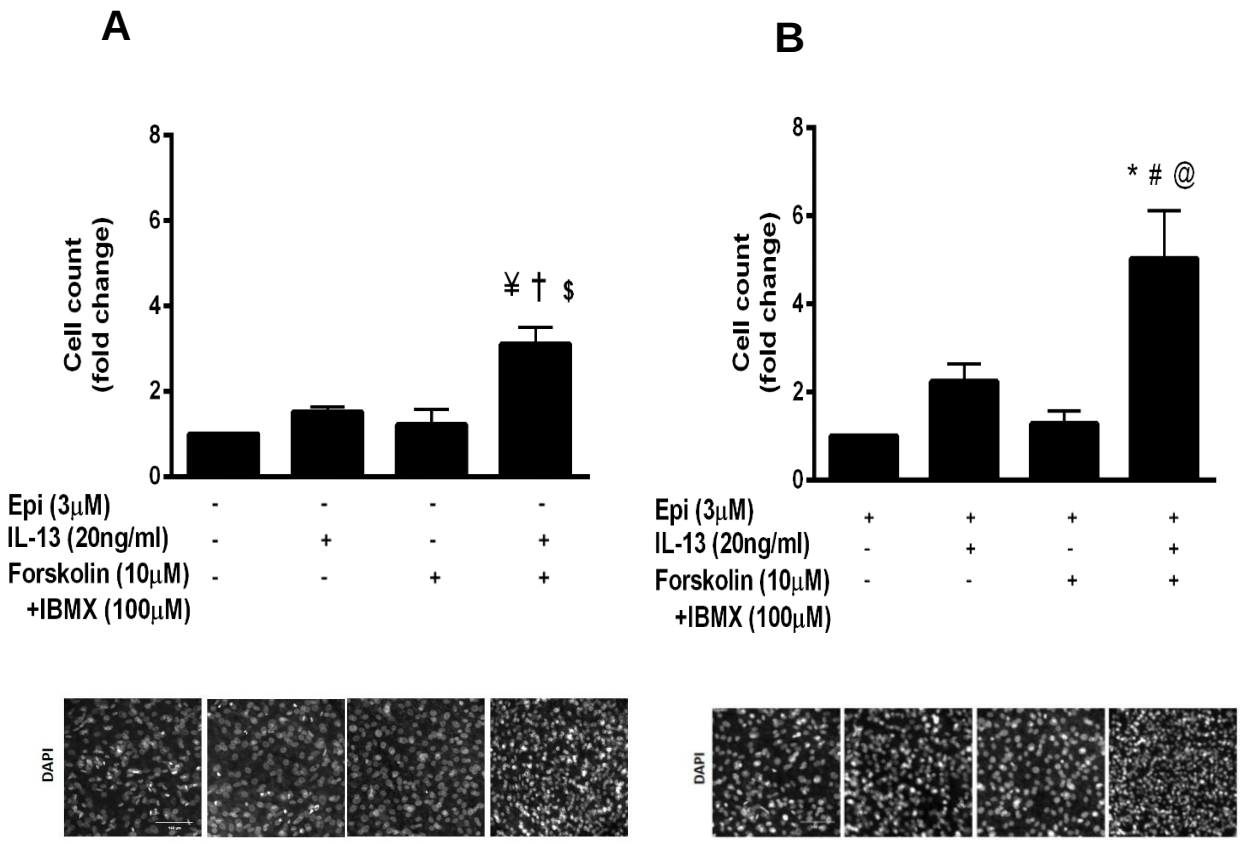



**Figure 77.** ***Role of intracellular cAMP elevation on cell proliferation in response to IL-13 in NHBE cells:*** Cells were grown in the absence of epinephrine 72 hours before ALI **(A)** or in the presence of epinephrine **(B),** then at ALI, they were incubated with or without 20 ng/ml IL-13 with or without 10 μM forskolin and 100 μM IBMX for 14 days. DAPI count was used as a marker of the cell number and the data presented as fold change compared to cells grown in the presence of epinephrine. Data are presented as means ± SEM from three donors. †, ¥,$ and *, #, @ indicate $p<0.05$ significance as compared to -epi, -epi+ IL-13,-epi+forskolin+IBMX, +epi and +epi+IL-13, +epi+forskolin+IBMX treated cells respectively.

## 5. Discussion:

Asthma, a chronic airway inflammatory condition, affects around 300 million people worldwide (Masoli, Fabian et al. 2004; 2007). Though there are several treatment modalities for asthma, it accounts for 250,000 deaths per year (2007). $\beta_2$-AR agonists are among the most widely used medications for asthma, both for acute relief and long term maintenance therapy. The chronic use of $\beta_2$-AR agonists is associated with increased risk of severe exacerbations and overall asthma related death (Currie, Lee et al. 2006; Nelson, Weiss et al. 2006; Salpeter, Buckley et al. 2006). Acute administration of β-AR blockers is associated with airway narrowing in asthmatic patients (Singh, Whitlock et al. 1976; Boskabady and Snashall 2000) and in murine model of asthma (Callaerts-Vegh, Evans et al. 2004). However, chronically blocking $\beta_2$-ARs by nadolol resulted in reduction in inflammation and mucus metaplasia in allergen driven murine model of asthma (Nguyen, Lin et al. 2009). Thus, $\beta_2$-AR signaling appears to contribute to asthma through its pro-inflammatory potential, and blocking these receptors may contribute to improvement in the disease status. Airway inflammation results in changes in airway epithelium that eventually causes mucus metaplasia. The increased mucus production that is associated with asthma and other airway inflammatory diseases, such as COPD, contributes significantly to airway obstruction and AHR. Autopsies of patients who died of

severe asthma attacks revealed goblet cell hyperplasia and mucus accumulation in peripheral airways (Aikawa, Shimura et al. 1992).

In our studies, we wanted to examine the role of $\beta_2$-AR signaling in mucus metaplasia. Due to the complexity of understanding the signaling pathways that are involved in mediating mucus production and the involvement of several inflammatory cells *in vivo*, it was essential to study the involvement of airway epithelial cells independent from other inflammatory and structural cells. In this study, we investigated the requirement for $\beta_2$-ARs in the expression of MUC5AC, the major mucin producing gene, and intracellular mucin accumulation in response to IL-13 in NHBE cells. Also, we further delineated the key signaling components involved such as MAPKs, CREB and STAT6. The major findings in this study are: 1) epinephrine-induced $\beta_2$-AR signaling is required for mucin production in NHBE cells in response to IL-13. 2) MAPKs are part of the required signaling cascade in IL-13 responses. 3) Signaling pathways downstream of cAMP and PKA play a role in mediating the effect of IL-13 on mucin parameters. 4) β-arrestin-2 signaling pathway does not modulate the effect of IL-13 on mucin parameters in NHBE cells. 5) $\beta_2$-AR signaling is not involved in IL-13 induced mucuciliary shift from ciliated cells to goblet cells in NHBE cells.

## 5.1 Epinephrine is required for mucin production in response to IL-13 in NHBE cells

Epinephrine, the endogenous ligand that activates all α and β adrenoceptors (Ahlquist 1948), is added to the culture medium of NHBE cells in most published studies (Atherton, Jones et al. 2003; Yoshisue and Hasegawa 2004; Fulcher, Gabriel et al. 2005; Zhen, Park et al. 2007; Kono, Nishiuma et al. 2010). When we removed epinephrine from the culture media 72 hours before cells reached ALI and throughout the treatment period, IL-13 was not able to induce MUC5AC expression or the accumulation of intracellular mucin 5AC and mucin glycoproteins in NHBE cells (Figure 15 and 16). On the other hand, in the presence of epinephrine, IL-13 significantly induced these three parameters of mucin expression (Figure 15 and 16). These data are consistent with our recent animal studies where genetic or pharmacological depletion of epinephrine in mice attenuated mucous metaplasia in an antigen-driven murine model of asthma (Thanawala, Forkuo et al. 2013).

Growing the NHBE cells at ALI caused an increase in the trans-epithelial electrical resistance (TEER) that peaked at ALI day 7 and then declined gradually (Figure 17A). TEER is an indirect indicator for the tight junction formation between cells and epithelial layer integrity (Stewart, Torr et al. 2012). This bell-shaped curve has been observed when retinoic acid is absent from the culture media (Murgia, Grosser et al. 2011), whereas most other papers used a

50 nM concentration of retinoic acid (Atherton, Jones et al. 2003; Fulcher, Gabriel et al. 2005; Zhen, Park et al. 2007). In our studies, we used 0.1 ng/ml (0.3 nM) retinoic acid, which is the concentration that is recommended by the vendor (Lonza®), and other studies have used this low concentration to support cell differentiation (Kafoury, Pryor et al. 1999; Lee, Yoo et al. 2005; Damera, Zhao et al. 2009). Moreover, The formation of tight junctions was also evaluated by showing the presence of ZO-1, a tight junction protein marker, on cell-junction by immunofluorescence (Stewart, Torr et al. 2012) (Figure 17B). IL-13 did not reduce TEERs values below control cells that were grown in the presence or absence of epinephrine.

We examined if the absence of epinephrine affected cell differentiation and if that was the underlying reason for MUC5AC expression not responding to IL-13 stimulation. Cells that were grown in the absence or presence of epinephrine have properties of airway epithelium at baseline (without IL-13 stimulation) at the level of MUC5AC transcripts, mucin 5AC and intracellular mucin glycoprotein expression (Figure 18A and B). Moreover, there was no significant difference at the level of FOXJ1 expression (hepatocyte nuclear factor homolog (HFH-4)/forkhead box protein J1), an early marker for ciliogenesis (Figure 18A). FOXJ1 is a transcription factor that is localized to proximal ciliated epithelial cells of the airways, as assessed by colocalization of FOXJ1 and β-tubulin IV (Blatt, Yan et al. 1999). Much evidence indicates that FOXJ1 plays a

central role in the process of ciliogenesis and it is expressed before the appearance of cilia during murine lung development (Tichelaar, Wert et al. 1999). The barrier function of cells grown in the presence or absence of epinephrine, as evaluated by TEER measurements and the cellular localization of ZO-1, were also at similar levels (Figure 17A and B). Differentiated airway bronchial epithelial cells secrete several characteristic chemokines such as *TSLP*, a well-studied epithelial cytokine, *MCP-1* and *MIP3α*, and the production of these were also unaffected by the presence or absence of epinephrine (Figure 18C). Taken together, these cells have levels of mucociliary differentiation that do not depend on the presence of epinephrine.

Our *in vitro* results are also consistent with our recent data where genetic or pharmacological depletion of epinephrine in mice resulted in attenuation of inflammation and mucous metaplasia in an antigen-driven model of asthma (Thanawala, Forkuo et al. 2013).

## 5.2 Epinephrine induced-$\beta_2$-AR signaling is required for mucin production in response to IL-13 in NHBE cells

$\beta_2$-AR is the principal subtype present in human airway epithelium (Davis, Silski et al. 1990). To verify the involvement of this subtype in the effects of IL-13 in cultured NHBE cells, we used ICI-118,551 and CGP 20712A. CGP 20712A is a highly preferential $\beta_1$-AR antagonist; its selectivity toward $\beta_1$-AR is ~500 fold

over $\beta_2$-AR and ~4000 over $\beta_3$-AR (Baker 2005). On the other hand, ICI-118,551 is a preferential $\beta_2$-AR antagonist; its selectivity to $\beta_2$-AR as compared to $\beta_1$-AR is ~500 fold and ~600 fold as compared to $\beta_3$-AR (Baker 2005). Treating NHBE cells with the preferential $\beta_2$-AR antagonist almost completely abolished MUC5AC expression in response to IL-13 and epinephrine (Figure 19A). On the contrary, the preferential $\beta_1$-AR antagonist did not alter the expression level under the same conditions. Also IL-13 induced accumulation of intracellular mucin 5AC and mucin glycoproteins were reduced by ICI-118,551 but not by CGP 20712A treatment (Figure 19B and Figure 20). Thus, $\beta_2$-AR activation is required in IL-13 induced mucin accumulation in NHBE cells. Our results are consistent with the murine model of asthma, in which blocking $\beta_2$-AR with ICI-118,551 (Nguyen, Omoluabi et al. 2008) or genetically ablating $\beta_2$-AR (Nguyen, Lin et al. 2009) resulted in attenuated mucus metaplasia in response to allergen. The concentrations of ICI-118,551 and CGP 20712A that were used in our studies did not affect the integrity of the monolayer, as evaluated by TEER measurement and ZO-1 apical localization, when compared to untreated control cells (Figure 21A and B).

Previous studies of the role of $\beta_2$-ARs in mucous secretion in the airways have produced varying results. An earlier study had shown that β-AR activation, through cAMP/PKA dependent mechanisms, enhanced the secretion of mucus cells from cat trachea *in vitro* (Liedtke, Rudolph et al. 1983). Moreover,

salbutamol, a $\beta_2$-AR agonist, increased the rate of mucus output from human bronchi *in vitro* (Phipps, Williams et al. 1982). In contrast, other *in vitro* studies on human bronchial airway preparations showed that β-AR stimulation, *via* isoproterenol (Shelhamer, Marom et al. 1980; Abdullah, Conway et al. 1997), or increasing the level of intracellular cAMP were not associated with increase in the release of mucin (Abdullah, Conway et al. 1997). It is noteworthy to mention that these studies focused on mucin secretion rather than on intracellular mucin accumulation, which we studied. Increased mucin accumulation possibly resulted from a change in the relative rates of mucin production and secretion. At the level of secretion, there appears to be no change in the molecular components or the rate of the secretion during mucus metaplasia (Evans, Kim et al. 2009). Therefore, the increase in mucin accumulation is most likely a reflection of increased mucin production (Young, Williams et al. 2007), specifically MUC5AC (Kirkham, Sheehan et al. 2002).

$\beta_2$-AR signaling can proceed from agonist-activated receptors and from constitutively active receptors (Bond, Leff et al. 1995). In two state receptor model receptors are at equilibrium between two conformations, active (R*) and inactive (R) (Kenakin 2001; Bond and Ijzerman 2006). The receptor in its active confirmation couples to G-protein and activates downstream signaling pathways in a ligand-independent fashion (Seifert and Wenzel-Seifert 2002; Milligan 2003). Ligands are classified based on their ability to bind and enrich one of these

conformations as outlined in Figure 78. An *agonist* binds and enriches the active conformation while an *inverse agonist* binds and stabilizes the inactive conformation, and thus it reduces the signaling induced by the constitutive active receptor (Milligan, Bond et al. 1995; Milligan 2003). A *neutral antagonist* theoretically binds without preference to both conformations (Parra and Bond 2007).

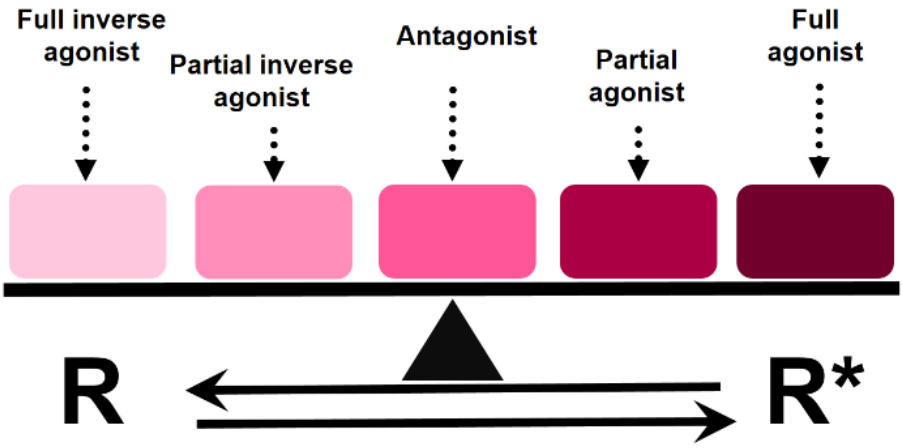



**Figure 78. Two state model of receptor theory**: The receptor exists in equilibrium between active (R*) and inactive state (R). *Full agonist* stabilizes more receptors in the active confirmation than the partial agonist. Full *inverse agonist* binds to and enriches the inactive conformation at a higher level as compared to partial inverse agonist. *Antagonist* binds to both receptor conformations and does not affect the equilibrium. Reproduced from Seifert and Wenzel-Seifert (2002).

We have shown that epinephrine activation of $\beta_2$-AR is required to induce mucin production in response to IL-13. To further evaluate whether agonist induced signaling or constitutive $\beta_2$-AR activity is sufficient for this effect, we treated the cells with nadolol and alprenolol in the presence of epinephrine. Nadolol is a non-selective β-AR blocker that has full inverse agonist activity at $\beta_2$-ARs (Callaerts-Vegh, Evans et al. 2004; Wisler, DeWire et al. 2007), thus it blocks both induced and constitutive $\beta_2$-AR signaling. Alprenolol lacks inverse agonist activity (Bond, Leff et al. 1995) and has weak $\beta_2$-AR agonist activity (Brodde, Daul et al. 1985; Lima 1996; Wisler, DeWire et al. 2007) behaving as an antagonist in the presence of epinephrine, thus preserving constitutive $\beta_2$-AR signaling. Both nadolol and alprenolol blocked the effect of IL-13 on the three parameters of mucin production to similar extents (Figure 22 and Figure 23).

The role of nadolol in reducing the mucin content of airway epithelium is consistent with our group's findings in the allergen driven murine model of asthma (Nguyen, Omoluabi et al. 2008). In contrast, alprenolol did not reduce mucus metaplasia in this model (Nguyen, Lin et al. 2009) while it reduced all three measured parameters (MUC5AC expression, intracellular mucin 5AC and mucin glycoproteins accumulation) in NHBE cells. This discrepancy in the results could be explained by differences between the *in vitro* and *in vivo* models. In the mouse model, mucus metaplasia is dependent on different mediators and

cytokines released from recruited inflammatory cells and not only on the direct effect of the ligand on airway epithelial cells. In our isolated system, we studied the effect of each ligand on NHBE cells independent from other cell types, either hematopoietic or parenchymal. The effect of alprenolol in NHBE cells may be explained based on its weak agonist properties. One proposed scenario is that alprenolol, the partial agonist, competes with epinephrine, full agonist, and displaces epinephrine from its binding sites on $\beta_2$-ARs. The resulting weak $\beta_2$-AR signaling may be insufficient for increased mucin expression in response to IL-13.

Thus, we provide evidence that constitutive $\beta_2$-AR receptor activity is not sufficient to drive the increase in mucin in response to IL-13 in human bronchial epithelial cells. Our *in vitro* results are also consistent with our recent animal data where genetic depletion of epinephrine resulted in attenuated mucus metaplasia which was restored by formoterol, a $\beta_2$-AR agonist, an antigen-driven murine model of asthma (Thanawala, Forkuo et al. 2013). More work in future is required to evaluate the contribution of different $\beta_2$-AR agonists in mediating the effect of IL-13 in NHBE cells.

## 5.3 MAPK signaling is involved in mucin production in response to IL-13 in NHBE cells

MAPKs play a major role in inflammation (Garrington and Johnson 1999), asthma pathogensis and in airway remodeling (Pelaia, Cuda et al. 2005). There are three principal families of MAPKs: ERK 1/2, JNK and p38. ERK 1/2 is mainly involved in cell growth and differentiation, while JNK and p38 play major roles in stress, inflammation and apoptosis signaling (Schaeffer and Weber 1999).

All MAPK pathways are activated through three core activation steps as summarized in Figure 79. We studied the involvement of the three MAPKs in mucin production in response to IL-13.

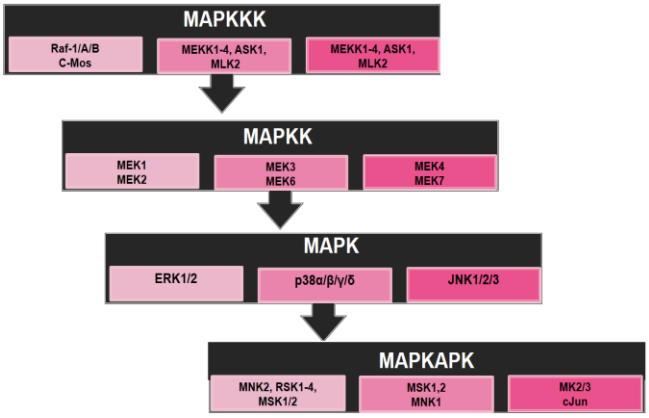



**Figure 79. Activation of MAPKs**. Briefly, different activators activate MAPK-kinase-kinases (MAPKKKs). These kinases activate MAPK-kinase (MAPKKs) which in turn phosphorylate MAPKs. The three major MAPKs, JNK, p38 and ERK1/2, then activate downstream substrates and eventually induce different cell responses. Reproduced and modified from Cargnello and Roux (2011).

### 5.3.1 Role of ERK1/2 signaling in mucin production in response to IL-13 in NHBE cells

In asthmatic patients, elevated levels of phosphorylated ERK1/2 were detected in airway epithelium and correlated positively with disease severity (Liu, Liang et al. 2008). Moreover, elevated levels of lung phosphorylated ERK1/2 are found in a murine model of asthma (Kumar, Lnu et al. 2003). IL-13 significantly increased ERK1/2 phosphorylation in the presence, but not in the absence, of epinephrine (Figure 24A). U0126, a specific MEK1/2 inhibitor, significantly downregulated ERK1/2 phosphorylation and MUC5AC expression induced by IL-13 (Figure 25A and B). U0126 is a selective non-ATP competitive inhibitor (Favata, Horiuchi et al. 1998) that binds to a pocket of MEK1/2 adjacent to ATP-binding site and thus inactivates its catalytic activity (Sebolt-Leopold and English 2006). Our result is consistent with a study by Atherton and colleagues in which IL-13 induced mucous hypersecretion manifested as an increase in goblet cell density and MUC5AC protein expression in NHBE cells, and these effects were attenuated with the use of MEK1/2 inhibitor (Atherton, Jones et al. 2003). In addition, U0126 lowered the level of mucus metaplasia in allergen driven murine model of asthma (Duan, Chan et al. 2004; Chialda, Zhang et al. 2005). Moreover, U0126 treatment reduces MUC5AC expression and the number of goblet cells in response to IL-13 in cultured mouse tracheal epithelial cells (mTECs), though ERK1/2 phosphorylation was not affected by IL-13 (Fujisawa, Ide et al. 2008).

These differences could be due to the differences in primary cells source as well the treatment protocol.

To confirm our finding of ERK-1/2 involvement, we also used FR180204, an ATP-competitive direct inhibitor of ERK1/2 (Ohori, Kinoshita et al. 2005). FR180204 reduced MUC5AC expression, intracellular mucin 5AC and mucin glycoprotein content in response to IL-13 (Figure 26A, B and Figure 27). Moreover, FR180204 did not affect the integrity of the monolayer as demonstrated by TEERs and ZO-1 localization (Figure 28 A and B). Thus, this is the second line of evidence toward the involvement of ERK1/2 signaling cascade in mediating IL-13 response.

ERK1/2 is activated by GPCRs, cytokines and growth factors such as epidermal growth factor (EGF) (Cargnello and Roux 2011). Also, IL-13 activates ERK1/2 through IRS1/2 /Grb-2 /SOS /Ras pathway (Jiang, Harris et al. 2000; Hershey 2003). cAMP causes phosphorylation of Rap-1/B-Raf, through PKA (Vossler, Yao et al. 1997) and Epac (Gerits, Kostenko et al. 2008), and consequently ERK1/2 activation (Busca, Abbe et al. 2000). β-arrestin, as a scaffolding protein, binds directly to Raf-1 and ERK1/2 and indirectly to MEK1 and consequently activates ERK1/2 (Pierce and Lefkowitz 2001; DeWire, Ahn et al. 2007). Moreover, β-arrestin recruits Src, a non-receptor tyrosine kinase, to the phosphorylated $\beta_2$-AR and phosphorylates ERK1/2 (Sun, McGarrigle et al. 2007;

Defea 2008). Src also activates ERK1/2 indirectly through transactivation of EGFR (Prenzel, Zwick et al. 1999).

Activated ERK1/2 phosphorylates a number of downstream substrates including membrane proteins such as calnexin (Roux and Blenis 2004) and other kinases such as the 90 kDa ribosomal S6 kinase (RSK) (Anjum and Blenis 2008). Of interest, ERK2 phosphorylates PDE4D3 and significantly reduces its activity (Hoffmann, Baillie et al. 1999), thus ERK2 can modify cAMP signaling. FR180204 did not influence ERK1/2 or p38 activation (Figure 29 A and C).

Of greatest relevance to our studies is that ERK1/2 has been shown to activate downstream signaling cascades that lead to activation or translocation of several transcriptional factors to bind to the MUC5AC promoter region and enhance MUC5AC transcription (Thai, Loukoianov et al. 2008) as outlined in Figure 80.

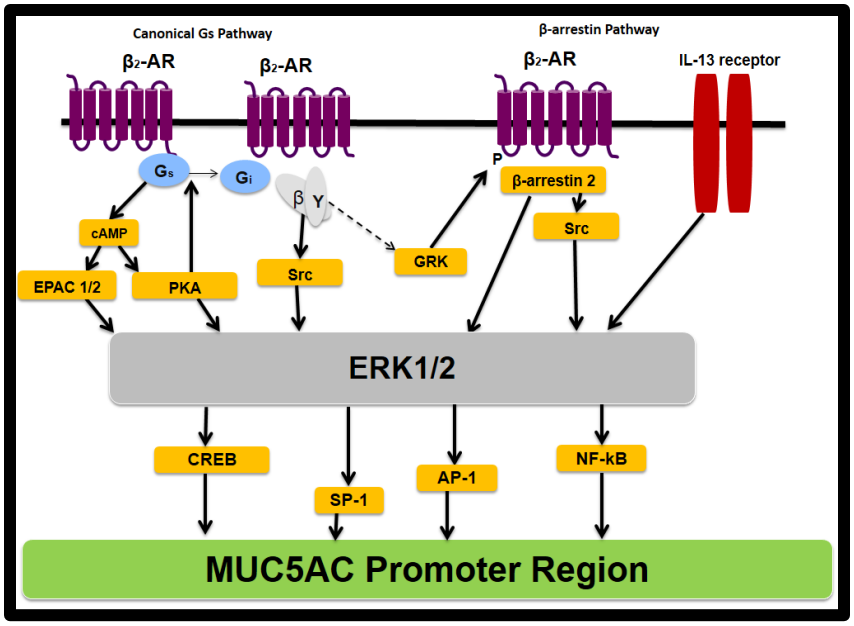



**Figure 80. Schematic illustration of MUC5AC expression by activated ERK1/2:** Several upstream signaling pathways result in ERK1/2 activation. Phosphorylated ERK1/2 activates several transcription factors, such as CREB, SP-1, AP-1 and NF-kB that bind to the MUC5AC promoter region and enhance its transcriptional activity.

ERK1/2 activation in bronchial epithelial cells results in phosphorylation of CREB *via* RSK (Kim, Hong et al. 2007) or mitogen- and stress-activated protein kinase 1 (MSK1) dependent signaling (Song, Lee et al. 2003). SP-1 (Hewson, Edbrooke et al. 2004) and NF-κB (Thai, Loukoianov et al. 2008) are also activated by ERK1/2. Moreover, ERK1/2 activates fos family proteins,

which are part of the AP-1 transcription factor complex with c-Jun proteins (Karin 1996; Gensch, Gallup et al. 2004).

In our studies, the level of phosphorylated RSK-1 or CREB were not affected by either IL-13 or FR180204 (Figure 30 A and B). The MUC5AC promoter region contains a TGACGTCA sequence (Johannessen, Delghandi et al. 2004) that is called the cAMP-response element (CRE) motif (Song, Lee et al. 2003; Kim, Hong et al. 2007; Thai, Loukoianov et al. 2008). Phosphorylation of the Serine-133 residue of CREB enhances its interaction with CREB-binding protein (CBP) and p300 transcriptional co-activators. The CREB /co-activator complex in turn enhances RNA polymerase complex assembly at the CRE motif of the MUC5AC promoter (Johannessen, Delghandi et al. 2004) and hence enhances gene transcription. The role of pCREB in mucous hypersecretion was suggested by the observation of elevated levels of pCREB in bronchial epithelial cells in asthma patients compared to normal individuals (Chiappara, Chanez et al. 2007). It has been shown that MUC5AC expression was induced in normal human bronchial cells by retinoic acid and prostaglandin F2α, via CREB dependent mechanisms and that MUC5AC expression was abolished by knocking down CREB (Kim, Hong et al. 2007; Chung, Ryu et al. 2009). In contrast, c-Jun phosphorylation was significantly induced by IL-13 and was inhibited by FR180204 treatment (Figure 29B). Thus, our results support the role of ERK1/2 in mediating IL-13 effect on mucin production through AP-1 nuclear

translocation and subsequent activation of MUC5AC transcription. Further studies are required to dissect the involvement of other transcription factors that might be downstream of ERK1/2 such as NF-κB and SP-1.

### 5.3.2 Role of JNK signaling in mucin production in response to IL-13 in NHBE cells

JNK is activated by environmental stress and pro-inflammatory cytokines (Pelaia, Cuda et al. 2005) such as TNF and IL-1 (Kyriakis and Avruch 2001). JNK is involved in regulating pro-inflammatory genes and airway remodeling in airway diseases (Bennett 2006). Three JNK isoforms are present in mammalian cells; JNK1, 2 and 3. JNK1 and 2 are expressed in most tissues, while JNK3 is only expressed in heart, brain and testes (Cargnello and Roux 2011). The most important substrate for activated JNK is the AP-1 transcription factor complex, composed of members of the fos and Jun families of transcription factors (Karin 1995). Different scaffold proteins are known to modulate JNK activity and target JNK to specific cellular locations. JNK-interacting protein (JIP1/2) and β-arrestin-2 are examples (Qi and Elion 2005). Several studies have shown that JNK plays a role in MUC5AC expression in response to cigarrete smoke extract (Yu and Zhou 2010), live *Legionella pneumophila* (Morinaga, Yanagihara et al. 2012) and *Streptococcus pneumoniae* pneumolysin (Ha, Lim et al. 2008) in cultured airway epithelial cells.

Phosphorylated JNK activates different transcription factors that belong to AP-1 transcription factor family (Gensch, Gallup et al. 2004) such c-Jun (Cargnello and Roux 2011), JunB and JunD (Davis 2000). In our study, IL-13 induced the phosphorylation of c-Jun, a distinct JNK downstream target, only in the presence of epinephrine (Figure 24B). These data point towards the involvement of IL-13 in stimulating the kinase activity of JNK. In the absence of epinephrine, IL-13 did not increase the phosphorylation of c-Jun (Figure 24B). Treating NHBE cells with 3 μM SP600125, an inhibitor of all three isoforms of JNK, reduced MUC5AC expression and mucin content in response to IL-13 in the presence of epinephrine (Figure 31A, B and Figure 32). This inhibitory effect of SP600125 on mucin production is mediated by downregulating c-Jun phosphorylation (Figure 34A). Our result is consistent with the attenuation of mucus metaplasia by SP600125, in an allergen driven murine model of asthma (Chialda, Zhang et al. 2005; Nath, Eynott et al. 2005). Moreover, SP600125 did not affect the integrity of the NHBE monolayer as demonstrated by TEERs and ZO-1 localization (Figure 33A and B). SP600125, at high concentrations, affects p38 activation through inhibiting the upstream MAPKKs (Cui, Zhang et al. 2007). Thus, it was necessary to evaluate the effect of SP600125 on p38 and ERK1/2 activation. SP600125 did not inhibit the phosphorylation of these MAPKs in response to IL-13 (Figure 34 B and C). Moreover, SP600125 did not affect CREB phosphorylation (Figure 35).

JNK is activated by $\beta_2$-AR via $G_s$-AC/cAMP dependent activation of the Rho family of small GTPases (Yamauchi, Hirasawa et al. 2001) and also by G protein-independent, βarrestin-2-dependent pathways (Defea 2008). In addition to AP-1 transcription factor family, JNK also activates SP-1 (Benasciutti, Pages et al. 2004). These transcription factors bind to specific sequences on MUC5AC promoter and enhance gene expression as outlined in Figure 81. Testing the involvement of SP-1 transcription factor is required in future studies.

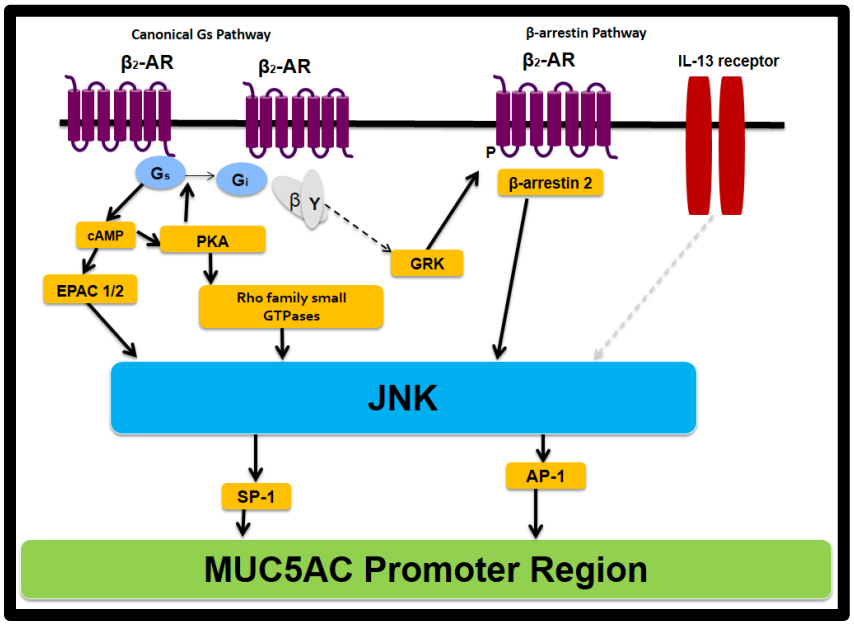



**Figure 81. Possible action of JNK on the MUC5AC promoter:** Several upstream signaling pathways activate JNK. Phosphorylated JNK activates a number of transcription factors, such as SP-1 and AP-1, that bind to the MUC5AC promoter region and increase its expression.

### 5.3.2 Role of p38 signaling in mucin production in response to IL-13 in NHBE cells

Elevated levels of phosphorylated p38, α, β and γ isoforms, are detected in the airway epithelium of asthmatic patients and the levels correlate with disease severity (Liu, Liang et al. 2008). Previous studies have illustrated the involvment of p38 in regulating MUC5AC expression in response to several inflammatory mediators such as IL-1β (Kim, Kwon et al. 2002), interleukin-17A (IL-17A) (Fujisawa, Velichko et al. 2009) and TNF-α (Song, Lee et al. 2003).

In our study, the phosphorylation of p38 was not induced by IL-13 either in the presence or absence of epinephrine (Figure 24C). The inability of IL-13 to induce p38 phosphorylation is inconsistent with other studies. In one study, incubation of NHBE cells with 20 ng/ml IL-13 for 20 and 60 minutes resulted in increased p38 phoshorylation (Kono, Nishiuma et al. 2010). Moreover, 10 ng/ml IL-13 treatment for 48 hours produced an increase in p38 phosphorylation in cultured primary mTECs (Fujisawa, Ide et al. 2008). The difference bewteen our results and theirs could be attributed to species differences, or to donors profiles.

To further explore a role for p38, NHBE cells were treated with 3μM SB203580, a specific ATP-competeitive inhibitor of both α and β isoforms of p38 (Lisnock, Tebben et al. 1998; English and Cobb 2002; Chung 2011) in combination with IL-13 and epinephrine. Among the four p38 isoforms, α (Rouse, Cohen et al. 1994), β (Jiang, Chen et al. 1996), γ (Li, Jiang et al. 1996) and δ

(Wang, Diener et al. 1997), the transcript levels of α and β isoforms in the human lung are higher than the other isoforms (Jiang, Gram et al. 1997). SB203580 significantly reduced MUC5AC expression, intracellular mucin 5AC and mucin glycoprotein content in response to IL-13 (Figure 36 A, B and Figure 37). Our result is consistent with other studies where inhibiting p38 activity resulted in attenuated IL-13 induced goblet cell density in NHBE cells (Atherton, Jones et al. 2003), mucin 5AC positive cells and MUC5AC expression in cultured mTECs (Fujisawa, Ide et al. 2008).

All MAPKs inhibitors that we used in our studies did not reduce the monolayer integrity compared to control cells that were grown in the presence of epinephrine, however SB203580 appeared to increase the barrier function of cultured NHBE cells (Figure 38A and B).

The role of p38 is also suggested in an allergen driven murine model of asthma, in which inhaled p38 α antisense oligonucleotide (Duan, Chan et al. 2005) or inhibiting p38α with a specific p38α inhibitor, SD-282,(Ma, Medicherla et al. 2008) attenuated mucus production in response to allergen and IL-13 adminstration respectively. The level of p38 phosphorylation was not affected by SB203580 since this inhibitor inhibits the kinase activity of p38 rather than its upstream activators (Kumar, Jiang et al. 1999) (Figure 39C). Thus, we tested the phosphorylation of MAP kinase activated protein kinase-2 (MAPKAP kinase-2) (Cuenda, Rouse et al. 1995), a downstream subtstrate of p38, and its

phosphorylation level was not affected by IL-13 or by the p38 inhibitor (Figure 40A). SB203580 did not affect the activation of other MAPKs such as ERK1/2 or cJun, the specific JNK downstream target (Figure 39A and B).

p38 is activated by $\beta_2$AR in a $G_s$-AC (Zheng, Zhang et al. 2000) and βarrestin-2 dependent fashion (DeWire, Ahn et al. 2007). The effect of p38 on MUC5AC gene expression is mediated by activation of different transcription factors that bind to MUC5AC promoter. Of these transcription factors; CREB (Song, Lee et al. 2003), NF-κB (Pelaia, Cuda et al. 2005) and SP-1 (Thai, Loukoianov et al. 2008) as outlined in Figure 82. CREB phosphorylation was not affected by p38 inhibitor (Figure 40B). Thus, we were not able to determine the mechanism by which p38 affect mucin production in response to IL-13 and more studies are required to examine the involvement of SP-1 as a downstream target of p38.

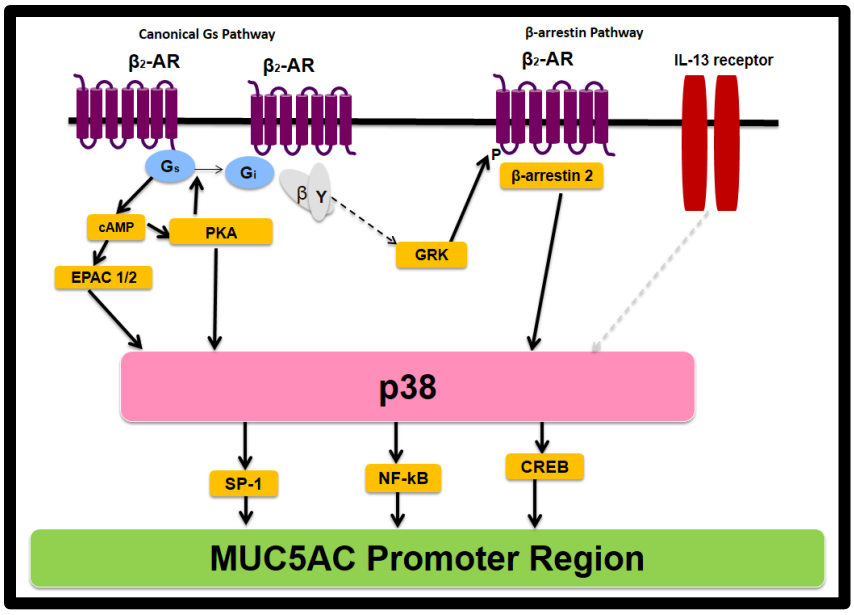



**Figure 82. Schematic illustration of p38 activity on MUC5AC promoter:** Several upstream signaling pathways activate p38. Phosphorylated p38 activates several transcription factors, such as CREB, SP-1 and NF-kB, that bind to the MUC5AC promoter region and enhance its transcriptional activity.

## 5.4 Role of PKA signaling in mucin production in response to IL-13 in NHBE cells

$\beta_2$-AR can signal through heterotrimeric G-proteins, and also through β-arrestin. The Gs pathway increases adenylyl cyclase activity and thereby intracellular cAMP concentrations, leading to activation of cAMP-dependent protein-kinase A (PKA) and exchange protein activated by cAMP (Epac). To test for a possible role of cAMP in mediating the effect of IL-13 + epinephrine, we first

tried a widely-used inhibitor of PKA, H89. This drug binds to the ATP binding site in the catalytic subunit of PKA and thus inhibits its activity (Chijiwa, Mishima et al. 1990; Lochner and Moolman 2006; Cho, Woo et al. 2009). Treating NHBE cells with 3μM H89 in combination with IL-13 and epinephrine resulted in a reduction in MUC5AC expression (Figure 41A). However, H89 did not reduce ERK1/2 phosphorylation (Figure 41B), thus either ERK1/2 is not a downstream substrate of PKA or the effect of H89 on MUC5AC expression is mediated by inhibiting MSK-1, which is downstream of ERK1/2, rather than PKA. Though H89 is used widely as PKA inhibitor, with a $K_i$ ~48 nM (Chijiwa, Mishima et al. 1990), it also blocks $\beta_2$-AR, at $K_i$ ~180 nM (Penn, Parent et al. 1999). Moreover, H89 inhibits MSK-1 and other kinases with almost with simliar potency as compared to PKA inhibition (Penn, Parent et al. 1999; Lochner and Moolman 2006). Therefore, another PKA inhibitor was utilized to study the involvement of PKA.

Rp-cAMPS is a cAMP analog that binds to cAMP binding site on PKA's regulatory subunit, preventing the dissociation of the catalytic subunits and inhibiting PKA activity (Botelho, Rothermel et al. 1988; Dostmann 1995; Lochner and Moolman 2006). MUC5AC expression and intracellular mucin 5AC protein levels induced by IL-13 and epinephrine were inhibited by 100 μM Rp-cAMPS, but not 50 μM Rp-cAMPS, while the mucin content of NHBE cells was inhibited at both concentrations (Figure 42A, B and Figure 43). Though Rp-cAMPS is believed to be specific toward PKA, it may also inhibit Epac, though the data are

contradictory (Christensen, Selheim et al. 2003; Rehmann, Schwede et al. 2003; Holz 2004). Therefore, we can not completely exclude the role of Epac in mediating the effect on MUC5AC expression induced by IL-13 and further studies are required utilizing reagents, such as the Epac activator 8-pCPT-2-O-Me-cAMP (Gloerich and Bos 2010).

The effect of Rp-cAMPS on MUC5AC expression may be explained by reference to the pathways shown in Figure 83.

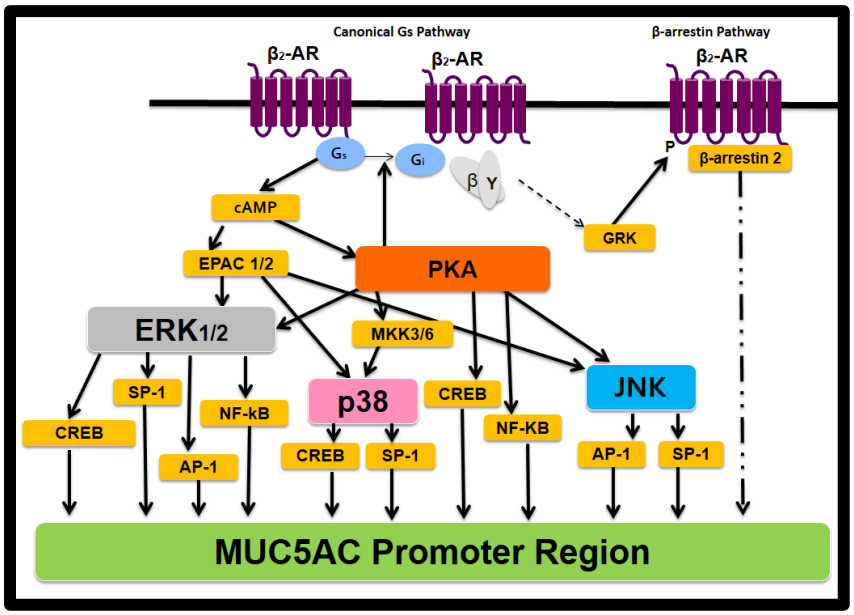



**Figure 83. Schematic diagram of possible PKA effects on MUC5AC promoter:** Activated PKA in the Gs/AC pathway may lead to activation of several downstream transcription factors that bind to MUC5AC promoter region and enhance its transcriptional activity.

The two concentrations of Rp-cAMPS that we used in our studies did not affect the integrity of NHBE cells monolayer as evaluated by TEER and ZO-1 localization (Figure 44A and B).

Inhibiting PKA by 100 μM Rp-cAMPS increased ERK1/2 phosphorylation in NHBE cells in response to IL-13 in the presence of epinephrine (Figure 45A). Therefore, PKA activation has an inhibitory effect on ERK1/2 phosphorylaion. PKA can either activate or inhibit ERK1/2 phosphorylation based on the isoform of interacting Raf and the cell type (Gerits, Kostenko et al. 2008). Activation of PKA, by $\beta_2$-AR, activates Rap-1/B-Raf signaling cascade and results in ERK1/2 activation (Schmitt and Stork 2000). Raf-1 is expressed in most tissues while A-Raf and B-Raf are restricted in their expression (Chen, Gibson et al. 2001). B-Raf is expressed at low levels in the lung (Stork and Schmitt 2002). On the other hand, PKA reduces ERK1/2 activation through phosphorylating and inactivating Raf-1 which reduces ERK1/2 phosphorylation (Cook and McCormick 1993; Mischak, Seitz et al. 1996). Moreover, Rap-1 activation by PKA enhances its association with Raf-1 and this complex sequesters Raf-1 and reduces its availability to bind and to Ras and consequently reduces ERK activation (Schmitt and Stork 2001). It's important to note that activation of Rap-1 can stimulate B-Raf and increase ERK phosphorylation (Gerits, Kostenko et al. 2008). The cross-talk between ERK1/2 and PKA is complex: ERK1/2 can modify the activity of cAMP/PKA through affecting the activity of PDE4 (Gerits, Kostenko et al. 2008).

In our hands, Rp-cAMPS inhibited MUC5AC expression while it increased ERK1/2 phosphorylation. This discrepancy could be due to the complexity of the signaling pathways that are involved in regulating MUC5AC promoter activity. For example, even though ERK1/2 phosphorylation was increased with Rp-cAMPS, it is possible that Rp-cAMPS inhibited other signaling pathways that we did not examine in this study and these pathways have greater influence on MUC5AC promoter than ERK1/2. In addition to that, the cellular localization, in the cytoplasm or translocated to the nucleus, of phosphorylated ERK1/2 by Rp-cAMPS could affect the cellular response.

PKA also affects p38 activity through different complex mechanisms such as regulating Rap-1 and different phosphatases that affect kinases upstream of p38 (Gerits, Kostenko et al. 2008). PKA not only affects ERK1/2 and p38 activation, but also JNK. The effect of PKA on JNK is not yet completely resolved, but cAMP/PKA might inhibit JNK activity through inducing the synthesis of JNK inhibitors while Epac could activate JNK-1(Gerits, Kostenko et al. 2008). But in our studies, inhibiting PKA did not alter the effect of IL-13 on p38 or JNK activity Figure 45B and C). Additionally, CREB or STAT6 phosphorylation were not affected by Rp-cAMPS (Figure 46A and B). Therefore, we were not able to determine the signaling pathway downstream of PKA.

## 5.5 Effect of cAMP elevating agents in mucin production in response to IL-13 in NHBE cells

### 5.5.1 Effect of forskolin+IBMX in mucin production in response to IL-13 in NHBE cells

To further establish the involvment of cAMP, forskolin and IBMX in combination were used to raise intracellular cAMP levels independent of $\beta_2$AR activity. Forskolin activates the catalytic activity of all AC isoforms except AC9 (Hurley 1999), through assembling the catalytic domains of AC (Zhang, Liu et al. 1997) and hence increasing intracellular cAMP levels (Seamon and Daly 1981). IBMX reduces the degradation of cAMP by inhibiting all PDEs isoenzymes except PDE8 (Bender and Beavo 2006). Combining both reagents substantially increases the intracellular cAMP levels, and this treatment increased mucin production in response to IL-13 even in the absence of epinephrine (Figure 47, Figure 48 and Figure 49).

In patients with mild to moderate asthma, forskolin is effective in preventing the acute attacks and did not cause any worsening in pulmonary function, as evaluated by forced expiratory volume in 1 second (FEV1) (Gonzalez-Sanchez, Trujillo et al. 2006). This effect of forskolin is due to its ability in reducing the release of inflammatory mediators from several structural and inflammatory cells that are present in the airways. For example, forskolin reduces the release of eotaxin from human ASM cells (Pang and Knox 2001), IL-

13 from peripheral blood mononuclears cells (Yoshida, Shimizu et al. 2001), leukotriene B4 from alveolar macrophages (Fuller, O'Malley et al. 1988). However, in our *in vitro* studies, forskolin resulted in an increase in mucin production in response to IL-13. Our *in vitro* model studied the direct effect of forskolin+IBMX on one specific parenchymal cell type, independent from other types of cells, and under the influence of one cytokine. Therefore, the results that are obtained from whole body or murine models should not be compared side by side to *in vitro* cultures. Forskolin+IBMX concentrations that we used in our study did not alter the barrier function of cultured NHBE cells in the presence or absence of epinephrine (Figure 50A, B). In NHBE cells, the forskolin+IBMX combination caused an increase in accumulated intracellular cAMP levels, by more than 400 fold as compared to cells grown in the presence or absence of epinephrine (Figure 51). However, this increase in cAMP does not mimic the effect mediated by epinephrine. Intracellular cAMP levels in cells grown in the absence or presence of epinephrine were not different. In our studies, cAMP levels were measured 48 hours after the last treatment point on ALI day 12. It has been shown previously that 10 µM isoproterenol treatment for 5 minutes results in significant increase in cAMP accumulation in cultured cell lines. However, when these cells were incubated with 10 µM isoproterenol for 24 hours the cAMP level was similar to untreated cells, probably due to receptor desensitization (Peng, Bond et al. 2011). Moreover, treating human bronchial

epithelial cells with salbutamol results in increased accumulation of cAMP that reaches maximum between 30-60 minutes (Dent, White et al. 1998). Formeterol, which has similar efficacy to epinephrine (Moore, Millman et al. 2007), increases cAMP levels in NHBE cells within 10 minutes (Korn, Jerre et al. 2001). Thus, epinephrine is expected to increase cAMP levels transiently and initiate a downstream signaling cascade that eventually affects MUC5AC expression. It is possible that chronically incubating NHBE cells with epinephrine causes downregulation and loss of $\beta_2$-ARs, but enough receptors remain to drive MUC5AC expression in response to IL-13. Additionally, phosphorylated $\beta_2$-ARs can affect MUC5AC expression by activation of other signaling cascades. $\beta_2$-ARs phosphorylated by PKA at serine 262, couple to Gi which activates ERK1/2 whereas phosphorylation of $\beta_2$-ARs by GRK at other receptor sites initiates a second wave of signaling through β-arrestin. Additional studies are required to evaluate the receptor levels after chronic exposure to epinephrine in NHBE cells. It is important to mention that it has been reported recently that internalized $\beta_2$-AR are capable in inducing a second wave of cAMP signaling through activation of $G_s$ pathway from the endosome (Irannejad, Tomshine et al. 2013).

Increased intracellular cAMP affects several downstream targets that can eventually increase MUC5AC expression as outlined in Figure 84. Forskolin+IBMX did not affect the level of ERK1/2, p38 or c-Jun phosphorylation

in response to IL-13 as compared to cells treated with IL-13 alone (Figure 52 and Figure 53).

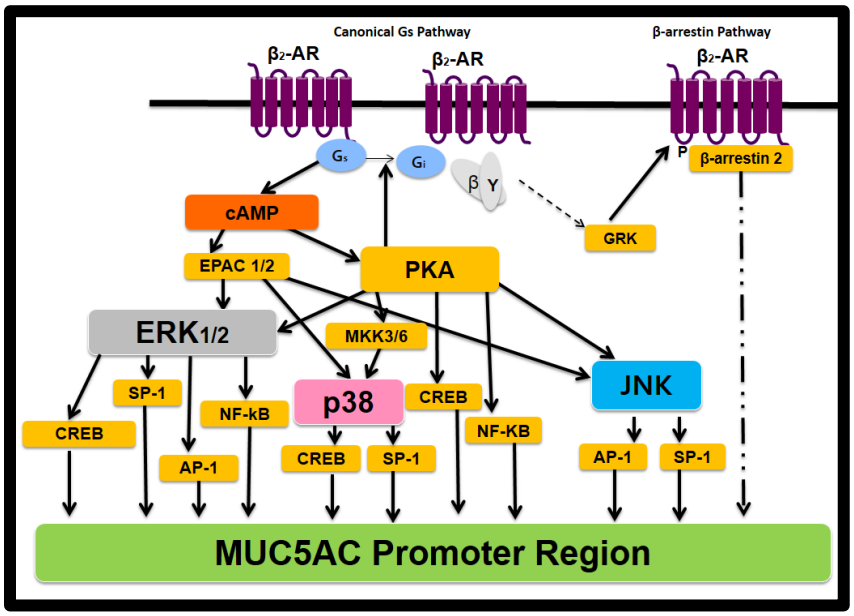



**Figure 84. Possible action of cAMP on the MUC5AC promoter:** Increased intracellular cAMP activates PKA and Epac signaling molecules. PKA and Epac phosphorylate several downstream signaling proteins that activate several transcription factors that are capable to enhance MUC5AC transcriptional activity. CREB, SP-1, AP-1 and NF-kB are examples of these transcription factors.

Only in the presence of epinephrine did forskolin+IBMX in combination with IL-13 increase CREB phosphorylation (Figure 54A and B). Other candidates that might mediate the effect of forskolin+IBMX in combination with IL-13 in the

absence of epinephrine and require further examination include HIF-1, SMAD-4 and NF-κB.

Additionally, the forskolin+IBMX combination increases STAT6 phosphorylation in response to IL-13 in the presence of epinephrine (Figure 54C). Since the MUC5AC promoter region lacks a STAT6 consensus motif, STAT6 may indirectly contribute to MUC5AC expression through the activation of other transcription factors such as HIF-1 and SMAD4 (Young, Williams et al. 2007; Thai, Loukoianov et al. 2008). It is noteworthy that even though forskolin+IBMX increased cAMP levels in the presence or absence of IL-13, mucin parameters *only* increased in the presence of IL-13. Thus, cAMP by itself is not enough to drive MUC5AC expression, and IL-13 activation of other signaling pathways is necessary. This interaction might be at the level of STAT6, thus an inhibitor of STAT6 could be used in future studies to further delineate the cross-talk between $\beta_2$-AR and IL-13 signaling.

### 5.5.2 Effect of a specific PDE inhibitor on mucin production in response to IL-13 in NHBE cells

One major limitation of forskolin+IBMX is that these agents result in a global and diffuse increase in intracellular cAMP levels throughout cells. Recently, it has become appreciated that compartmentalized factors are responsible for spatially and temporally controlling the generation and

degradation of cAMP (Arora, Sinha et al. 2013). This compartmentalization phenomenon is the underlying reason for the differences in cell responses to variety of ligands that work through the same second messenger. For example, differences in the effect of $\beta_2$-AR ligands and prostaglandin on heart contractility are due to differences in the compartmentalization of cAMP and consequently the phosphorylation of specific downstream substrates (Di Benedetto, Zoccarato et al. 2008). The spatially confined subcellular compartmentalization of cAMP signaling elements includes PKA, PDEs, phosphatases, specific substrates (Wong and Scott 2004) and ACs (Dessauer 2009) that are anchored to A kinase anchoring proteins (AKAPs). This compartmentalization creates different cAMP pools, each with its specific set of substrates and unique cell responses, as shown in the Figure 85. For all of the aforementioned reasons, we used a more specific PDE inhibitor.

cAMP compartmentalization in the subcortical region of human airway epithelial cells has been reported to be involved in PKA mediated control of cystic fibrosis transmembrane conductance regulator (CFTR) activity, and alteration in this compartmentalization has been reported in cystic fibrosis (CF) (Monterisi, Favia et al. 2012).

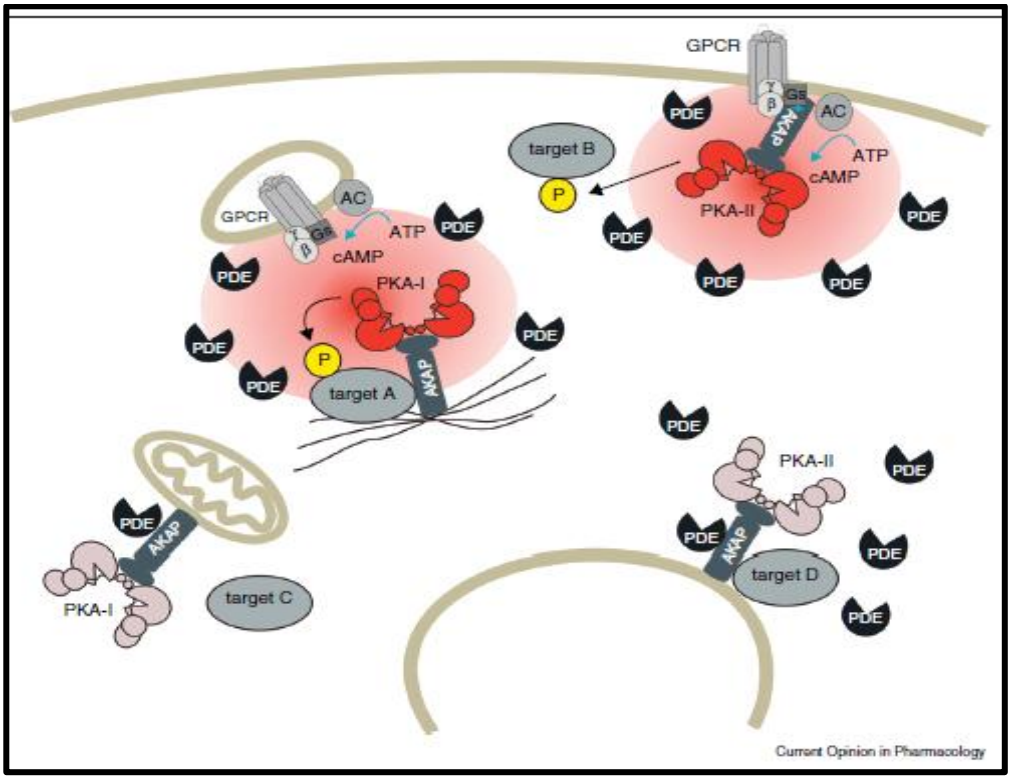



**Figure 85. Schematic illustration of compartmentalized cAMP signaling**: Two different cAMP pools (red) are generated by AC based on the localization of the enzyme (anchored to plasma membrane or attached to the internalized receptor), GPCR (on the cells surface or internalized) and PDEs where they degrade the cAMP to prevent its leak to other compartments. Each compartment activates different subsets of substrates. Moreover, the differences in the localization of different PKA (PKAI and PKAII) and their variability in cAMP sensitivity contribute to activation of different subset of substrates. Adapted from Zaccolo (2011).

In humans, there are 11 different families of PDEs (Omori and Kotera 2007). In the airways, each cell type expresses a predominant PDE isoenzyme. For example, ASM cells express PDE3, 4, and 5 while airway epithelial cells and inflammatory cells express mainly PDE4 (Barnes 1995). Most inhibitors that act on PDE4 interfere with the catalytic activity of the enzyme (Page and Spina 2012). The compartmentalization of PDEs is especially important to degrade cAMP in specific microdomains and prevent the spread of cAMP to other compartments which might affect other substrates. The importance of this is revealed by an elegant study by Jurevicius and Fischmeister, where they measured L-type calcium current ($I_{Ca}$) by whole-cell patch-clamp recording in cardiac myocytes. Exposing half of the cell surface to isoproterenol or forskolin resulted in a local and global effect on $I_{Ca}$ respectively. However, isoproterenol combined with IBMX resulted in a *global* effect, indicating the importance of PDE in compartmentalized $\beta_2$-AR signaling (Jurevicius and Fischmeister 1996). Beside the role of each PDE isoenzyme in compartmentalized signaling, there are different PDE4 isoforms (PDEA, B, C and D) and at least 18 splice variants (Kodimuthali, Jabaris et al. 2008). The main isoform that mediates anti-inflammatory effects is PDE4B while PDE4D contributes to the adverse effects, mainly emesis and nausea (Lipworth 2005). Roflumilast inhibits all PDE4 isoforms (Lipworth 2005). Thus, it is expected that each of these isoforms have its role in a specific compartmentalized signaling.

Treating NHBE cells with 100 nM roflumilast in the absence of epinephrine did not increase MUC5AC expression, intracellular mucin 5AC or mucin glycoproteins in response to IL-13 (Figure 55A, Figure 56A and Figure 57A).

In the presence of epinephrine, roflumilast did not affect MUC5AC expression in response to IL-13 (Figure 55B). However, mucin 5AC and intracellular mucin were reduced significantly in response to IL-13 (Figure 56B and Figure 57B). The levels of cAMP were not increased in roflumilast treated cells in response to IL-13 either in the presence or absence of epinephrine (Figure 59A and B). Human primary bronchial epithelial cells show similar activities of both PDE4 and PDE1 and lower activity of PDE3 and PDE5 isoforms (Dent, White et al. 1998). However, in another study, cultured human epithelial cells showed the presence of the PDE4 and 5 isoenzymes (Fuhrmann, Jahn et al. 1999). Therefore, it is possible that inhibiting PDE4 only is not enough to increase intracellular cAMP levels. In addition, the effect of roflumilast on intracellular mucin 5AC and mucin glycoproteins production induced by IL-13 in the presence of epinephrine could be cAMP independent.

Roflumilast reduced muc5ac expression and mucus producing cells, in a dose-dependent fashion, in mouse lungs in response to bleomycin (Cortijo, Iranzo et al. 2009). Thus, it is possible that we could observe more reduction of MUC5AC expression if we used higher concentrations of roflumilast.

The discrepancy in the effect of PDE4 inhibition on mucus is also observed in in murine models. Roflumilast did not affect mucus metaplasia in chronic murine model of asthma (Kumar, Herbert et al. 2003). In contrast, rolipram, another PDE4 inhibitor, decreased mucus metaplasia in an acute murine model of asthma (Kanehiro, Ikemura et al. 2001). *In vitro* studies with roflumilast have revealed its inhibitory effects toward human inflammatory cells such as eosinophils, neutrophils and macrophages (Hatzelmann and Schudt 2001). Moreover, it has been shown that the increase in cAMP concentration caused by PDE4 inhibition suppresses eosinophil activity (Dent, Giembycz et al. 1994). The use of roflumilast in inflammatory airway diseases, such as asthma and COPD, results in a significant improvement in the disease condition (Cowan 2005; van Schalkwyk, Strydom et al. 2005; Bateman, Izquierdo et al. 2006). Roflumilast attenuated sputum levels of eosinophils and neutrophils (Gauvreau, Boulet et al. 2011) and also reduced AHR (Louw, Williams et al. 2007; Gauvreau, Boulet et al. 2011) in mild asthmatic patients. Thus, it is possible that the usefulness of PDE4 inhibitors *in vivo* is mediated by the reduction in the inflammatory mediators released from parenchymal cells and inflammatory cells. This could explain the discrepancy between *in vivo* results and our *in vitro* results, where we focused on one cell type and studied the direct effect of roflumilast independent from its other anti-inflammatory effects on other cells.

Roflumilast did not reduce the barrier function as compared to cells grown in the presence or absence of epinephrine (Figure 59A and B).

Examining the signaling pathways (Figure 84) downstream of roflumilast revealed that the roflumilast and IL-13 combination did not affect ERK1/2 or cJun phosphorylation in the absence or presence of epinephrine (Figure 60A, B and Figure 61A, B). On the other hand, the roflumilast and IL-13 combination increased p38 phosphorylation in the absence of epinephrine as compared to control cells, yet this increase was not associated with any effect on MUC5AC transcription (Figure 60C). Other studies have shown that the anti-inflammatory effect of roflumilast in A549 cells is mediated by inhibition of ERK1/2 and p38 activation (Mata, Sarria et al. 2005) and, in a murine macrophage cell line, through inhibiting NF-κB, p38 and JNK (Kwak, Song et al. 2005). In our study, we were not able to determine how roflumilast inhibits mucin production in response to L-13. Therefore, testing the involvement of NF-κB is required and further studies using donors whose cells respond to IL-13 by increasing MAPKs phosphorylation are required.

Roflumilast did not increase STAT6 or CREB phosphorylation in response to IL-13 in the presence of epinephrine (Figure 62B-C), in contrast to the forskolin+IBMX combination. This provides an explanation for the differences in mucin parameters that we saw with the global increase in cAMP by

forskolin+IBMX combination versus roflumilast, though both are expected to increase intracellular cAMP levels in NHBE cells.

## 5.6 Role of β-arrestin signaling in mucin production in response to IL-13 in airway epithelial cells

### 5.6.1 Effect of carvedilol on mucin production in response to IL-13 in NHBE cells

In addition to its signaling action through G proteins, $\beta_2$ARs also signal through β-arrestin dependent pathways. We performed studies which were intended to provide evidence for or against a role of β-arrestin in the promotion of mucin production by $\beta_2$ARs. For this purpose, we employed a biased $\beta_2$AR agonist.

Biased agonism describes the ability of a ligand to preferentially activate a specific signaling pathway that is known to be activated by the receptor (Rajagopal, Rajagopal et al. 2010; DeWire and Violin 2011; Kenakin and Christopoulos 2013), as shown in Figure 86A below. This modifies the two state receptor theory, in which there are a number of unique active conformations (R*), each having its own preferential stimulation of G-protein or β-arrestin signaling (Walker, Penn et al. 2011), as outlined in Figure 86B.

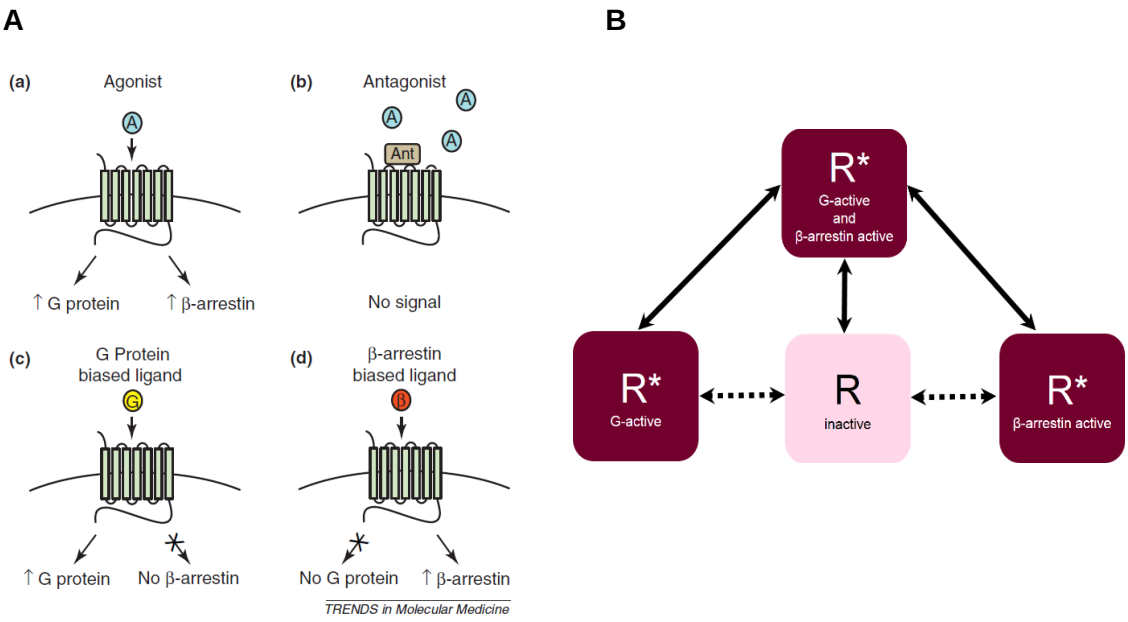



**Figure 86. Biased agonism concept: A) Agonist, antagonist and biased ligand concept**. a) Binding of non-biased agonist to the receptor activates all downstream signaling cascades, in this example, β-arrestin and G-protein signaling. b) Non-biased antagonist binds receptors and inhibits all pathways. c) G-protein biased ligand binds to the receptor and only activates G-protein signaling. d) β-arrestin biased ligand binds to the receptor and only activates β-arrestin and inhibits G-protein signaling. Adapted from Whalen, Rajagopal (2011) .

**B) Proposed conformations of $\beta_2$-AR based on biased agonism concept.** The receptor exists at equilibrium between the inactive conformation (R) and different active conformations (R*). R* could be unbiased and signal through G-protein or β-arrestin signaling (R* **G-active and β-arrestin active**), or biased toward G-protein (R* **G-active**) or β-arrestin signaling (R* **β-arrestin active**). Reproduced from Walker, Penn et al. (2011).

Different β-AR ligands have different signaling profiles. Alprenolol is a weak partial agonist on both the $G_s$/AC and β-arrestin-2 pathways (Wisler, DeWire et al. 2007). Nadolol is an inverse agonist with respect to Gs/AC signaling, while not affecting β-arrestin signaling (as assessed by ERK1/2 phosphorylation) (Wisler, DeWire et al. 2007). Carvedilol and propranolol, non-selective β-AR blockers, are inverse agonists of the $G_s$/AC pathway and agonists of the β-arrestin-2 pathway (Galandrin and Bouvier 2006; Wisler, DeWire et al. 2007; Stallaert, Dorn et al. 2012). Carvedilol induces $\beta_2$-AR phosphorylation, recruitment of β-arrestin-2 and internalization of the receptors to initiate ERK1/2 activation through β-arrestin-2 dependent mechanisms (Wisler, DeWire et al. 2007). Carvedilol neither increased mucin production in NHBE cells in response to IL-13 in the absence of epinephrine nor altered the mucin production induced by IL-13 in the presence of epinephrine (Figure 63A, B, Figure 64A, B and Figure 65A, B). Though carvedilol is a full inverse agonist at $G_s$/AC pathway, intracellular cAMP levels in carvedilol treated groups were not lower than control cells (Figure 68A, B). This could be due to the time point at which we measured cAMP levels in these cells as discussed previously.

We then studied the effect of carvedilol on different downstream signaling molecules, as outlined in Figure 87. Unexpectedly, carvedilol did not increase ERK1/2 phosphorylation either in the presence/absence of epinephrine or presence/absence of IL-13 (Figure 69A and 70A). Instead, carvedilol in

combination with IL-13 reduced ERK1/2 phosphorylation as compared to cells grown in the absence of epinephrine (Figure 9A). Carvedilol activates ERK1/2 through Gs-independent and β-arrestin-2 dependent pathway (Galandrin and Bouvier 2006; Wisler, DeWire et al. 2007; Stallaert, Dorn et al. 2012). Thus, variability in donors' responses and profiles could be the underlying reason for not observing any difference in ERK1/2 activation by IL-13 or IL-13+carvedilol treatment. Further studies using donors whose cells respond to IL-13 by increasing ERK1/2 phosphorylation are required. Phosphorylation of cJun, the downstream substrate of JNK, was not affected by carvedilol in the absence of epinephrine (Figure 69B). However, in the presence of epinephrine, carvedilol + IL-13 treatment increased cJun phosphorylation as compared to cells grown in the presence of epinephrine (Figure 70C). The phosphorylation of cJun could be the underlying reason for the lack of difference in mucin production between cells treated with epinephrine+IL-13 and epinephrine+IL-13+carvedilol. It is noteworthy, that all signaling molecules that were affected by different activators or inhibitors did not affect MUC5CA expression in the absence of IL-13.

The levels of p38 or CREB phosphorylation were not affected by carvedilol either in the presence or absence of epinephrine (Figure 69C, Figure 70C and Figure 71A, B), though it has been reported that β-arrestin-2 activates ERK1/2 and CREB through PKA independent pathways in CF (Manson, Corey et al. 2011). Thus, we could not establish a role for β-arrestin-2 in mediating the IL-13

response and further experiments on NHBE cells from different donors may be required, as well as reducing the expression of β-arrestin-2 using shRNA or knockdown similar approach

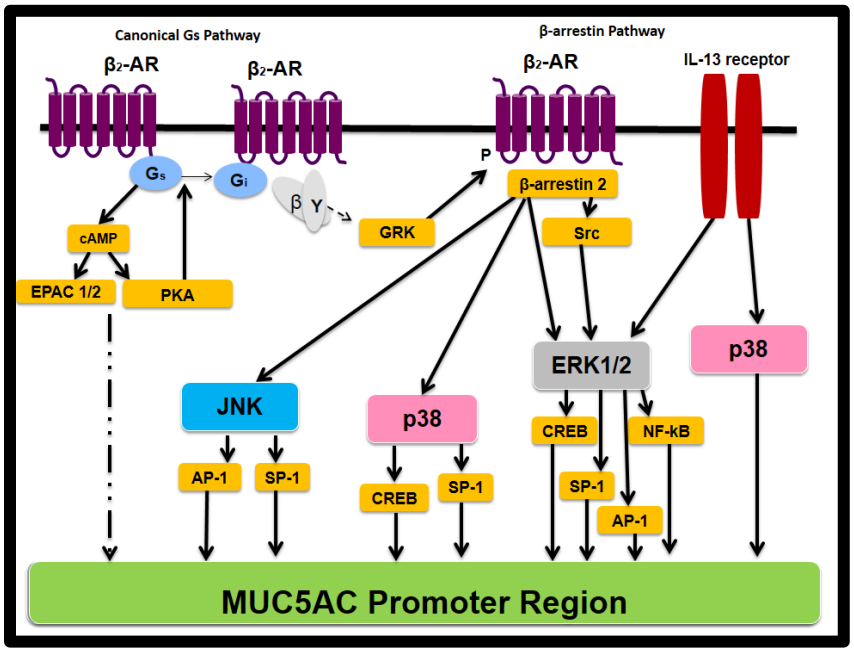



**Figure 87. Schematic illustration of possible action of β-arrestin-2 on MUC5AC promoter:** Activated β-arrestin-2 signaling cascade result in phosphorylation of MAPKs. Activated MAPKs in turn activate several transcription factors, such as CREB, SP-1, AP-1 and NF-kB,that enhance MUC5AC transcriptional activity.

### 5.6.2 Effect of β-arrestin-2 on mucin production in response to IL-13 from isolated mouse tracheal epithelial cells (mTECs).

The intracellular content of mucin 5ac in mTECs cultured from β-arrestin-2 KO mice was increased in response to IL-13 as compared to untreated cells from β-arrestin-2 KO, while it was significantly lower as compared to IL-13 treated cells from WT mice (Figure 72). On the other hand, intracellular mucin glycoprotein was significantly higher in IL-13 treated cells from WT mice as compared to IL-13 treated cells from β-arrestin-2 KO (Figure 73). Several studies suggest the involvement of β-arrestin-2 in a murine model of asthma. β-arrestin-2 KO mice fail to develop AHR and airway inflammation in response to allergen (Walker, Fong et al. 2003). Additionally, chimeric mice with β-arrestin-2 expression in hematopoietic cells but not in structural cells ($S^-/H^+$) did not show any decrease in mucus metaplasia, as reported by periodic acid-Schiff score, in response to IL-13 or ovalbumin challenge compared to wild type mice (Hollingsworth, Theriot et al. 2010). Also, exclusive β-arrestin-2 expression in structural cells but not in hematopoietic cells ($S^+/H^-$) revealed a reduction in mucus metaplasia in response to IL-13 or ovalbumin challenge compared to wild type mice (Hollingsworth, Theriot et al. 2010). These data illustrate the importance of β-arrestin-2 signaling in hematopoietic cells in mediating mucus metaplasia in response to IL-13 or allergen. One suggested scenario is that the inflammatory mediators released from hematopoietic cells, via β-arrestin-2

dependent mechanisms, act on airway epithelial cells to enhance mucus metaplasia. β-arrestin-2 in mouse airway epithelial cells do not appear to have a direct involvement in mediating mucus production in response to IL-13 in vivo, consistent with our NHBE cells findings but not with mTECs results. This discrepancy could be due to differences between *in vitro* and *in vivo* models. In mTECs studies, the isolated cells were cultured in the absence of any other inflammatory cell types and were treated for 14 days with IL-13 while it was for 7 days in *in vivo* study.

The inconsistency of the findings from NHBE cells and mTECs can be attributed to differences in species, culture media and growth supplements and most importantly, the starting time point of IL-13 treatment. IL-13 treatment started on ALI day 1 in NHBE cells and on ALI day 14 in mTECs after the cells were fully differentiated. Starting the IL-13 treatment after the cells are fully differentiated resembles the situation *in vivo*, in which the cells are already differentiated and the treatment does not affect the differentiation process. Finally, it may be that the KO mice have adapted to the absence of β-arrestin-2 and β-arrestin1 or other proteins were capable in mediating the increase in mucin 5ac levels in response to IL-13 but to a significantly lower levels as compared to IL-13 treated mTECs from WT mice.

Evaluating the barrier function revealed that treating mTECs from β-arrestin-2 KO mice had lower TEER values after 14 days of treatment as

compared to untreated cells (Figure 74A), with leaking of the media from the basal compartment of the insert to the apical compartment. Moreover, ZO-1 apical and cell-cell junction localization was also disturbed in these cells (Figure 74B). A smaller, but not significant, reduction in TEERs was observed in IL-13 treated mTECs from WT as compared to untreated cells. However, in cultured NHBE cells we did not observe this difference. This increase in epithelial permeability by IL-13 in cells from β-arrestin-2 KO means that β-arrestin-2 molecule might play a role in maintaining the integrity of the monolayer. Therefore, it is necessary to evaluate the expression and localization other molecules that are known to be involved in the formation of tight junction such as occluding and claudin (Shin, Fogg et al. 2006). During inflammation, cytokines such as IL-4 and IL-13, reduce the barrier function of epithelial cells (Forster 2008). Moreover, airway epithelial cells from asthmatic patients have a disrupted barrier function and lower TEER measurements as compared to cells from normal individuals (Xiao, Puddicombe et al. 2011). However, in cultured NHBE cells we did not observe any reduction in TEER of IL-13 treated cells as compared to control cells. This difference in the IL-13 effect on the integrity of the monolayer between cultured mTECs and NHBE cells could be explained by differences at the level of cell culture. mTECs were fully differentiated when IL-13 was administered. The response of fully differentiated cells mimic the behavior of *in vivo* bronchial epithelial cells. Whereas IL-13 was added at the

beginning of the differentiation phase in NHBE cells and hence IL-13 affects the differentiation of these cells and the way these cells respond to IL-13. Thus, evaluating the barrier function in fully differentiated NHBE cells in response to IL-13 is important for establishing a better *in vitro* model for studying asthma.

### 5.7 Effect of $\beta_2$-AR signaling on IL-13 modulation of mucociliary differentiation of NHBE cells.

Growing NHBE cells to ALI provokes polarization and differentiation to form pseudostratified epithelium with tight junctions. Airway epithelium is composed of a heterogeneous population of basal, ciliated and goblet cells. The goblet cell phenotype can be assessed by immunohistochemical detection of mucin 5AC or MUC5AC expression, the main mucin-producing gene (Atherton, Jones et al. 2003; Yoshisue, Puddicombe et al. 2004). The ciliated cell phenotype can be assessed by the expression of specific markers such as tektin, FOXJ1 and β-tubulin IV. Previous reports pointed toward an effect of IL-13 in upregulating goblet cell numbers at the expense of ciliated cells and causing the ciliated cells to transdifferentiate to mucin-producing cells. Chronic administration of IL-13 during mucociliary differentiation resulted in increased goblet cell numbers in cultured bronchial epithelium leading to mucous hypersecretion (Atherton, Jones et al. 2003; Yoshisue, Puddicombe et al. 2004). However, this increase in goblet cells was associated with a reduction in ciliated cell population

as represented by downregulation of tektin (Yoshisue, Puddicombe et al. 2004) and FOXJ1 (Yoshisue, Puddicombe et al. 2004; Turner, Roger et al. 2011) expression.

To examine if epinephrine is necessary for mediating the shift of ciliated cells to goblet cells, we correlated MUC5AC and FOXJ1 expression in response to IL-13 in the presence and absence of epinephrine. In the presence of epinephrine, IL-13 induced MUC5AC expression in NHBE cells, but the expression of FOXJ1 was not downregulated (Figure 75 A). Our results are inconsistent with other reports that demonstrated the downregulation of FOXJ1 expression in response to IL-13 (Yoshisue, Puddicombe et al. 2004; Turner, Roger et al. 2011). In the absence of epinephrine, IL-13 did not induce MUC5AC expression and FOXJ1 expression showed a non-significant increase (Figure 75A).

Next, we examined if the reduction in MUC5AC expression during blockade of $\beta_2$-ARs was associated with upregulation of FOXJ1. Specifically, we asked if $\beta_2$-AR signaling is required for the mucociliary shift that is induced by IL-13. In the presence of epinephrine, IL-13 or CGP 20712A did not affect FOXJ1 expression. Moreover, ICI-118,551 did not increase the FOXJ1 expression significantly (Figure 75B). Though previous reports showed that IL-13 decreased FOXJ1 on day 14 ALI (Turner, Roger et al. 2011), but donor variations might attribute to these differences.

Other cells that could transdifferentiate to mucus producing cells are basal cells and club cells. Several studies have shown that goblet cells originate directly from club cells in a murine model of asthma (Reader, Tepper et al. 2003; Evans, Williams et al. 2004; Hayashi, Ishii et al. 2004; Chen, Korfhagen et al. 2009; Pardo-Saganta, Law et al. 2013). Club cells are the main non-ciliated secretory cells throughout mouse airways but these cells are found only in the bronchioles in human lungs (Liu, Driskell et al. 2006). Therefore, the involvement of club cells in goblet cell formation is not expected and testing whether basal cells play a role is recommended.

The increase in mucus production could be due goblet cell hyperplasia, which is the increase in goblet cell numbers in areas where goblet cells are usually present (Rogers 2003; Mary Mann-Jong Chang 2008). Accordingly, the proliferation of existing goblet cells causes an increase in mucin production. Since the cell seeding density for all treatment groups were the same, counting the cell nuclei provides a clue as to whether $\beta_2$-AR signaling is involved in cell proliferation. In the presence of epinephrine, IL-13 significantly increased the cell number as compared to cells grown in the presence or absence of epinephrine (Figure 76A). Treating NHBE cells with IL-13 and CGP 20712A resulted in a significant increase in the cell number. ICI-118,551 reduced the cell number as compared to CGP 20712A + IL-13 treated cells (Figure 76B). Therefore, a more

specific way to evaluate cell proliferation may be required, such as assessing proliferating cell nuclear antigen (PCNA).

Forskolin+IBMX in combination with IL-13 increased the DAPI count significantly compared to all other groups, either in the absence or presence of epinephrine (Figure 77A and B). Interestingly, forskolin+IBMX alone without IL-13 did not induce an increase in cell number. IL-13 can initiate cell proliferation through releasing transforming growth factor-alpha (TGF-α) from airway epithelial cells, which then activates EGFR signaling (Booth, Adler et al. 2001; Booth, Sandifer et al. 2007). Moreover, it has been reported that epinephrine and cAMP elevating agents enhance cell division and proliferation and inhibit squamous cell differentiation (Pfeifer, Lechner et al. 1989). Thus, a global increase in cAMP may result in a synergistic increase in the IL-13 effect on cell proliferation and mucociliary differentiation toward more goblet cells. Nonetheless, we cannot assume that goblet cells are the proliferating cells and not the precursor cells that differentiate/transdifferentiate to mucus producing cells.

## 6. Summary and Conclusions:

Our study examined the involvement of $\beta_2$-ARs signaling in mucin production parameters (MUC5AC expression, intracellular mucin 5AC and mucin glycoproteins accumulation) that are induced by IL-13 in NHBE cells. The main findings in our study are:

1. IL-13 induces mucin production in NHBE cells only in the presence of epinephrine. Growing the cells in the absence or presence of epinephrine throughout ALI does not affect the levels of expressed mucociliary differentiation markers, the released chomkines or the barrier function. The only difference is their response to IL-13.

2. Epinephrine-induced $\beta_2$-AR signaling is required for IL-13 induced mucin production in NHBE cells. Whereas constitutive $\beta_2$-AR signaling is not sufficient to mediate the effect of IL-13 on mucin production in bronchial airway epithelial cells.

3. Mitogen activated protein kinases (MAPKs) are involved in mucin production in NHBE cells in response to IL-13. Only in the presence of epinephrine, IL-13 induces ERK1/2 phosphorylation and JNK activity, but does not alter p38 activation. ERK1/2 and JNK signaling molecules affect MUC5AC expression through activating cJun, a member of AP-1 transcription factor family.

4. Protein Kinase A (PKA), as a part of the canonical Gs/adenylyl cyclase pathway of $\beta_2$-AR signaling, is involved in IL-13 induced mucin production in NHBE cells.

5. cAMP elevating agents potentiates mucin production in response to IL-13 in NHBE cells, in the presence or absence of epinephrine. In the presence of epinephrine, forskolin+IBMX combination enhances MUC5AC expression through CREB and STAT6 signaling molecules. However, in the absence of epinephrine, forskolin+IBMX combination does not alter the levels of CREB phosphorylation.

6. Roflumilast, a PDE4 inhibitor, does not reduce MUC5AC expression, but it attenuates intracellular mucin accumulation in the presence of epinephrine. Whereas it has no effect on mucin parameters in response to IL-13 in the absence of epinephrine.

7. Activation of β-arrestin signaling, by carvedilol, does not alter the induced levels of mucin parameters in response to IL-13 in the presence of epinephrine but it induces cJun phosphorylation. In the absence of epinephrine, carvedilol neither induces mucin production in response to IL-13 nor affects MAPKs or CREB activation. Therefore, β-arrestin does not seem to be involved in IL-13 induced mucin in NHBE cells.

8. IL-13 treatment of *mouse* tracheal *epithelial cells* (mTECs) from β-arrestin-2 KO mice results in a lower level of mucin production as compared to IL-13 treated cells from WT mice. Therefore, β-arrestin-2 is involved in mediating the cell response to IL13 in mice airway epithelium.

9. Epinephrine, epinephrine-induced $\beta_2$-AR signaling or IL-13 are not involve in mucocilary shift from ciliated cells to goblet cells in IL-13 treated NHBE cells.

10. IL-13 increases cell proliferation only in the presence of epinephrine. However, $\beta_2$-AR signaling does not seem to be involved in this effect. Increasing intracellular cAMP by forskolin+IBMX combination induces cells proliferation in response to IL-13, either in the presence or absence of epinephrine.

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## 8. Appendix:

The requirement of epinephrine in inducing mucin production in response to IL-13 in NHBE cells is consistent with our recent data where genetic or pharmacological depletion of epinephrine in mice resulted in attenuation of inflammation and mucous metaplasia in an antigen-driven allergic model of asthma (Thanawala, Forkuo et al. 2013). To determine if this requirement for epinephrine is specific to the $T_H2$ driven model of asthma, we tested the involvement of epinephrine in innate immunity model, induced by toll-like receptor (TLR) ligands (Pam2CSK4, TLR2/6 agonist, and ODN2395, TLR9 agonist) (Duggan, You et al. 2011). TLRs recognize pathogen-associated molecular patterns (PAMPs) (Medzhitov 2001), conserved features of pathogens (Takeda, Kaisho et al. 2003), and induce innate immune responses. Activation of TLR upon its binding to PAMPs results in a initiating downstream signaling cascade through the Toll/IL-1 receptor (TIR) domain and results ultimately in inducing the expression of inflammatory mediators and antimicrobial proteins (Takeda and Akira 2004). Recently, a synergistic interaction between TLR2/6 and TLR9 has been reported to provide a protection against lung infections (Duggan, You et al. 2011; Tuvim, Gilbert et al. 2012).

For establishing an innate immunity model, we treated WT mice and epinephrine-ablated mice (these mice lack phenylethanolamine N-methyltransferase (PNMT) enzyme which is required for epinephrine synthesis)

with 4μM Pam2CSK4 and 1μM ODN2395 for 20 minutes by nebulization and then sacrificed 24 hours after the treatment. Total inflammatory cells and neutrophil numbers in bronchoalveolar lavage fluid were counted.

In our study, neither the total inflammatory cells nor neutrophils recruitment to the airways was affected by the presence or absence of epinephrine (Figure A1). In contrast to the previously published data, where epinephrine ablated mice had complete attenuated asthma phenotype, airway eosinophilic inflammation beside AHR and mucus metaplasia, in an antigen-driven allergic model of asthma (Thanawala, Forkuo et al. 2013). Therefore, we cannot extrapolate the requirement of epinephrine to other murine models of airway inflammation besides those driven by $T_H2$ lymphocytes.

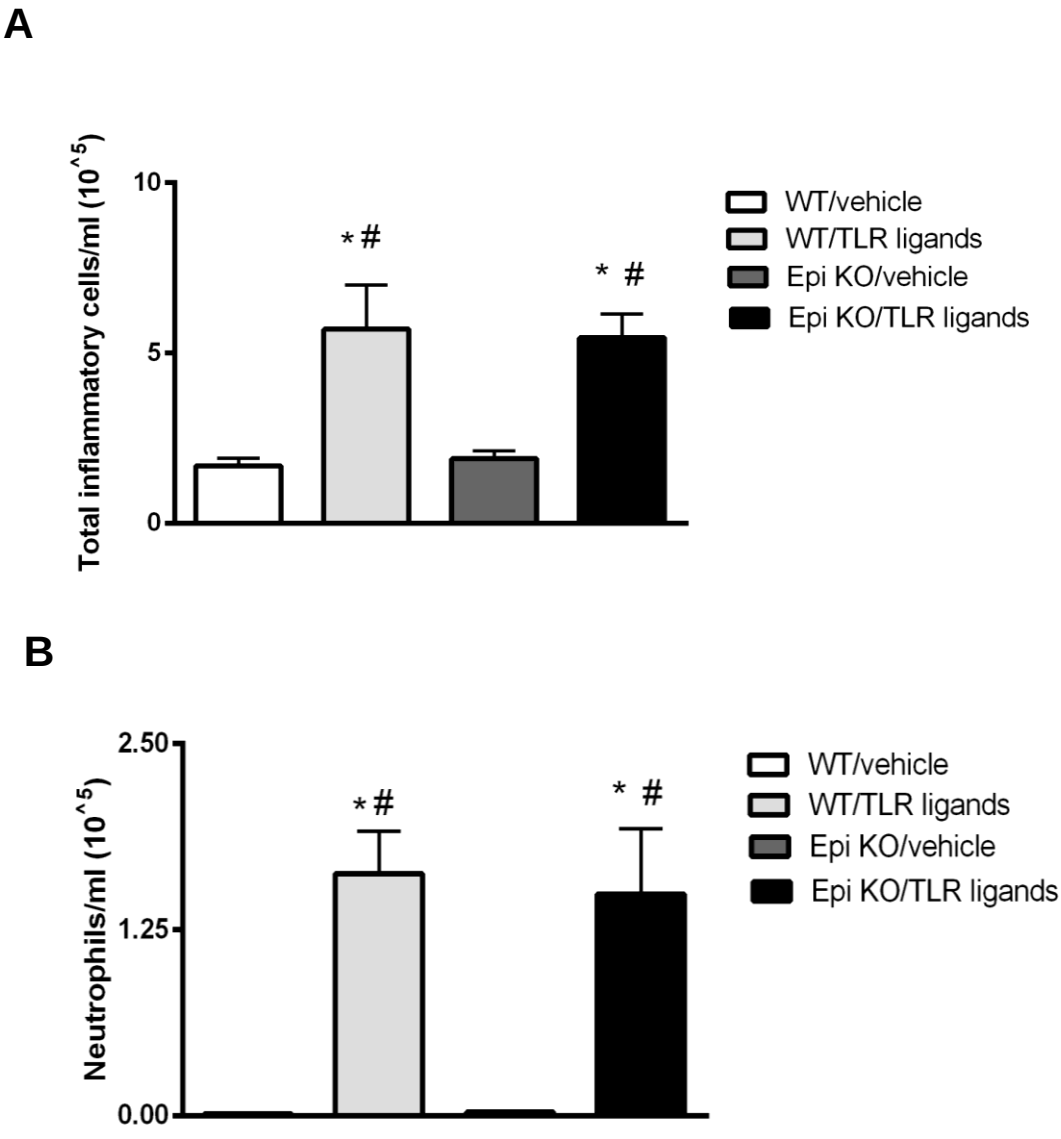


**Figure A1.** ***Role of epinephrine in a neutrophil model of airway inflammation***. Wild type (WT) and epinephrine knockout (Epi KO) mice were treated with PBS (vehicle) or toll-like receptor (TLR) ligands (4µM Pam2CSK4, TLR2/6 agonist, and 1µM ODN2395, TLR9 agonist) for 20 minutes by nebulization and then sacrificed 24 hours after the treatment. Bronchoalveolar lavage was performed and the recovered total cells **(A)** and differential neutrophil count **(B)** were counted. Data are presented as means ± SEM from N=4-5 mice. * and # indicate $p<0.05$ significance between WT/vehicle and Epi KO/vehicle respectively.