# THE ANTI-INFLAMMATORY EFFECTS OF LONG-ACTING BETA-AGONISTS ON BRONCHIAL EPITHELIUM

by

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## Abstract

Long-acting β2-agonists (LABAs) are a staple of Chronic Obstructive Pulmonary Disease (COPD) maintenance treatment frequently prescribed to patients for prolonged bronchodilation. Recent studies have suggested that they may also be anti-inflammatory, as demonstrated by lowered neutrophil infiltration and lower mucus secretion. The mechanism of action behind these effects are unknown and further study is needed to elucidate the pathways involved. The purpose of this project was to study the effect of the LABA olodaterol on an *in vitro* model of the airway epithelium in response to an inflammatory stimulus.

Primary airway epithelial cells derived from COPD and control non-COPD patient bronchial brushings were grown into air-liquid interface (ALI) cultures. Cells underwent priming with olodaterol in addition to respiratory syncytial virus (RSV) infection. Culture media was quantified for interleukin-8 (IL-8) secretion via ELISA. Whole cultures underwent immunohistochemistry for quantification of Muc5AC staining, an airway mucin protein. The 1HAEo- and the NCI-H292 cell lines were used to model the bronchial epithelium. Olodaterol was used to pre-treat both cell lines, after which either RSV or lipopolysaccharide (LPS) was added as an inflammatory stimulus and the IL-8 content was measured. Protein lysates were characterized using western blotting for the $\beta_2$-adrenergic receptor (β2AR), the binding receptor for LABAs. siRNA was used to silence the β2AR. RT-qPCR was used to quantify the knockdown efficiency.

ALI cultures showed that COPD subjects secreted higher baseline IL-8 levels than non-COPD controls, indicating that COPD subjects have an altered inflammatory phenotype. When both COPD and non-COPD ALIs were treated with olodaterol and RSV, olodaterol attenuated RSV-induced secretion of IL-8 and Muc5AC in both subject groups. In both 1HAEo- and NCI-

H292 cells, RSV and LPS were able to stimulate high IL-8 secretion that was inhibited by treatment with olodaterol. siRNA gene silencing of β2AR in NCI-H292 cells negated olodaterol-mediated attenuation of LPS and RSV-induced IL-8 secretion, suggesting that the anti-inflammatory effect of olodaterol proceeds through the canonical binding receptor. LABAs such as olodaterol have clear anti-inflammatory effects *in vitro* and may hold clinical relevance. Further studies are needed to determine if these effects have a significant impact on patient therapy.

## Lay Summary

Chronic obstructive pulmonary disease is a debilitating illness that affects millions worldwide and is driven by a strong inflammatory component. Recent studies have suggested that a common subset of bronchodilators used to treat this disease, long-acting β2-agonists (LABAs), may have anti-inflammatory effects in addition to their bronchodilation effects, but this has not been well-studied. This project examines the effect of the LABA olodaterol on cell culture models in inhibiting interleukin-8 secretion, a marker of inflammation, when the cells are treated with an inflammatory stimuli. It is important to study how LABAs may affect inflammatory responses in order to improve current understanding of pharmacotherapy for management of airway inflammation. Furthermore, LABAs as a potential anti-inflammatory agent in addition to bronchodilation may have a significant impact on patient therapies.

## Preface

This thesis was funded by a CIHR grant (20R25167) held by Dr. Don Sin and the Michael Smith Foundation for Health Research (MSFHR, NJZY 20R7746), AllerGen-Networks of Excellence (NMJK 20R12545), and the British Columbia Lung Association (NRLQ 20R00475) awards held by Dr. Del Dorscheid. Boehringer Ingelheim also provided funding for this project. Identification and design of the research program was accomplished by Dr. Del Dorscheid, Dr. Don Sin and myself.

COPD and control subject recruitment and bronchial brushing collection were performed by the Pacific Lung Health Centre patient coordinators and Dr. Janice Leung, respectively. The experiments and data in Chapter 3 (air-liquid interface cell culture, histology, enzyme-linked immunosorbent assays) were performed and generated by Dr. Gurpreet K. Singhera and Roxana Yan. I performed statistical analysis on the data. Dr. Gurpreet K. Singhera, Roxana Yan, Dr. Del Dorscheid and I interpreted the data.

Olodaterol hydrochloride was provided by Boehringer Ingelheim.

A manuscript containing the research and data documented in Chapters 3 and 5 is currently being revised for submission. The first draft of the manuscript containing only the data from Chapter 3 was written by Roxana Yan and Dr. Gurpreet K. Singhera. All subsequent re-writes and revisions were completed by myself. Dr. Michael P. Pieper reviewed the manuscript and subsequent data on behalf of Boehringer Ingelheim prior to submission for publication.

Ethics approval for the research conducted in Chapter 3 was granted by the University of British Columbia Ethics Board in collaboration with Providence Healthcare, as specified in the certificates: REB# H11-02713 and REB # H15-01778.

All experimental procedures (cell culture, western blotting, enzyme-linked immunosorbent assays, siRNA gene silencing, RT-qPCR) in Chapters 4 and 5 were performed by myself. Analysis of the research data from these chapters was performed by myself. Dr. Del Dorscheid and Dr. Don Sin assisted me in the interpretation of the data.

All components of this thesis was written by myself.

# Table of Contents

## List of Tables

## List of Figures

## List of Abbreviations

(+): GAPDH positive control siRNA

AAT: alpha-1-antitrypsin

ALI: Air-liquid interface

ATCC: American Type Tissue Culture Collection

β2AR: β2-adrenergic receptor

BCA: Bicichoninic acid

cAMP: Cyclic adenosine monophosphate

CD14: Cluster of differentiation 14

COPD: Chronic Obstructive Pulmonary Disease

Ctrl: Control

DAMPS: Damage-associated molecular pattern

DMEM: Dulbecco's Minimum Eagle Medium

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

$FEV_1$: Forced expiratory volume in 1 second

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GPCR: G-protein coupled receptor

GR: glucocorticoid receptor

GREs: glucocorticoid response elements

HRP: horseradish peroxidase

HPRT: Hypoxanthine-guanine phosphoribosyltransferase

ICS: Inhaled corticosteroids

IL-8: interleukin-8

LABA: long-acting β2 agonist

LDH: lactate dehydrogenase

Lipo: Lipofectamine RNAiMAx

LPS: Lipopolysaccharide

mCD14: membrane bound cluster of differentiation 14

mRNA: messenger ribonucleic acid

Muc5AC: Mucin 5AC

NF-κB: Nuclear Factor kappa-light-chain

Olo: Olodaterol

O+L: Olodaterol + LPS treatment

O+R: Olodaterol + RSV treatment

PBS: Phosphate-buffered saline

Pool: Pool of β2AR-targeting siRNA

RPMI: Roswell Park Memorial Institute Medium

RSV: Respiratory syncytial virus

RT-qPCR: Reverse transcription quantitative polymerase chain reaction

SABA: Short-acting β2 agonist

sCD14: soluble cluster of differentiation 14

Scr: Scrambled non-targeting siRNA

SDS: sodium dodecyl sulfate

siRNA: Short interfering ribonucleic acid

TBS-T: Tris-buffered saline with 0.01% tween

TLR: Toll-like receptor

TLR4: toll-like receptor 4

## Acknowledgements

First and foremost, I would like to thank my supervisors Dr. Don Sin and Dr. Del Dorscheid for the tremendous opportunity to work on this project. Dr. Sin has provided me with invaluable support and guidance during my time as a student in his lab. His insightful feedback has encouraged me to strive for higher standards and his trust in me has allowed for an unparalleled amount of independence and control in driving this project. I am grateful to Dr. Dorscheid for his immense commitment to mentoring his students and could not have finished this project without his guidance. I would like to thank him for all of the thought-provoking feedback that has challenged me and shaped me into becoming a better scientist.

I would also like to thank Dr. Gurpreet K. Singhera for mentoring me in the lab and always being a source of support to turn to. I owe much of my wet lab expertise to her training.

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*To my parents*

# Chapter 1: Introduction

## 1.1 Background on COPD

Chronic Obstructive Pulmonary Disease (COPD) is a highly debilitating illness and one of the leading causes of death worldwide [1]. The disease is characterized by progressively worsening airflow limitation that cannot be cured with current therapies [2] and thus, treatment focuses on alleviating patient symptoms and improving quality of life. COPD is poised to become the third leading cause of death worldwide by 2020 [3, 4] and remains a steadily increasing healthcare burden. In fact, compared to other common causes of death, it is the only one with an increasing prevalence and mortality rate [3]. Current therapeutics remain insufficient to address the growing problem as they are unable to reverse the disease progression [1].

A variety of factors play a part in the development of COPD. The disease has been primarily described as smoking-derived, but can also arise from continuous exposure to pollution and biomass fumes as well as agricultural pesticides [1, 3]. Clinically, the key features include chronic bronchitis, small airways disease, and emphysema [1]. Chronic bronchitis is defined as chronic coughing and sputum production lasting more than three months in a year and recurring for at least two consecutive years [5]. The small airways (defined as less than 2 mm in diameter) are the sites of airflow obstruction in COPD [6] and disease results from obstruction due to increased mucus production and inflammatory cell congregation, fibrosis, and narrowing of the airway lumen [7]. Finally, emphysema is the result of parenchymal lung destruction in the alveoli due to inflammatory cell infiltration [1, 8] leading to a decrease in the forced expiratory volume in one second ($FEV_1$) [6]. There is a greater degree of airway inflammation in COPD patients which contributes greatly to the development and worsening of these symptoms [9].

## 1.2 COPD Pathology

Defining characteristics of COPD pathology is increasing airway obstruction and a runaway inflammatory effect in the lungs [10]. In the airways greater than 2 mm in diameter, there is an increase in the infiltration of immune cells such as macrophages and T lymphocytes [10, 11]. Neutrophils are present in the lumen or in cases of severe disease [12]. The central airways also remain the primary site of mucus hypersecretion as a result of the increase in goblet cell numbers, leading to production of excessive amounts of mucus [10, 11].

The small airways account for about 98.8% of the total lung volume [13] and exhibit clear damage from cigarette smoke exposure in the form of an inflated inflammatory response [14]. Infiltration of white blood cells such as macrophages into the airway walls are prominent [14] and the airways tend to thicken and develop epithelial cell hyperplasia [15]. Increased smooth muscle is directly correlated with worsening airway flow and decreased $FEV_1$ [14] as the airway wall increases in thickness [16]. This increase is precipitated by a host of changes in the epithelium including the increased density of immune cell infiltration and development of fibrosis [16]. Small airways disease, observed in the disappearance of terminal bronchioles, appears to begin in the early stages of COPD and precede the development of more significant markers of the disease such as emphysema and airflow obstruction [13, 17]. The resulting emphysema also leads to loss of alveolar attachments, which results in hyper-inflation of the lungs as gas remains trapped within [17] and lead to collapse of the distal airways during expiration [13]. Furthermore, mucus hypersecretion arising from goblet cell hyperplasia in the small airways physically block airflow through mucus plugs and can retain trapped pathogens, promoting increased levels of inflammation [16]. All these symptoms characterize the physiological changes in the peripheral airways due to COPD.

The lung parenchyma is typically where emphysema develops through the enlargement of the airspaces and tissue destruction [11]. Destruction can follow one of three types: centrilobular emphysema, panlobular emphysema and paraseptal emphysema. In centrilobular emphysema, tissue destruction is mostly relegated to the respiratory bronchioles and the central regions of the affected lobes, with the surrounding regions mostly intact [11]. This pattern of emphysema is primarily associated with cigarette smoking and localized to the upper lobes of the lung [18]. In contrast, panlobular emphysema follows a more uniform destruction of the lung parenchyma [11], generally localized in the lower lobes, and is strongly tied to α1-antitrypsin deficiency [18]. Paraseptal emphysema is more irregular than either centrilobular or panlobular with the destruction relegated to the outer portions of the lung lobes [18]. Emphysematous destruction significantly impacts gas exchange and worsens with disease progression.

### 1.3 The Airway Epithelium

The airway epithelium functions as a barrier against inhaled pathogens and irritants and is the primary site of pathological changes in COPD as well as a key initiator of inflammatory responses [19]. Of the bronchial epithelial cells that line the airway lumen, they are subdivided into three categories: basal cells, ciliated cell and secretory cells [20]. Basal cells are involved in cell adhesion and play an important role in differentiation and regeneration after injury as they have stem cell-like properties, and are also able to produce signaling molecules such as cytokines [20, 21]. The ciliated cells play an important role in the mucociliary clearance system as they are able to clear away the mucus (comprised largely of the Muc5AC mucin in the human airway) secreted by goblet cells [20, 21]. Secretory cells in the airways refer to non-ciliated cells known as club cells that are responsible for maintaining the integrity of the epithelium through secreting surfactants and antiproteases [20]. They may also act as progenitors for ciliated cells and goblet

cells. Of interest within the small airways, terminal bronchioles are typically covered in ciliated columnar epithelial cells while the more distal respiratory bronchioles feature both columnar and cuboidal epithelial cells [16]. The alveolar attachments are, conversely, lined with flattened epithelial cells [16]. Finally, the extracellular matrix forms a basement membrane below the epithelium as an anchor for the cells, maintaining cellular polarity, and as an additional barrier separating the epithelium from the underlying compartments [21]. Existing pores in the basement membrane allow immune cells recruited by the epithelium to traverse through [21].

The integrity of the epithelial layer is maintained by apical junction complexes that guard against microbes and other environmental insults, while also tightly regulating the movement of immune cells [19, 20]. Notably, the epithelial surface is covered in a layer of periciliary fluid to maintain hydration while an additional mucosal layer on top traps potential pathogens and other inhaled substances [19]. Importantly, the airway epithelium is able to launch immune responses against airway insults through pattern recognition receptors that recognize microbial-exclusive molecules [19, 20]. These receptors include the Toll-like receptors, Nod-like receptor that are able to sense microbial derivatives as well as infection or damage to the airway in order to generate the necessary cytokines in response to recruit immune cells.

As a key player in innate immunity, the airway epithelium generates many antimicrobial peptides and inflammatory mediators to overcome infections [19]. However, repeat exposure to inflammatory stimuli, as in COPD, impair the ability of the epithelium to heal after injury and this can lead to remodeling [19]. In COPD airways, the remodeling of the airway leads to basal cell and goblet cell hyperplasia and a significant decrease in barrier integrity, with cigarette smoke in particular being implicated in breaking down tight junctions [19]. Furthermore, the COPD epithelium is noted to have decreased cilia length and beatings that impair mucus

clearance and leads to subsequent airway obstruction [19, 20]. In response to injury, the airway epithelium inadvertently triggers airway remodeling in the process of wound healing and re-epithelialization. This is observed in the changes of different epithelial cell type density, such as in goblet cell metaplasia [16]. Additionally, basal cell repair and differentiation processes are noted to be impaired due to cigarette smoke, which significantly alters its transcriptome [16]. Hence, dysregulation of the airway epithelium is a prominent factor in COPD disease pathogenesis.

### 1.4 The Role of Inflammation

Airway inflammation is a key driving feature behind COPD disease progression [22]. Inflicted injuries on the airway epithelium triggers repair mechanisms that include the migration of neighboring cells into the wound, whereby they proliferate and re-differentiate to regenerate the injured portion of the epithelium [23]. Continued exposure to cigarette smoke and other irritants lead to dysregulated airway repair mechanisms and subsequent remodeling [24]. This is seen in the form of increased fibrosis, goblet cell hyperplasia and increases in smooth muscle mass [23]. Exposure to cigarette smoke also causes necrotic cell death, activating the damage-associated molecular patterns (DAMPs) which include the TLR family of receptors, leading to NF-κB-mediated transcription of inflammatory cytokines [22]. Further evidence of dysregulated airway repair is that COPD airway epithelial cells show goblet cell hyperplasia and increased mucus production when cultured in a differentiated air-liquid interface model [25] and exhibit decreased cellular proliferation and wound closure rates compared to non-COPD patients [23].

All smokers (either current or ex), who are the predominant population of COPD patients, feature increased levels of inflammation in their lungs [1, 4] even though not all smokers will develop clinically relevant disease symptoms [26, 27]. Damage to the airway

epithelial cells due to inhaled irritants lead to the activation of innate immune responses which produce cytokines [28] . One such cytokine, interleukin-8 (IL-8), is a chemoattractant for neutrophils [1]. IL-8 is able to up-regulate expression of adhesion molecules on endothelial cell surfaces [29]. As shown in Figure 1, this promotes attachment of circulating neutrophils through selectin molecules [29-31]. Once bound, the neutrophils enter the “rolling” phase of Muc5AC

by which they undergo transendothelial migration to the site of inflammation [31]. Rolling neutrophils will encounter IL-8 molecules that bind to the CXCR1/2 receptors (G-protein coupled receptors) on their cell surface which then stimulates increased integrin expression [31]. Arrest of the neutrophil at the site of inflammation results from integrin interactions, as the LFA-1 integrin expressed on neutrophils bind with high affinity to ICAM-1 receptors [31]. Interactions between CD11b/CD18 on the neutrophil surface and ICAM receptors on the endothelium then mediate neutrophil crawling across the endothelium [21]. It is believed that existing pores in the endothelial and epithelial basement membranes then allows the migration of the neutrophils through the interstitial spaces and epithelium [21]. Once recruited, IL-8 also stimulates the degranulation of neutrophils [29, 30].

Patients with COPD present elevated levels of neutrophils in their sputum and bronchoalveolar lavage fluid [1] as neutrophil-mediated inflammation is one of the driving features behind COPD pathogenesis [22, 32]. Neutrophils are able to release elastases and proteases upon activation [33]. These molecules are released in response to invading pathogens such as bacteria and viruses but during chronic inflammation, lead to damage to host tissue and emphysema. Extensive neutrophil infiltration has also been linked to mucus hypersecretion in the airways, another characteristic of chronic airway inflammation, due to the stimulation upon goblet cells by neutrophil-released enzymes [9].

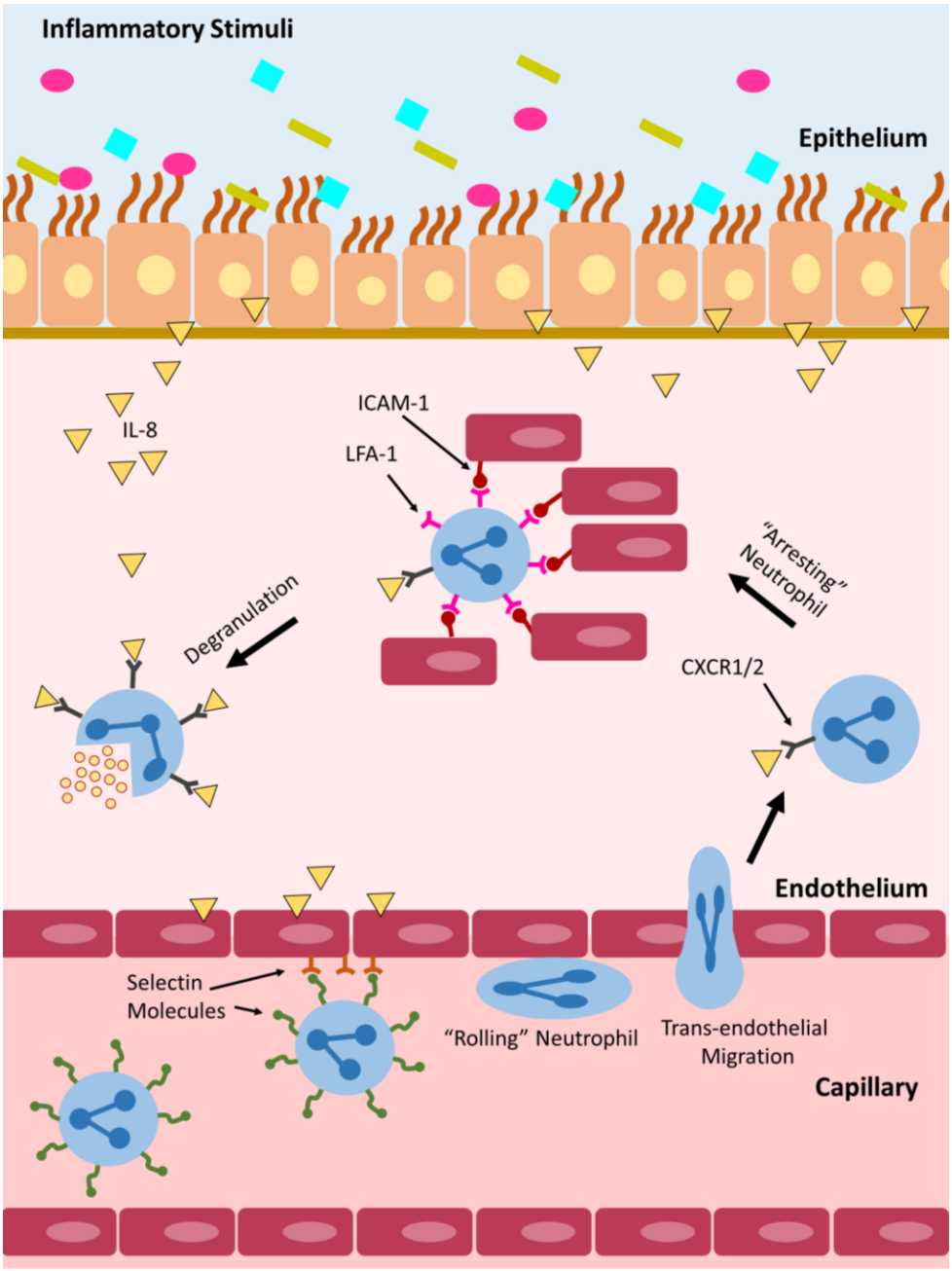



**Figure 1. Visual depiction of neutrophil chemotaxis and transendothelial migration via a chemokine gradient.** Inflammatory stimuli from the airway trigger the release of IL-8 from the airway epithelium which diffuses down to upregulate expression of selectin molecules on the endothelium surface. Circulating neutrophils in the capillaries are captured via the selectin molecules whereby they crawl along the endothelium before migrating through to reach the area of inflammation. Binding of IL-8 to CXCR1/2 receptors on the neutrophil surface triggers arrest of the neutrophil through upregulation of adherin molecules. Further binding with IL-8 causes neutrophil degranulation.

Another type of inflammation found in COPD to a lesser degree is eosinophil-driven inflammation. Eosinophils, like neutrophils, are also capable of cytotoxic effects against parasitic pathogens, but may inadvertently harm host tissue as well [34]. Several studies have also indicated an association between eosinophil levels and a greater risk of severe exacerbation [35]. Many other inflammatory mediators feature prominently in COPD airway inflammation. These include tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) [36]. TNF-α is able to activate NF-κB, increase expression of adhesion molecules, and stimulate the production of matrix metalloproteinases by macrophages while IL-1β functions similarly to TNF-α and induces the release of neutrophils from the bone marrow [37]. Additionally, reactive oxygen species are produced in elevated quantities in COPD patients, contributing to increased oxidative stress that leads to tissue damage [36].

The protease-antiprotease imbalance theory is widely accepted to be one of the contributing factors for development of emphysema [38-41]. This theory arose from observations that patients with a deficiency in α1-antitrypsin (AAT) were more susceptible to developing lung diseases [38, 40]. Inflammatory stimuli can lead to recruitment of immune cells such as neutrophils. Since neutrophils are capable of releasing enzymes that affect the proteolytic breakdown of extracellular matrix components, they require the action of antiproteases to neutralize their effect and maintain lung homeostasis [41, 42]. Serine proteases are the largest class of proteases and AAT is the most abundant serine antiprotease in the lungs. Hence, mutations in the α1-antitrypsin gene lead to a depletion of the antiprotease in the lungs and greatly increase the risk of COPD and $FEV_1$ decline [38], contributing to the disease phenotype [43]. Furthermore, new studies have shown evidence that AAT is also able to inhibit neutrophil

migration [44]. Thus, AAT and neutrophils play a key role in airway inflammation and progression of COPD.

Aside from neutrophils, IL-8 and AAT, other danger associated molecular patterns (DAMPs) are implicated in airway inflammation in COPD. These signals are released from injured or dying cells, such as in response to cigarette smoke and other environmental insults [45]. Binding of DAMPs to pattern recognition receptors (eg. Toll-like receptors, RIG-I-like receptors, NOD-like receptors, advanced glycation end-products receptors) lead to activation of downstream pathways that upregulate expression of inflammatory mediators including NF-κB and mitogen activated protein kinases [45]. This further stimulates production of proinflammatory cytokines such as IL-8, IL-6, type 1 interferons and tumor necrosis factor proteins [45]. Expression levels of these mediators and pattern recognition receptors are altered in COPD. For example, COPD patients exhibit increased expression of advanced glycation end-products receptors (RAGE) in their airway mucosal, bronchial epithelial and smooth muscle cells and increased levels of several endogenous ligands (eg. S100 proteins, LL-37, HMGB1) in the sputum and bronchoalveolar lavage fluid [22, 45]. COPD patients' sputum also show increased levels of IL-6 and TNF-α [46].

### 1.5 Other Inflammatory Cells in COPD

In addition to neutrophils, several other inflammatory cell types play key roles in driving the disease pathogenesis. One such cell type are macrophages, which are a type of leukocyte that is increased in number in the airways, bronchoalveolar lavage fluid and sputum of patients with COPD [32, 46]. Macrophages are able to secrete several different types of inflammatory mediators including IL-1β, TNF-α, IL-8, matrix metalloproteinases, and reactive oxygen species [32]. The large amount of matrix metalloproteinases that macrophages produce, much like

neutrophils, contribute to alveolar destruction and airway remodeling [32]. Macrophages can also recruit other inflammatory cells such as T lymphocytes and monocytes [32, 46].

T lymphocytes are part of the adaptive immune response and can be found in the airways of COPD patients, with a direct correlation between the number of cells and worsening airflow and alveolar destruction [46, 47]. They are able to facilitate the apoptosis of epithelial and endothelial cells, leading to airway damage [47] either directly, or through activation of macrophages [32]. They have also been suggested to promote neutrophilic inflammation [32].

Dendritic cells are a type of cell that bridges the innate and adaptive immune system through presenting antigens to T lymphocytes and are in higher concentration in the lungs with increasing COPD severity [46]. Some COPD patients may also see increased levels of eosinophils in their lungs, which is usually more characteristic of asthma-COPD overlaps [46]. Nonetheless, higher levels of eosinophils in COPD patients has been noted to be predictive for better corticosteroid and bronchodilator response [46].

Nevertheless, neutrophils remain one of the key inflammatory cells driving COPD pathogenesis and is the focus of many studies. All COPD patients are characterized by heightened neutrophil levels in both the blood and sputum [32] and this number is further increased in the event of an exacerbation [46]. A large amount of the proteinases in the lung that result in structural damage is facilitated by neutrophils' secretion of matrix metalloproteinases, elastases and myeloperoxidase, resulting in extensive lung destruction and mucus hypersecretion [32, 46]. Furthermore, cytokines secreted by other inflammatory cells such as IL-1β and IL-8 can further recruit neutrophils, in addition to what is secreted by the epithelium [32]. The number of activated neutrophils in both bronchoalveolar lavage fluid and sputum is directly reflected in a

patients' disease severity and hence, inflammation in COPD is characterized as largely neutrophilic [46].

### 1.6 Infection in COPD

In addition to inflammatory cell infiltration, infection theory also helps drive COPD pathogenesis and exacerbation. There is little doubt about the importance of infection, as the majority of COPD exacerbations are the result of respiratory infections [48]. During an infection, the immune system senses both viruses and bacteria, signaling the recruitment of immune cells to the lungs [48]. However, in smokers and patients with COPD, these innate signaling pathways are impaired. For example, expression of pathogen-sensing molecules such as toll-like receptors 2 and 4 are both noted to be decreased in severe COPD [48]. Furthermore, the ability of lung macrophages to phagocytose pathogens is also decreased while expression of NF-κB is increased [48]. Significant evidence suggests that repeated infections (both viral and bacterial) continually disrupt the immunogenic responses in the lung, leading to chronic infections [48]. This vicious cycle is purported to be a cause of worsening lung function.

The respiratory syncytial virus (RSV) is an enveloped negative-sense single-stranded RNA [49, 50]. Of the many viral infections that patients with COPD face, RSV is one of the most common and makes up approximately 56% of virus-related acute exacerbations [51]. Infection by RSV leads to an increase in airway inflammation, cytokine release and neutrophil recruitment to the lungs [50]. Patients infected with RSV experience an increase in sputum production, dyspnea, coughing, muscle aches, potential fever, increased risk of exacerbations and require longer hospitalizations [52]. The seasonal nature of the virus makes it particularly prevalent in the winter months, with a corresponding spike in exacerbations and hospitalizations during this time [52]. The recurrence of RSV-mediated acute exacerbations leads to detrimental

effects on the patient health outcome as more frequent exacerbations are associated with a faster decline in lung function [49].

Lipopolysaccharide is an important component of Gram-negative bacteria's outer membrane [53]. It is present in cigarette smoke and pollution and much like RSV, it can lead to acute exacerbations [54]. Recognition of LPS by the innate receptors is dependent upon binding by the LPS-binding protein, passing the LPS to the molecule CD14, which then presents the LPS to TLR4 [53]. Binding of LPS to TLR4 then leads to immunogenic responses such as the activation of NF-κB and the release of various cytokines [53]. LPS remains a common inflammatory stimulus in airway inflammation research as it can help mimic bacterial infection responses.

## 1.7 COPD Treatments

Many treatments are currently available for symptomatic management of COPD. Two of the most commonly prescribed pharmacologic treatments for COPD are bronchodilators and inhaled corticosteroids, both of which alleviate patient symptoms and reduce the frequency of exacerbations [55].

### 1.7.1 Bronchodilators

A mainstay of COPD treatment, bronchodilators are commonly prescribed to patients to treat their symptoms of dyspnea and have a direct effect on the airway smooth muscle [56, 57]. The primary categories of inhaled bronchodilators currently prescribed are the β2-agonists and the muscarinic antagonists [2, 57, 58]. They can be prescribed as a monotherapy, in combination therapy with each other, or inhaled corticosteroids [59]. β2-agonists function by targeting the β2-adrenergic receptors in the airway smooth muscle to facilitate muscle relaxation. Muscarinic antagonists inhibit the muscarinic receptors in the airway smooth muscle to prevent binding by

acetylcholine, which causes bronchoconstriction [60]. In addition, regular use of β2-agonists and muscarinic antagonists are linked to a reduced risk of COPD exacerbations [61]. This may be because of the potential of muscarinic antagonists to be anti-inflammatory as they can block the release of pro-inflammatory mediators that result from activation of the receptor by acetylcholine [62, 63]. β2-agonists have also been postulated to have anti-inflammatory effects but the current literature is limited [64].

#### 1.7.1.1 Treatment Guidelines

The Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) 2019 guideline has provided recommendations for selection of the appropriate pharmacological treatment based on each patients' symptoms [65]. According to the GOLD guidelines, patients are stratified into four categories (GOLD 1-4) according to their FEV1 as shown in Table 1, and assessed for symptoms severity.

**Table 1. COPD Patient Severity As Categorized by GOLD.**

| | | |
|---|---|---|
| GOLD 1 | Mild | FEV1 $\geq$ 80% predicted |
| GOLD 2 | Moderate | 50% $\leq$ FEV1 < 80% predicted |
| GOLD 3 | Severe | 30% $\leq$ FEV1 < 50% predicted |
| GOLD 4 | Very Severe | FEV1 < 30% predicted |

The initial patient treatment, as advised by GOLD, is divided into four groups (A-D). Group A, in which patients report 0-1 moderate exacerbations a year that do not lead to hospital admission and have mild symptoms are recommended a single bronchodilator. This can be either short-acting or long-acting, depending on the effectiveness at controlling breathlessness. Group B patients differ from Group A in that they exhibit more severe symptoms and are thus

recommended a long-acting bronchodilator as they are more effective than short-acting bronchodilators. Group C patients have two or more exacerbations in a year or at least one exacerbation a year that requires hospitalization but generally mild symptoms. Such patients are prescribed a long-acting muscarinic antagonist (LAMA) as studies have indicated that it is better at reducing exacerbation frequency than a long-acting beta 2 agonist (LABA). Finally, Group D patients exhibit the same exacerbation characteristics as Group C, coupled with more severe symptoms. Such patients are treated with LABA/LAMA if they are highly symptomatic or inhaled corticosteroids (ICS)/LABA if their blood eosinophil count is $\geq$ 300 cells/μL.

If the patients' symptoms are insufficiently controlled via the initial therapy, GOLD recommends that follow-up treatments be prescribed based on whether patients are experiencing insufficient dyspnea control or exacerbation control. In the case of single bronchodilator regimens, treatment may be escalated to dual bronchodilator therapy. Similarly, those on LABA/ICS therapy may be escalated to LABA/LAMA/ICS triple therapy. For patients with poorly controlled exacerbations, treatment may also be escalated from a single bronchodilator to dual bronchodilator or triple therapy. Alternatively, they may be also be prescribed roflumilast (for further inflammation control) or azithromycin (for bacterial infections). De-escalation from ICS combination therapies should be considered if the patient is not responding to ICS treatment or there are adverse side effects such as development of pneumonia.

#### 1.7.1.2 β2-Agonists

β2-adrenergic receptor agonists (β2-agonists) target the β2-adrenergic receptors in the airway epithelium in order to elicit downstream muscle relaxation [66]. The time of onset for different β2-agonists is dependent on the water solubility of the drug and diffusion into smooth muscle while the magnitude of the effect is due to the receptor binding affinity [67] and other

side-chain modifications that change the rate of migration and retention in the lipid bilayer [68]. The β1, 2, and 3 receptors share 60-70% sequence homology [69] and as such, even the most selective β2-agonists may have some cardiovascular side effects due to varying degrees of affinity for the β1-adrenergic receptors present on heart muscle through systemic absorption and may lead to issues such as tachycardia [67].

Selectivity of the β2-agonists for the β2-adrenergic receptor over the β1 or α receptors are conveyed by specific chemical modifications. On the phenylethylamine base that makes up all β2 agonists, a hydroxy group at the β carbon position and at carbon 3 or 4 of the benzene ring is required for activity at the β subtype receptor [70]. Additionally, larger substituents on the end of the side chain further confer selectivity. Increasing size from a small hydrogen to a methyl group to an even larger isopropyl group leads to greater selectivity for the β receptor and decreasing selectivity for the α receptor [70]. Further substitution with a tert-butyl group at the end makes the compound selective for β2 over β1 receptors [70]. Addition of an ethyl group on the α carbon of the phenylethylamine base can further enhance β2 selectivity [70]. While all β2-agonists achieve their effects through the same biological mechanisms, they are further subdivided into two categories, short-acting and long-acting, based on their duration of effectiveness.

#### 1.7.1.2.1 Short-Acting

Short-acting β2-agonists (SABAs), such as salbutamol, are typically used as rescue medication to combat acute exacerbations [59]. They have a fast onset and their effects generally last 4-8 hours [71]. However, this short duration makes them ineffective for long-term treatment use and they are mostly prescribed to patients with mild to moderate COPD [72].

#### 1.7.1.2.2 Long-Acting

Long-acting β2-agonists (LABAs) are more convenient to use than SABAs due to their longer period of efficacy and frequently prescribed to those with severe or very severe COPD for symptoms management and exacerbation prevention [56, 58]. Their effects last much longer than that of SABAs and can range anywhere from 12-24 hours of duration [73]. Despite the name, LABAs do not necessarily have a slower onset of action than SABAs. For example, the LABA formoterol has a rapid time of onset comparable to the SABA salbutamol; approximately 5 minutes [74].

#### 1.7.1.2.3 Olodaterol

Olodaterol is part of a small subset of ultra-LABAs known for having an extended duration of effect, with a rapid onset of action comparable to formoterol [75]. Produced by Boehringer Ingelhiem and first approved in 2013, olodaterol is a once-daily LABA with up to 24 hours of treatment duration [76]. Compared to older LABA formulations, olodaterol also has a much higher selectivity for the β2-adrenergic receptor (241-fold greater selectivity over β1-adrenergic receptors) [77].

#### 1.7.1.2.4 Mechanistic Pathway

Once inhaled, all β2-agonists bind to the β2-adrenergic receptors, a type of G-protein coupled transmembrane receptor present in the airway [66]. As shown in Figure 2, binding of the LABA causes a conformational change in the receptor, leading to activation of the coupled G-protein which stimulates adenylyl cyclase, drastically increasing production of cAMP in the cytosol [66]. This increase of cAMP then activates protein kinase A, an effector molecule that phosphorylates a number of downstream pathways while simultaneously exerting an effect on the cytosolic concentration of calcium ions to sequester ions back into sarcoplasmic reticulum

[66, 78]. One of the pathways that is phosphorylated by protein kinase A is the myosin light chain pathway [78]. Phosphorylation of myosin light chain kinase by protein kinase A inhibits it from efficiently phosphorylating myosin light chain, thereby reducing the cell's ability to contract [79]. Furthermore, calcium ion ATPase pumps and sodium/calcium exchangers work to shuttle calcium out of the cytoplasm, amplifying the bronchodilation effect [80]. It is unclear which inflammatory pathways may be affected by agonist activation of β2-adrenergic receptors.

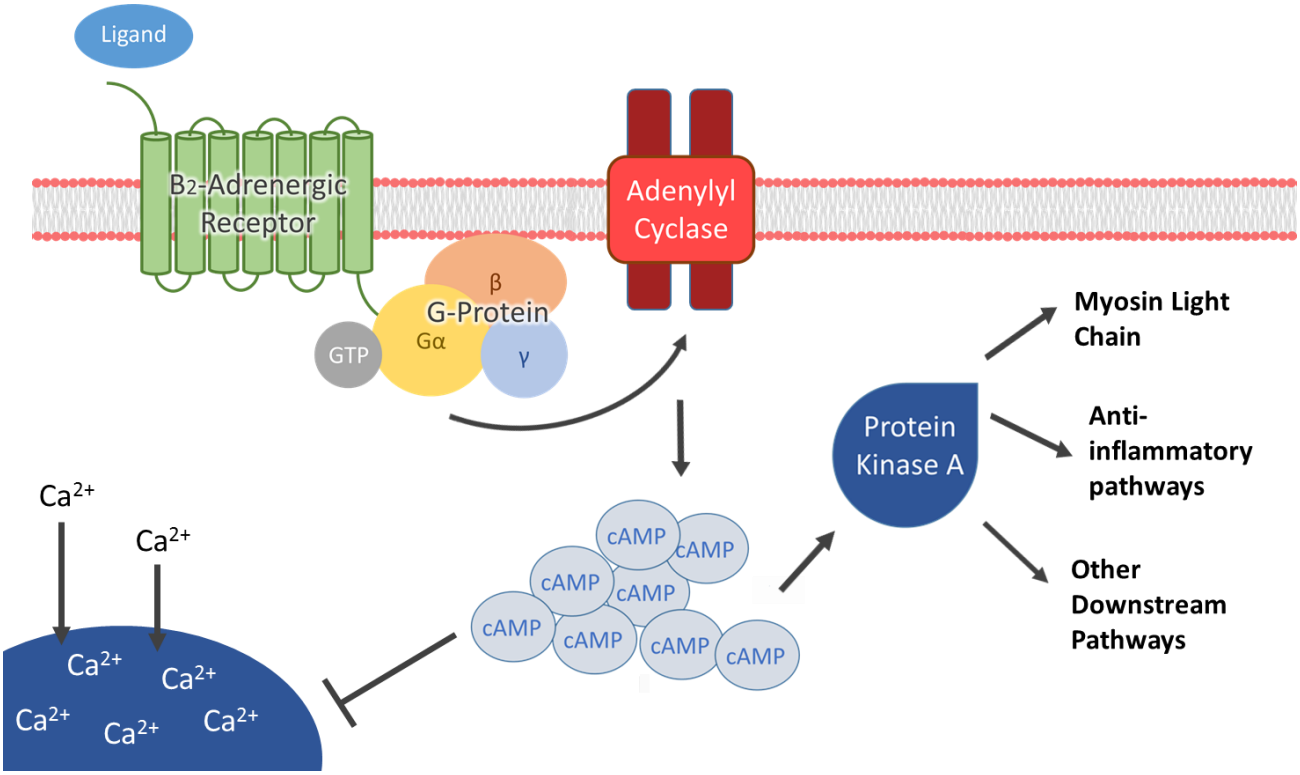



**Figure 2. Schematic representation of the activation of the β2-adrenergic receptor.**

Additionally, the different modifications on each agonist determine whether it is short-acting or long-acting, and also its method of interaction with the receptor binding site. For example, the SABA salbutamol is hydrophilic, and therefore diffuses quickly into the receptor binding site, subsequently facilitating a fast onset of action [81]. However, its hydrophilicity also

limits its duration of action as the drug re-equilibrates quickly and diffuses out of the lipid bilayer [81].

There is currently three models to explain LABAs' long duration of action. The microkinetic model states that depending on the lipophilicity of the molecule, the surrounding lipid bilayer can act as a reservoir for the drug from which the agonist can be continually released to interact with the activation site in the receptor, as is visualized with the LABA formoterol [74]. The exosite model suggests that the lipophilic side chain of a LABA such as salmeterol may bind to a secondary site (an exosite) that is either close to the receptor or located on the receptor but away from the activation site [74]. This would keep the molecule in close proximity to the activation site for continued receptor stimulation. Finally, the rebinding model suggests that a dissociated LABA molecule can be re-shuffled to bind with the same receptor or the β2ARs in the same vicinity and thereby delay its release from the lipid bilayer and maintain bronchodilation effect [74].

### 1.7.2 Inhaled Corticosteroids

Inhaled corticosteroids (ICS) are also a frequently prescribed therapeutic for COPD patients to treat airway inflammation and for control of exacerbations [58, 82]. This drug functions by binding to the glucocorticoid receptors (GRs) present in the cytosol, which induces translocation of the ligand-receptor complex into the nucleus where it binds to the glucocorticoid response elements (GREs) [83]. As a result, expression of certain genes can be up-regulated or down-regulated, with the overall effect being anti-inflammatory [83]. Long-term use of ICS is troublesome due to a host of unpleasant local and systemic side effects [84]. One of the main concerns is the increased incidences of pneumonia, which may result in complications and even death [84, 85]. In addition, other prevalent side effects include muscle weakness, steroid

myopathy, oropharyngeal candidiasis, increased risk of tuberculosis and even potential respiratory failure in cases of severe COPD [2, 84].

### 1.7.3 Combination Therapy

For patients whose symptoms are not successfully managed by bronchodilator monotherapy, there exists combination therapy of one or more bronchodilators for added efficacy. Dual bronchodilator combination therapy may have an advantage over monotherapy as they are able to facilitate bronchodilation via two different pathways [86]. This may allow for lower drug dosing as well [87], potentially decreasing the prevalence of side effects [88]. This is because β2-agonists function to stimulate adrenergic receptors, which are part of the sympathetic nerve system while muscarinic antagonists inhibit muscarinic receptors, which are part of the parasympathetic system [89]. Indeed, clinical trials have shown that many patients see better symptom control on dual bronchodilator therapy [62].

However, inflammation can stimulate the parasympathetic nervous system, leading to release of acetylcholine. This can result in bronchoconstriction, airway remodeling and increased mucus secretion as acetylcholine activates muscarinic receptors [90, 91]. As such, some studies have shown that use of muscarinic antagonists may have beneficial anti-inflammatory effects through inhibiting muscarinic receptors [90, 92]. Interestingly, β2-agonists have been observed to induce release of acetylcholine from parasympathetic nerves [91]. Thus, one problem with β2-agonist monotherapy in COPD patients is the concern that it could offset bronchodilation effects and potentially mediate increased inflammation [91]. Regardless, this is not conclusive as some *in vitro* models have not demonstrated this to be the case [91].

#### 1.7.3.1 Hypothesized Synergy between LABA and ICS

LABAs and ICS combination therapy is one of the leading treatment options for patients with poorly controlled COPD exacerbations [93]. It has been suggested that the increased symptom control is due to the activated GRs upregulating β2-adrenergic receptor gene expression while existing β2-adrenergic receptors are able to induce translocation of GRs into the nucleus [73]. Patients on ICS/LABA combination therapy report better lung function, less exacerbations or breathlessness compared to either monotherapy [58, 73].

The improvement of COPD patients' symptoms while on ICS/LABA combination treatment seem unlikely to be explained by bronchodilation alone and thus led to initial theories regarding synergy between LABAs and ICS. It was hypothesized that LABAs were able to induce an off-target effect to boost the efficacy of ICS treatment through uptake into the cytosol where it could enhance GR translocation into the nucleus [87, 94] as supported by some studies [95, 96]. However, another more recent study by Dr. Robert Newton's group has demonstrated no LABA-induced nuclear translocation of GRs through nuclear staining, indicating a clear contention in the literature regarding the mechanism of LABA/ICS synergy [97]. Regardless of the potential role of LABAs to boost ICS functionality, there is support for LABAs having an inherent anti-inflammatory effect in addition to bronchodilation. Studies have shown that while LABA/ICS together had synergistic effects, LABAs alone induced the expression of certain anti-inflammatory genes [98, 99] as well as decrease production of IL-8 and other cytokines [100, 101] but more information is needed to better understand these effects.

# Chapter 2: Experimental Aims

## 2.1 Hypothesis

The hypothesis of this project is that the long-acting β2-agonist olodaterol reduces airway inflammation in airway epithelial cells (AEC) resulting from exposure to an inflammatory stimulus such as respiratory syncytial virus or lipopolysaccharide via the β2-adrenergic receptor (β2AR).

## 2.2 Specific Aims

In order to test the hypothesis stated above, the project focused on accomplishing the following specific aims:

1) Measure the levels of IL-8 secretion from both primary AEC and a model cell line in response to olodaterol and an inflammatory stimulus to determine the presence of an anti-inflammatory effect (Chapter 3).
2) Establish and optimize a working model of the airway epithelium for investigation of the mechanistic pathway of the anti-inflammatory effect (Chapter 4).
3) Determine whether the effect observed is an off-target effect or is mediated through the β2AR via gene silencing (Chapter 5).

# Chapter 3: The Effect of Olodaterol on Primary Airway Epithelial Cells

## 3.1 Materials and Methods:

### 3.1.1 Reagents and Virus Preparation

Olodaterol hydrochloride was provided by Boehringer Ingelheim Pharma GmbH & Co. KG, Germany.

Human Long Strain Type A respiratory syncytial virus purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) was propagated in-house using the cell line Hep-2 (ATCC) using the methods described by Hegel, *et. al.* [102]. Hep-G cells were grown in Dulbecco's Minimum Eagle Medium (DMEM) supplemented with heat inactivated 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin, non-essential amino acids and sodium pyruvate while kept at 37$^{o}$C in a 5% $CO_2$ humidified incubator. After four days, the culture media containing the virus is collected and centrifuged at 10 000 X g for 10 minutes at 4$^{o}$C to obtain the cell-free supernatant, which was then concentrated by ultrafiltration through a 100 kDa cut-off membrane (Millipore, MA, USA). Viral infection was administered to the apical surface of the differentiated air-liquid interface cell culture (ALI) and performed at multiplicity of infection 1 ($MOI_1$) and allowed to incubate with the cells for 90 minutes. The culture plate was shaken every 20 minutes to disperse the virus. After infection, the ALIs were rinsed with PBS to remove any free virus and further exposed to air for 16 hours before sample collection.

### 3.1.2 Bronchial Brushings Acquisition

Ten subjects were divided into COPD and control groups based on evaluations of their spirometry results according to the Global Initiative for Chronic Obstructive Lung disease [103]. Subjects with normal lung function were deemed controls. Subject age, sex and lung function are listed in Table 2. All subjects underwent bronchoscopy whereby human bronchial epithelial cells

were collected using cytology brushes (Cat #4206, Primed Canada). Samples were collected from the small airways (<2 mm in diameter) in the upper left or right lobes. The sites of collection were free of any nodules or masses as noted on chest thoracic computed tomography scans.

**Table 2. BEC subject demographics information.**

| | **Non-COPD** | **COPD** | **P-Value** |
|---|---|---|---|
| Age (Years $\pm$ SD) | 66 $\pm$ 8.8 | 64.5 $\pm$ 9.9 | ns |
| Sex (Male:Female) | 1:4 | 3:2 | ns |
| $FEV_1/FVC$ (Mean % $\pm$ SD) | 76.5 $\pm$ 3.3 | 55.9 $\pm$ 11.4 | $P < 0.0047$ |

### 3.1.3 ALI Cultures

Primary cells from bronchial brushings were grown as ALIs on 24-well culture inserts (Cat #3470, Corning) using Pneumacult-ALI media from STEMCELL Technologies (Cat #0051). The cultures were maintained as ALIs for 3-4 weeks and olodaterol treatment and viral infection was administered on the apical side. Olodaterol pre-treatment of the cultures was performed for 8 hours, after which the drug-containing media was removed and the cultures were exposed to air for 16 hours. This process was repeated across three days for sufficient drug-priming and to prevent the apical surface from being in continuous contact with media, which would lead to de-differentiation.

Olodaterol was administered onto the ALIs at 10 µM. This concentration of the drug was determined to be the best reflection of a single dose of the drug as prescribed for patient use. The calculation for this concentration was based on the calculation performed by Dorscheid et al. in deriving an experimental concentration of dexamethasone reflective of one inhalation of the drug

[104]. Given the data sheet of olodaterol indicates 10 µg of olodaterol hydrochloride is administered per dose and assuming 10-30% deposition into the average perciliary fluid volume of a patient, the resulting concentration was approximately 10 µM.

### 3.1.4 Immunohistochemical (IHC) Analysis

ALI cultures were fixed in 10% formalin, before being embedded in paraffin blocks and subsequently cut into 4 µm sections. The sections were de-paraffinized in CitriSolv (Fisher Scientific, Ontario, Canada) before being rehydrated. The resulting slides then underwent staining with an anti-Muc5AC antibody (Cat #ab24071, Abcam, Cambridge, UK) using a Leica autostainer at 1:1000 dilution with a red substrate. A matched isotype control antibody was used as the negative control and hematoxylin was used for counterstaining the nuclei. The slides were analyzed via ImagePro (Media Cybernetics, MD, USA) whereby the positive staining area was normalized to the total epithelial surface.

### 3.1.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Condition media from the ALI culture plates were centrifuged at 6000 rpm for 5 mins to obtain the cell-free supernatant. IL-8 secretion in the samples were quantified via a commercially available IL-8 ELISA (Invitrogen) as per manufacturer's instructions.

### 3.1.6 Statistical Analysis

Graphical data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, CA, USA). Two-tailed unpaired Student *t*-tests were used to compare the means between two groups while one-way ANOVA with Bonferroni correction was used for multiple group comparisons. Error bars on the graphs indicate mean $\pm$ standard deviation.

## 3.2 Results

### 3.2.1 Baseline Secretion of IL-8 in ALIs

The supernatants from untreated COPD and control ALIs were collected and quantified for IL-8 in order to compare the production of this cytokine at baseline between the disease and control phenotype. As demonstrated in Figure 3, COPD-ALIs constitutively expressed four times higher IL-8 secretion than the control ALIs ($P < 0.01$). This appears to indicate that the COPD-ALIs have an altered, inflammatory phenotype compared to the non-COPD controls.

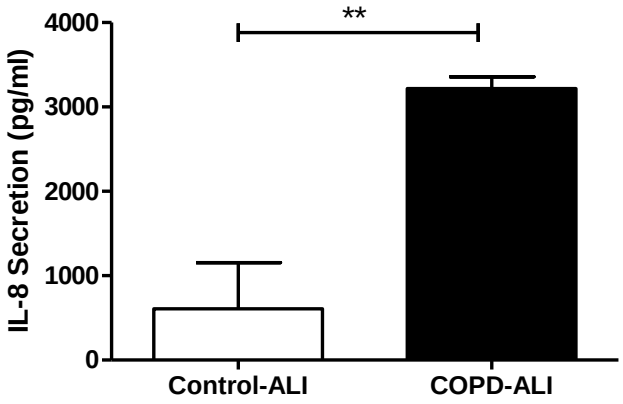



**Figure 3. Basal concentrations of IL-8 is higher in COPD-ALIs compared to control ALIs.**

At baseline, COPD-ALIs produced four times greater concentrations of IL-8 than control ALIs (n=3, $P < 0.01$).

### 3.2.2 IL-8 Secretion of ALIs in Response to Olodaterol

We next examined the secretion of IL-8 in a primary cell culture in response to olodaterol with and without RSV infection. As shown in Figure 4, when the cells were treated with olodaterol and RSV, olodaterol was able to significantly inhibit RSV-mediated IL-8 secretion in COPD and control ALIs compared to the RSV-only condition (approximately 30%), indicating that olodaterol had a notable anti-inflammatory effect.

**(A) Control** **(B) COPD**

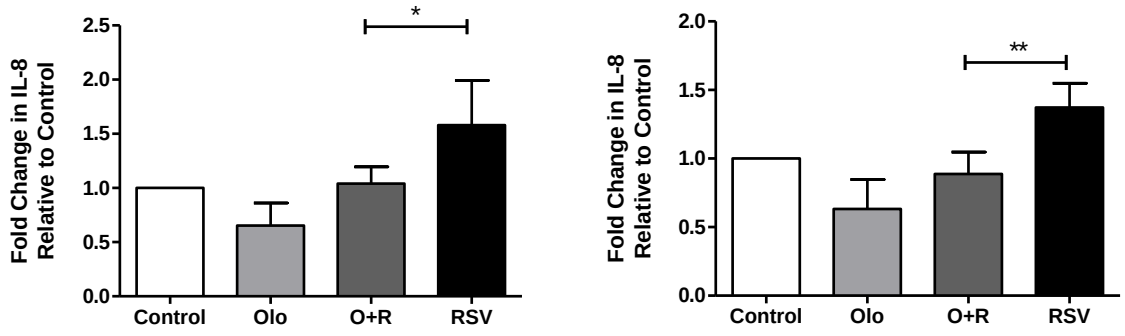



**Figure 4. Fold change in IL-8 secretion of ALIs treated with olodaterol and RSV.**

ALI cultures were treated with 10 μM olodaterol and/or RSV infected at a MOI of 1. (A) In ALIs grown from control bronchial brushings, olodaterol treatment (O+R) attenuated RSV-mediated IL-8 secretion ($P < 0.05$). (B) In ALIs grown from COPD patient bronchial brushings, O+R also significantly inhibited IL-8 compared to the RSV-only group ($P < 0.01$). Experiments were conducted at n=4.

### 3.2.3 Muc5AC Expression

Increased mucus secretion has been correlated with higher levels of inflammation in the airways. Therefore, the expression of Muc5AC, a prominent mucin, was measured in response to olodaterol and RSV treatment. For both COPD and control ALIs, there was a trend towards increasing Muc5AC expression with RSV infection (Figure 5). In COPD-ALIs, RSV-only treatment elicited significantly higher Muc5AC secretion than non-stimulated cultures (Figure 5, $P < 0.01$). In COPD-ALIs, olodaterol was able to significantly inhibit RSV-induced expression of Muc5AC ($P < 0.05$). The results further suggest that in COPD subjects, olodaterol has significant anti-inflammatory effects and that it extends to the mucosal pathway.

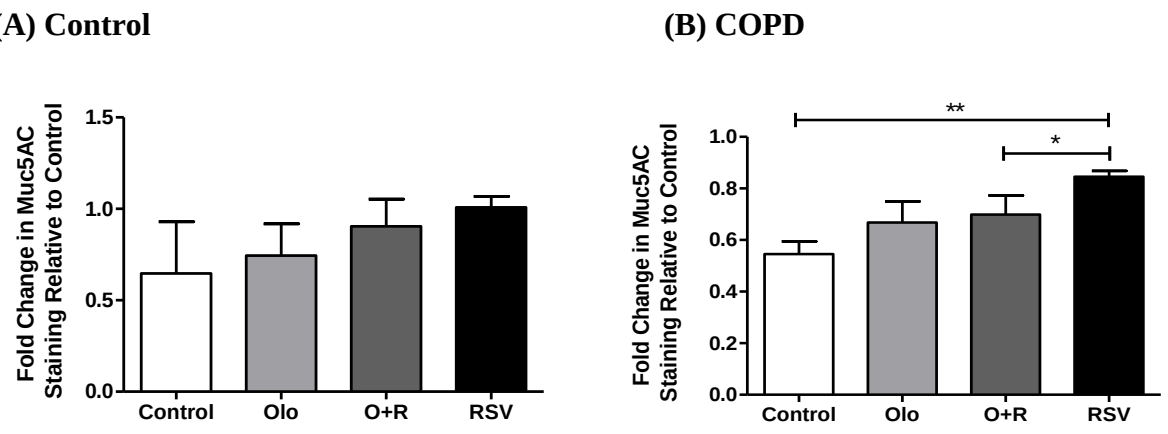



**Figure 5. Fold change of Muc5AC staining in control and COPD ALIs.**

Muc5AC staining was used to quantify mucus secretion in ALI samples. (A) Control ALIs produced significantly more Muc5AC when stimulated with RSV than in baseline controls ($P < 0.05$). n=4. (B) COPD ALIs produced significantly more Muc5AC with RSV-only stimulus compared to control ($P < 0.01$) and as compared to O+R treatment ($P < 0.05$). n=3.

## 3.3 Discussion

In COPD ALIs, when stimulated with an RSV challenge, olodaterol was able to attenuate IL-8 secretion and inhibit Muc5AC staining. At baseline, COPD patient-derived samples provided a clearly altered inflammatory phenotype when compared to the control samples as indicated by secreting large quantities of IL-8. With the application of a viral challenge, the LABA olodaterol mediated a significant reduction in inflammation resulting from the infection in both the COPD and control-derived patient cells in the form of lowered IL-8 and Muc5AC secretion. The effects are significant in the COPD-derived samples while the control-derived patient samples only trended towards a reduction. This is likely a result of the COPD samples being in a heightened inflammatory state, which increased the visible margin of change in response to the drug treatment and viral challenge. Both IL-8 and Muc5AC are key features of

the chronic inflammation present in COPD. Thus, the results indicate that olodaterol demonstrates clear anti-inflammatory properties on primary airway epithelial cells.

The airway epithelium is a significant site of disease pathogenesis and the small airways are noted to be especially prone damage and destruction, even prior to development of more obvious symptoms such as emphysema and airflow obstruction. As chronic inflammation is a key player in the destruction of alveolar attachments, olodaterol's ability to curb IL-8 mediated inflammation may be important in inhibiting the progression of small airways disease. In addition, as demonstrated in this chapter, olodaterol also appears to have salutary effects on the mucosal secretion pathway. As the small airways are also the sites of mucus hypersecretion and goblet cell hyperplasia, mucus plugs trigger significant airway blockages that may be alleviated by olodaterol. Patients may therefore experience reduced dyspnea through more than just a direct bronchodilation effect.

To our knowledge, this is the first study to show an altered inflammatory phenotype between baseline COPD and control-derived ALI cultures via IL-8 secretion. Previously, LABAs have demonstrated anti-inflammatory effects on animal models [100] but its effects on human airway epithelial cells have been less clear. The results presented in this chapter show that the anti-inflammatory effects of olodaterol can be extended to primary human epithelial cells, which is significant as they are the primary site of disease in COPD. There may be a role for LABAs to promote airway epithelial cell health in response to a viral infection. However, the mechanism by which olodaterol mediates these effects is still unknown and the following chapters will focus on studying the mechanism of action of this drug.

# Chapter 4: Establishing a Mechanistic Model of Airway Inflammation

## 4.1 Materials & Methods

### 4.1.1 Cell Line Tissue Culture

The 1HAEo- human lung epithelial cell line was a gift from Dr. Dieter Gruenert (University of California, San Francisco) and the NCI-H292 pulmonary carcinoma cell line was purchased from the American Type Tissue Culture Collection (ATCC, Virginia, USA). 1HAEo- cells were cultured in Dulbecco's Minimum Eagle Medium (DMEM) and NCI-H292 cells were cultured in Roswell Park Memorial Institute Medium (RPMI) purchased from Thermofisher (Massachusetts, USA). Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermofisher) and grown in tissue culture flasks in a 37$^{o}$C-humidified incubator with 5% $CO_2$. Culture medium was replaced three times a week and cells were passaged once they reached 90-100% confluence.

### 4.1.2 Olodaterol Drug Treatment

Olodaterol drug treatment was administered once cells reached 70-80% confluence. Media was removed and the cells washed with phosphate buffered saline (PBS) before the addition of fresh media containing olodaterol. Cells were incubated with the drug for 8 hours prior to any additional treatments. Olodaterol was maintained in the culture overnight for a full 24-hour exposure.

### 4.1.3 RSV Infection

Cells were infected with in-house grown RSV viral particles at $MOI_1$. Initial drug-containing media from each culture well was removed and saved and fresh media containing the virus was added at a reduced volume (100 µL/well of a 24-well plate) to facilitate viral contact. The plate was hand-shaken to disperse the virus and then allowed to incubate for 90 minutes in a

37ºC humidified incubator with 5% $CO_1$. Every 20 minutes, the plate was hand-shaken to re-distribute the virus. After 90 minutes, the media containing the virus was removed and the cells washed with PBS prior to the re-addition of the saved media. Cells were returned to the incubator and samples were collected the following morning (14.5 hours later).

### 4.1.4 Lipopolysaccharide (LPS) Stimulation

Following 8 hours of olodaterol pre-treatment, stock LPS (Sigma-Aldrich, Missouri, USA) was added directly into the condition media for a final concentration of 2 µg/mL. The plate was hand-shaken to ensure even mixing of the LPS. Cells were returned to the 37ºC incubator overnight and samples were collected the following morning (16 hours later).

### 4.1.5 Protein Lysate Collection

Cells were washed with PBS and then lysed in the tissue culture plate using lysing buffer from Cell Signaling (Massachusetts, USA). After addition of lysing buffer, plates were allowed to shake on ice for 30 minutes before the samples were centrifuged at 6000 rpm for 5 minutes to obtain the cell-free supernatant.

### 4.1.6 Protein Quantification

Bicichoninic acid (BCA) assay (Thermofisher) was used to quantify the protein concentration in protein lysates as per manufacturer's instructions.

### 4.1.7 Western Blotting

Cell lysates were loaded at 20 µg each in a 10% SDS gel under denaturing conditions. Samples were boiled with sample buffer containing β-mercaptoethanol prior to loading. Gels were transferred onto nitrocellulose membranes, which were blocked in 5% skim milk in tris-buffered saline with 0.01% tween (TBS-T) for 1 hour. All washes were conducted using TBS-T. Membranes were incubated with a rabbit polyclonal antibody against human β2AR (#PA5-

27083, Invitrogen, California, USA) as the primary antibody diluted 1:1000. Secondary goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated antibody (#12-348, Sigma Millipore, Massachusetts, USA) was used for detection at a dilution of 1:2500. Mouse anti-β-actin-HRP (#sc-47778, Santa Cruz, Texas, USA) antibody served as the loading control. Protein abundance was visualized using a chemiluminescent reagent (Femto ECL, Thermofisher).

#### 4.1.8 Enzyme-Linked Immunosorbent Assay (ELISA)

IL-8 secretion was quantified via ELISA as per the methods documented in Chapter 3.

#### 4.1.9 Lactate Dehydrogenase (LDH) Assay

The percent cytotoxicity of the cell-free supernatants was measured via an LDH assay (Promega, Wisconsin, USA) as per manufacturer's instructions. Samples were diluted 1:10 prior to assaying and control protein lysates diluted 1:20 was used as the maximum LDH release control.

#### 4.1.10 Statistical Analysis

Graphical data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, CA, USA). One-way ANOVA with Bonferroni correction was used for multiple group comparisons. Error bars on the graphs indicate mean $\pm$ standard deviation.

### 4.2 Results

#### 4.2.1 $B_2$-Adrenergic Receptor Protein Expression

B2AR is the known binding receptor of β2 agonists. In order to establish a working model with which to test the potential anti-inflammatory effects of olodaterol, initial experiments examined the expression of β2AR in the 1HAEo- and the NCI-H292 cell lines. As shown in

Figure 6, 1HAEo- and NCI-H292 cells constitutively express β2AR across all treatment conditions.

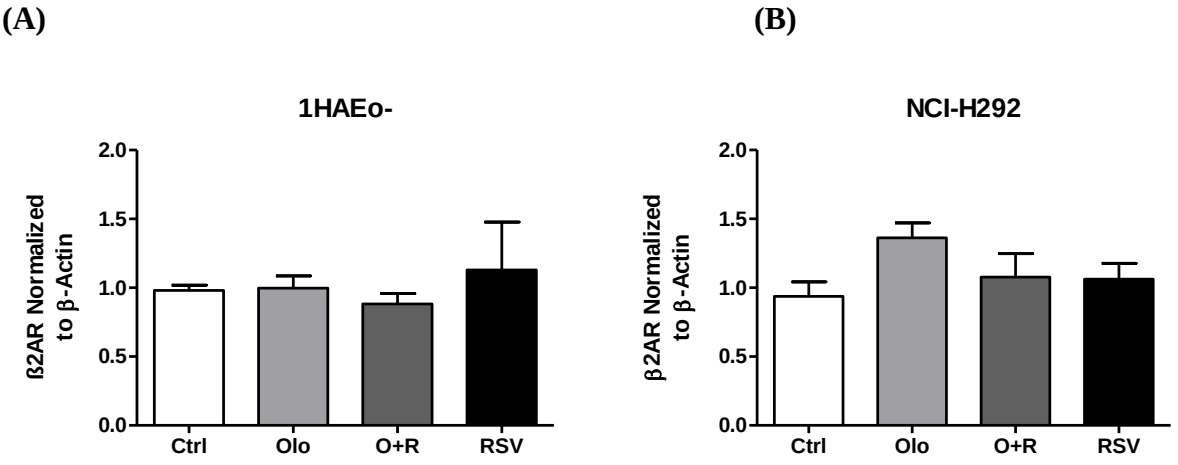



**Figure 6. Expression of β2AR in 1HAEo- and NCI-H292 cells with and without olodaterol and RSV treatment.**

Western blot analysis of 1HAEo- and NCI-H292 cells were quantified via densitometry. Cells were either untreated controls (Ctrl), treated with 10 μM olodaterol, olodaterol and RSV at a $MOI_1$ (O+R) or RSV alone. β-actin served as the loading control between conditions. No significant different was observed in either (A) 1HAEo- cells or (B) NCI-H292 cells between test conditions.

When the protein expression of β2AR in 1HAEo- and NCI-H292 cells were normalized to the loading control β-actin, there does not appear to be any significant change in β2AR expression across different treatment conditions (Figure 6).

### 4.2.2 The Effect of Olodaterol on RSV-mediated IL-8 Secretion in a Cell Line

To determine whether olodaterol was able to attenuate inflammation in the 1HAEo- and NCI-H292 cells lines, both cell lines were pre-treated with olodaterol and/or infected with RSV and quantified for IL-8 secretion. As shown in Figure 7, RSV stimulated a high IL-8 response in both 1HAEo- and NCI-H292 cells. Cells in either cell line treated with only olodaterol and no

inflammatory stimuli showed no discernable change in IL-8 secretion compared to the untreated controls, indicating the drug treatment itself did not stimulate an inflammatory response. In 1HAEo-, pre-treatment with olodaterol did not show a noticeable difference in IL-8 secretion compared to RSV-only treated cells in this experiment (Figure 7A). In NCI-H292 however, pre-treatment of the cells with 10 µM of olodaterol was successful at attenuating IL-8 secretion compared to the RSV-only treated cells ($P < 0.05$). It appears that olodaterol has an anti-inflammatory effect on NCI-H292 cells when the cells were infected with RSV.

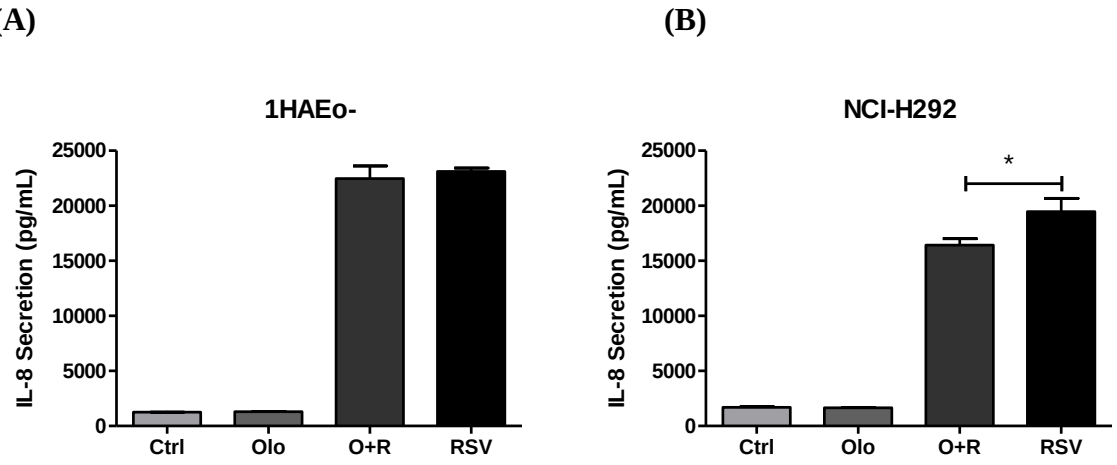



**Figure 7. IL-8 Secretion as a measure of inflammatory response.**

Olodaterol and RSV treatment (O+R) on NCI-H292 cells showed a significant decrease in IL-8 secretion compared to RSV-only treated NCI-H292 cells ($P < 0.05$). Olodaterol (Olo) treatment alone had no effect on IL-8 secretion compared to the control (Ctrl) in both 1HAEo- and NCI-H292 cells.

### 4.2.3 Optimization of Olodaterol Concentration

While Figure 7 showed attenuation of RSV-mediated IL-8 secretion by olodaterol treatment, the difference was only 20%. The initial starting concentration of 10 µM olodaterol

was chosen based on the concentration used in the primary ALI cultures, determined to be the most effective at inhibiting IL-8 secretion while minimizing cytotoxicity as well as being at a clinically relevant concentration. Use of 10 μM olodaterol approximated the concentration of the drug that would be dissolved in the average patient's periciliary fluid volume per single dose, as previously noted in Chapter 3. As cell lines are generally more hardy towards external stimuli compared to primary cells, additional olodaterol concentrations were tested in order to optimize the dosage for the highest IL-8 suppression. As seen in Figure 8, increasing dosages of olodaterol in NCI-H292 cells exhibited a decreasing trend in IL-8 secretion. However, 30 μM of olodaterol, while attenuating IL-8 to a greater degree than 10 μM, did not present statistical significance. Although 100 μM of olodaterol did significantly attenuate IL-8 secretion to a higher degree than 10 μM, 10 μM remains the chosen concentration for experimental procedures to maintain consistency with the ALI cultures and for its clinical significance. It should be noted that in the ALI study documented in Chapter 3, use of both 30 μM and 100 μM of olodaterol demonstrated significantly compromised cell viability in primary ALI cultures (data not shown).

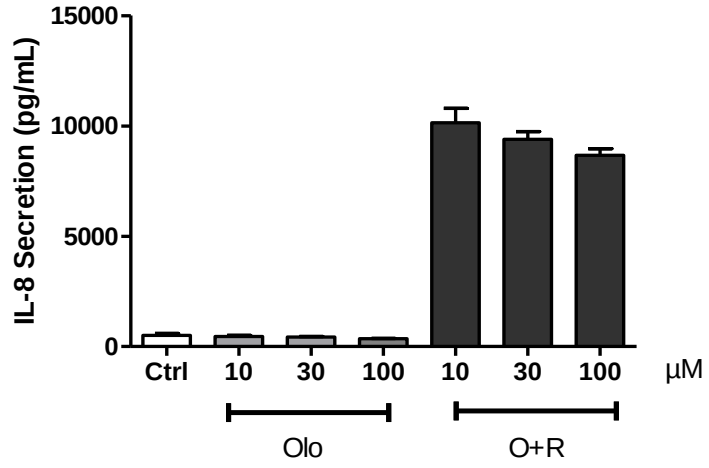



**Figure 8. IL-8 secretion NCI-H292 cells pre-treated with varying concentrations of olodaterol and stimulated with RSV**.

Increasing concentration of olodaterol mediated a greater decrease in IL-8 secretion with RSV infection. Increasing concentration of olodaterol (Olo) sequentially decreased IL-8 secretion resulting from RSV infection (O+R).

### 4.2.4 Duration of Olodaterol in Condition Media

The effect of prolonged exposure to olodaterol in the cell condition media on IL-8 secretion was also examined. We wished to study whether the 8 hours of olodaterol pretreatment alone was sufficient to be anti-inflammatory or if olodaterol was needed to actively inhibit inflammatory processes during infection.

Media containing 10 µM olodaterol was saved prior to RSV addition and re-administered onto the cells after RSV treatment was completed (pre-condition media) to mimic the full 24 hour treatment cycle as seen in patients. For the 8 hours of olodaterol pretreatment alone, olodaterol-containing media was removed prior to RSV treatment and fresh complete media (no olodaterol) was added after RSV treatment. As shown in Figure 9, pre-condition media was able to inhibit RSV-induced IL-8 secretion ($P < 0.005$) and was able to do so significantly more

effectively than 8 hours of olodaterol pre-treatment alone ($P < 0.05$). Furthermore, 8 hours of olodaterol pre-treatment alone was insufficient at inhibiting IL-8 secretion compared to the RSV-only treatment condition, suggesting that the presence of olodaterol in the condition media after exposure to an inflammatory stimuli is necessary for successful inhibition of inflammation. This may be due to the wash step after RSV infection which may have removed the majority of the drug from the culture, indicating that it was not well-retained well in the lipid bilayer.

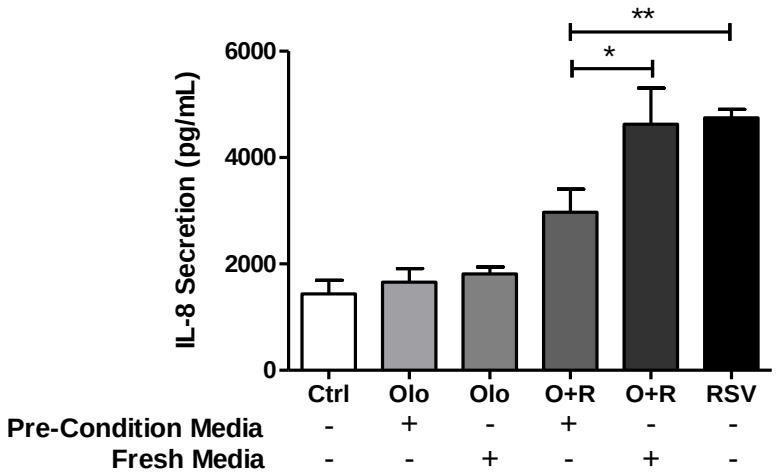



**Figure 9. The effect of prolonged exposure to olodaterol in condition media on IL-8 secretion.**

In the pre-condition media group, NCI-H292 cells have the olodaterol-containing pre-treatment media re-administered to the cells after RSV stimulation. In the fresh media condition, cells have fresh complete media (no olodaterol) added after RSV stimulation. Pre-condition media cells stimulated with RSV produced significantly less IL-8 than cells stimulated with RSV alone ($P < 0.005$). Pre-condition media also attenuated RSV-mediated IL-8 secretion more than fresh media ($P < 0.05$).

### 4.2.5 Cytotoxicity of Olodaterol on NCI-H292

NCI-H292 cells were treated with varying concentrations of olodaterol to determine the ideal concentration for use in a cell line. An LDH assay was performed to examine the degree of

cytotoxicity of the varying concentrations. Olodaterol administered at 10, 30 and 100 µM without RSV had no significant cytotoxic effect on the cells compared to the untreated control (Figure 10). Cells treated with all three concentrations of olodaterol and stimulated with RSV (O+R) showed higher degrees of cytotoxicity than the untreated control but less than the RSV-only treated cells. Furthermore, as seen in Figure 10, it does not appear that 30 or 100 µM of olodaterol had a significantly greater cytotoxic effect on the cells compared to only 10 µM olodaterol, suggesting that this drug is well-tolerated by the cell line.

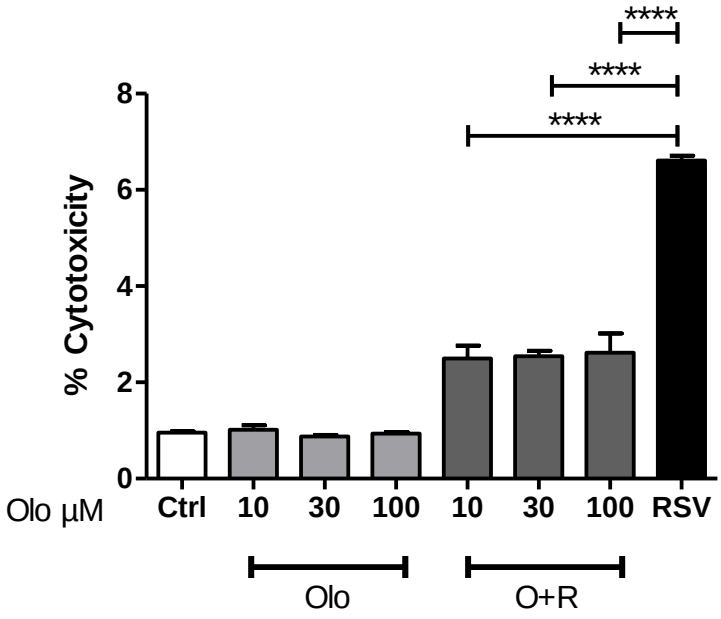



**Figure 10. Cytotoxicity of varying concentrations of olodaterol in NCI-H292 cells.**

Cells were treated with olodaterol at various concentrations (Olo), olodaterol at various concentrations and RSV at $MOI_1$ (O+R), RSV-alone or untreated. No significant difference in cytotoxicity was seen between different concentrations of olodaterol, with and without RSV infection. 10 µM, 30 µM and 100 µM all significantly decreased RSV-mediated cytotoxicity compared to RSV-only ($P < 0.0001$).

### 4.2.6 LPS as an Inflammatory Stimulus

While it has been determined that olodaterol does indeed have an anti-inflammatory effect in NCI-H292 cells in response to RSV, we also examined whether this effect carried over to a different stimuli. LPS was chosen as it is a common inflammatory stimulus that has been featured in previously published studies centered on LABAs' anti-inflammatory effects [100, 105].

To determine the most effective concentration of LPS for eliciting an IL-8 response, we tested LPS concentration both in the nanogram range and the microgram range as literature indicated a wide range of concentrations used for LPS stimulus. As seen in Figure 11A, increasing LPS concentration from 10 ng/mL to 100 ng/mL to 2000 ng/mL (2 µg/mL) saw a corresponding increase in IL-8 secretion. Use of 2000 ng/mL of LPS elicited significantly higher IL-8 response than 100 ng/mL, indicating that the peak effect has not been reached. Contrastingly, while LPS was able to elicit high IL-8 response at 2, 5 and 10 µg/mL, the differences between each concentration was not statistically significant (Figure 11B), suggesting that we are not able to elicit a significantly higher increase in IL-8 secretion with continued increase in LPS concentration. Hence, experimental procedures maintained 2 µg/mL as the LPS stimulus concentration for its large effect on IL-8 secretion while minimizing potential adverse effects to the cell culture with higher endotoxin concentration.

**(A)** **(B)**

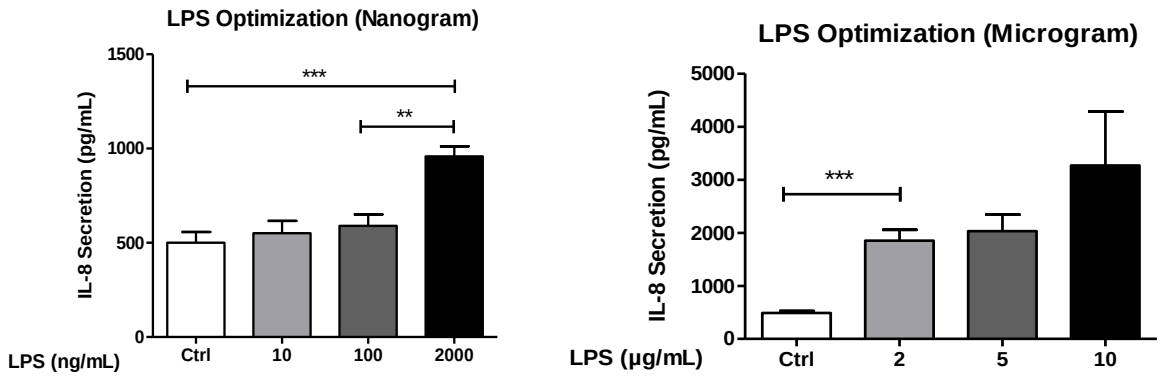



**Figure 11. Optimization of LPS concentration.**

(A) 1HAEo- was stimulated with LPS concentration in the nanogram range to examine the effectiveness of lower concentrations on IL-8 secretion. 2000 ng/mL of LPS stimulated a significantly higher increase in IL-8 than the unstimulated control ($P < 0.001$) and 100 ng/mL of LPS ($P < 0.002$). (B) 1HAEo- cells were stimulated with LPS in the microgram concentration range. 2 µg/mL LPS significantly increased IL-8 secretion compared to unstimulated control ($P < 0.0005$). No significant difference was detected between the different concentrations.

Having established the working concentration of LPS, both cells lines were stimulated with LPS and the resulting cell-free supernatant was measured via an IL-8 ELISA. As shown in Figure 12, the 1HAEo- cells exhibited a robust response to LPS stimulus. Use of 10 µM of olodaterol successfully attenuated LPS-mediated IL-8 secretion by approximately 50% ($P < 0.05$). Conversely, NCI-H292 did not initially respond to olodaterol and LPS treatment (data is not shown).

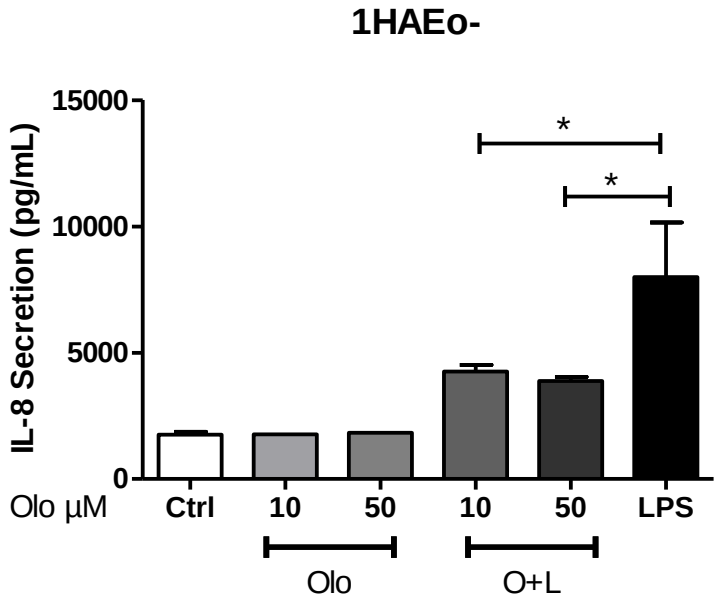



**Figure 12. IL-8 secretion of 1HAEo- cells in response to olodaterol and LPS treatment.**

LPS was administered directly into the culture media at a final concentration of 2 µg/mL Olodaterol (Olo) at 10 and 50 µM was able to significantly attenuated LPS-mediated IL-8 secretion (O+L, $P < 0.05$).

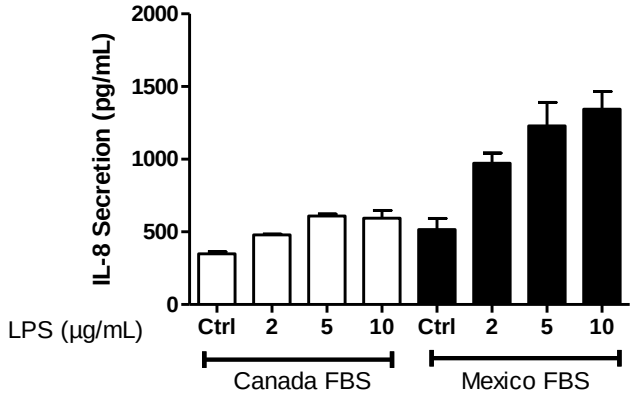
#### 4.2.6.1 Factors Affecting Cellular Response to LPS

Experimentally, several factors were found to have a measurable impact on the IL-8 secretion resulting from administration of LPS. Different cell lines responded to LPS stimulus at the same concentration with varying amounts of IL-8 secretion and this difference extended to higher passage numbers of the same cell line. However, the variations in IL-8 secretions were relatively minor and therefore the results are not shown.

A more compelling change was observed with the use of different FBS formulations. Canada-origin FBS (Gibco) and Mexico-origin FBS (Gibco) elicited different IL-8 secretion levels at the same serum and LPS concentrations. As shown in Figure 13A, 1HAEo- cells cultured in Mexico-made FBS showed a much more robust IL-8 response than 1HAEo- cells

cultured in Canada-made FBS both treated with 2 µg/mL of LPS. Furthermore, Mexico-made FBS was less cytotoxic to the cells compared to Canada-made FBS (Figure 13B). The results indicate that different FBS formulations indeed play a role in sensitivity to LPS. All experiments conducted up to this point and all subsequently experiments used Mexico-origin FBS.

**(A)**

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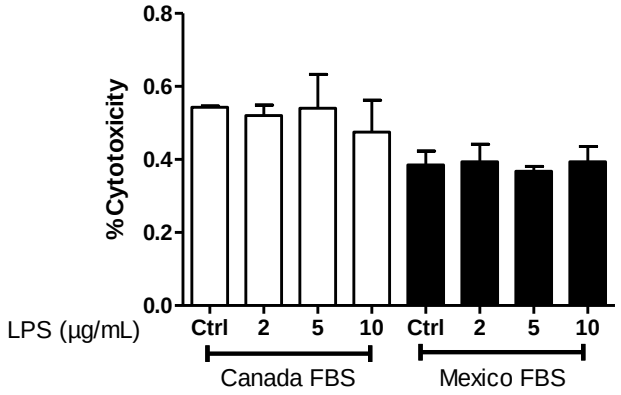



**Figure 13. Comparison of Canada-made FBS and Mexico-made FBS on IL-8 secretion of 1HAEo- cells stimulated with LPS.**

1HAEo- was divided and cultured in RPMI media containing 10% of either FBS formulation. LPS was stimulated at 2, 5 or 10 µg/mL. (A) The IL-8 secretion of 1HAEo- cells cultured in Canada-made FBS and Mexico-made FBS. (B) The corresponding cytotoxicity of both formulations.

Furthermore, it became clear that serum concentration also influences LPS sensitivity. Literature examination revealed observations by others that higher concentrations of serum in the culture media increased cell responsiveness to LPS. The presence of a TLR4 co-receptor found in serum, CD14, is crucial for binding of LPS and activation of TLR4 [106-108]. Physiologically, CD14 is present in both a phosphatidylinositol-linked membrane bound form (mCD14) as well as a soluble form (sCD14) [107]. As epithelial cells do not make notable amounts of mCD14, they are dependent on circulating sCD14 to facilitate activation of TLR4 in response to LPS [106]. Several studies have shown that high serum (10%) or addition of CD14 into the culture media is required for robust IL-8 signals in response to LPS [109].

To investigate the effect of serum on IL-8 secretion, olodaterol and LPS stimulation was conducted at 0%, 1% or 10% serum. In consideration of time, the experiment was only performed on the NCI-H292 cell line. Figure 14 demonstrates the clear trending towards increasing IL-8 secretion with increasing serum concentration. Furthermore, the effect of olodaterol to attenuate LPS-induced IL-8 secretion is also more marked in 10% serum compared to 0% or 1% serum. Even accounting for baseline differences of IL-8 secretion between the different serum concentrations, 10% serum appeared to elicit greater sensitivity to LPS than 0% or 1%. The data therefore suggests that serum concentration plays a significant effect on cell sensitivity to LPS.

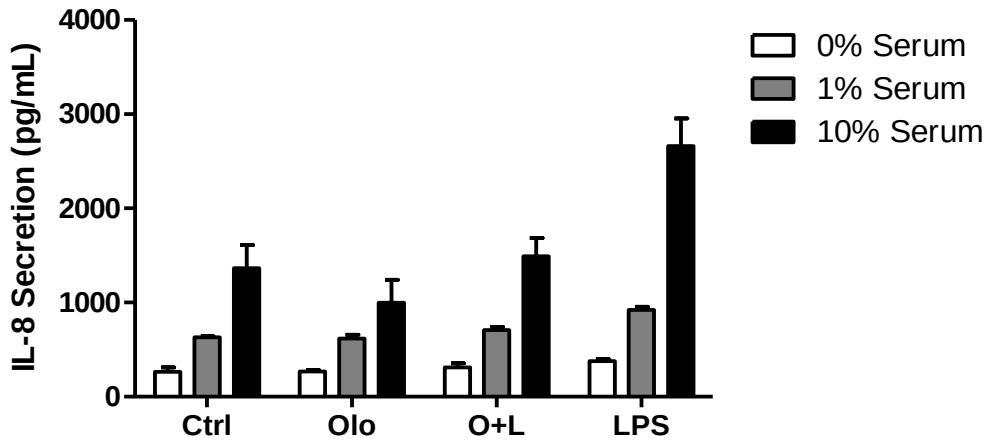



**Figure 14. The IL-8 response of NCI-H292 cells to olodaterol (Olo) and LPS with different serum FBS concentrations.**

Cells were cultured in 10% FBS RPMI media and underwent LPS stimulation overnight in 0%, 1% and 10% FBS RPMI media.

## 4.3 Discussion

The initial focus of this project aimed to establish a working model of airway inflammation in order to test our hypothesis and better understand the mechanisms of olodaterol's anti-inflammatory action. Several conditions required optimization in order to establish proper treatment protocols including comparisons of two different cell lines and two inflammatory stimuli. Various concentrations were compared for the most optimal inhibition of IL-8.

Both the 1HAEo- and the NCI-H292 cell lines were chosen for this study because of their long history of use in the lab and because they have been the subject of various published papers exploring the airway epithelium. As such, they were determined to be potential models for this project. Both cell lines were found to constitutively express detectable amounts of β2AR that did

not appear to vary with olodaterol or RSV treatment, indicating that receptor density does not appear to be changing in response to drug treatment. Furthermore, both cell lines reacted well to RSV stimulation by producing large amounts of IL-8. NCI-H292 showed a significant inhibition of IL-8 secretion with olodaterol treatment and RSV infection, corroborating results from Chapter 3. Although 1HAEo- did not show IL-8 inhibition in the presence of olodaterol, this may not indicate that 1HAEo- is ill-suited for modeling our study, but rather that more optimization is required. However, in the interest of time, this avenue was not pursued further.

Additional experiments suggested that 10 μM of olodaterol was the ideal drug concentration as it was capable of eliciting a significant inhibition of RSV-mediated IL-8 secretion. Higher concentrations did not elicit more detrimental cytotoxicity in the cell culture and produced slightly greater IL-8 inhibition. Nonetheless, 10 μM remained the ideal concentration as it is the clinically relevant *in vivo* concentration of one inhaled dose of the olodaterol, as detailed in Chapter 3.

Exposing the cell culture to the drug in the media for a full 24 hour cycle (thereby mimicking patient treatment regimen) was significantly better at attenuating IL-8 secretion than 8 hours of drug-priming alone. This appears to indicate that the anti-inflammatory effect of olodaterol did not come from the 8 hours of priming and that olodaterol was unlikely to be retained in the cell membrane in sufficient amounts to continue to trigger the response. The chemical structure of olodaterol lacks the lipophilic tail that other LABAs such as formoterol have, which has been documented previously to assist in the insertion and retention in the lipid bilayer [74]. Thus, the mechanism by which olodaterol retains long-running effects is unclear and may warrant additional experiments in a future study.

The data also showed that olodaterol is capable of inhibiting IL-8 secretion by both RSV and LPS, as seen in both 1HAEo- and NCI-H292 cells, indicating that the anti-inflammatory effects are not cell-line or stimuli-dependent but an inherent property of this drug. The inflammatory stimuli RSV and LPS both facilitate IL-8 secretion via different molecular pathways. RSV is able to drive IL-8 secretion via activation of Toll-like receptor 3 and the MyD88-independent pathway as well as the retinoic acid-inducible gene I receptor [110]. Meanwhile, LPS stimulation proceeds through TLR 4 and the MyD88-dependent and MyD88 independent pathways [111]. However, both stimuli lead to the eventual nuclear translocation of the transcription factor NF-κB and this shared overlap may suggest a common point of interest upon which olodaterol exerts its effect. Perhaps olodaterol helps to stabilize cytosolic NF-κB inhibition by the IκB proteins in order to suppress IL-8 transcription. However, additional studies are needed to investigate the validity of this hypothesis.

Interestingly, variations in serum formulations were found to play a large role in LPS sensitization. Mexico-origin FBS elicited greater LPS sensitivity than the Canada-origin FBS, potentially due to varying levels of cytokines and growth factors in the respective FBS. A literature review indicated CD14, commonly found in serum, is important for LPS sensitivity. One study has noted a correlation between higher amounts of soluble CD14 in COPD patient lungs and lower lung function [107]. This association was not seen in non-COPD patients. While the in-depth disease significance of CD14 in COPD has not been well-studied, it is clear that CD14 plays an important function in the immune response against infection by Gram negative bacteria as it helps TLR4 recognize bacterial pathogens [112, 113]. Because it is not significantly expressed in the membrane-bound form by airway epithelial cells, there remains a reliance on circulating CD14. It was found that addition of either CD14 or 10% serum was sufficient to

sensitive cells to LPS [109]. Thus, CD14 or 10% serum is required for consistent responsiveness to LPS.

The experiments in this study are limited by the use of only two model lung cell lines as different cell lines may react differently to the same stimuli. We were not able to elicit a proper inhibition of RSV-mediated IL-8 secretion in the 1HAEo- cell lines as we were able to do so for RSV and LPS stimuli in the NCI-H292, but this may simply be due to a lack of optimizing. Nonetheless, the results indicate olodaterol may affect each cell line slightly differently. Additionally, it would have been ideal to use primary cells as they are more translatable clinically, but the investigation using gene silencing in the next Chapter would have rendered the use of primary cells near impossible due to the inherent heterogeneity and difficulty of achieving sufficient levels of silencing.

In summary, the experiments up until this point have established a working model for the investigation of the mechanism behind olodaterol's anti-inflammatory effects. A concentration of 2 μg/mL of LPS and 10 μM of olodaterol and have been found to elicit and significantly inhibit the secretion of IL-8, respectively. The next chapter of this thesis will focus on the siRNA-directed knockdown of the β2AR in order to determine whether the anti-inflammatory effect seen is specific or off-target.

# Chapter 5: Knockdown of the β2-Adrenergic Receptor

## 5.1 Materials & Methods

### 5.1.1 siRNA Gene Silencing

Silencer Select siRNAs from Life Technologies (California, US) were purchased to target the β2AR mRNA sequence (s1121, s1122, s1123, s531994), GAPDH mRNA (4390849), and a scrambled non-targeting sequence (4390844). All four β2AR siRNAs were combined in-house in equi-molar proportions to create a custom pool. All siRNAs were delivered using Lipofectamine RNAiMax reagent (Life Technologies, California, USA) according to manufacturer's instructions.

Cells were seeded into 24-well culture plates (CORNING, New York, USA) at a density of $6x10^4$ cells per well and allowed to grow until 60% confluence before siRNA treatment. The cells were treated with the siRNA-lipofectamine transfection complex as per Ambion's standard protocol for 24 hours before the removal and replacement of the media with fresh complete media. Additional experiments were performed 48 hours after initial transfection.

### 5.1.2 Olodaterol, LPS Treatment and RSV Infection

Olodaterol (10 µM), LPS (2 µg/mL) and RSV ($MOI_1$) were administered as per previously established methods in Chapter 4. The condition media and protein lysates were obtained for ELISA assay and Western blotting, respectively, as per the methods documented in Chapter 4.

### 5.1.3 Western Blotting

Western blotting was performed as per the methods documented in Chapter 4 using the same reagents. For visualizing siRNA knockdown efficiency a rabbit Anti-GAPDH-HRP

antibody (#14C10, Cell Signaling) was used. Protein abundance was visualized using a chemiluminescent reagent (Femto ECL, Thermofisher).

### 5.1.4 RNA Extraction and Quantification

Total RNA was extracted from cultures using the RNeasy Mini Kit as per manufacturer's instructions. Quantification of the total RNA concentration was performed using the Nanodrop Spectrophotometer.

### 5.1.5 cDNA Library Synthesis

The cDNA library was reverse synthesized from the total RNA isolates using the iScript cDNA Synthesis Kit by Biorad Laboratories (California, USA) as per manufacturer's instructions. A total of 800 ng of RNA template was loaded per sample and the resulting solution was diluted 1:10 in pyrogen-free water.

### 5.1.6 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed using the iTaq Universal Sybr Green Supermix from Biorad. The results were normalized against the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) while Glycerol-3-phosphate dehydrogenase (GAPDH) was used to confirm the efficacy of the siRNA knockdown. Custom DNA primers targeting β2AR, HPRT and GAPDH cDNA transcripts were designed using PrimerBlast based on sequences obtained from GeneBank (Appendix B , Table 2). Knockdown efficiency of the target genes were analyzed via the ddCt method.

### 5.1.7 Enzyme-Linked Immunosorbent Assay (ELISA)

IL-8 secretion was quantified via ELISA as per the methods documented in Chapter 3.

### 5.1.8 Statistical Analysis

Graphical data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, CA, USA). One-way ANOVA with Bonferroni correction was used for multiple group comparisons. Two-way ANOVA with Bonferroni correction was used for multiple group comparisons with two variables (ie. Between siRNA-treated cells and non-siRNA treated cells). Error bars on the graphs indicate mean $\pm$ standard deviation.

## 5.2 Results

The establishment of an airway epithelium model of inflammation meant it was possible to investigation the specificity of olodaterol's anti-inflammatory effect. We wished to determine whether the effect observed is mediated through the β2AR, which required the optimization of siRNA gene-silencing.

### 5.2.1 Optimization of siRNA Transfection Protocol

To determine the concentration of siRNA and lipofectamine required to elicit sufficient knockdown, we tested a variety of conditions using GAPDH-targeting siRNA as it is a pre-validated siRNA targeting a well-established gene before optimizing our target. Different volumes of lipofectamine and concentrations of siRNA per well of a 24-well plate are shown in Table 3.

**Table 3. The tested ratios of lipofectamine RNAiMAX volume to GAPDH-targeting siRNA concentration per well of a 24-well plate.**

| | **Lipofectamine 2 µL** | | | **Lipofectamine 4 µL** | | |
|---|---|---|---|---|---|---|
| **siRNA Concentration (nM)** | 1 | 5 | 10 | 1 | 5 | 10 |

As shown in Figure 15, while some degree of protein knockdown was present for all ratios of lipofectamine and siRNA tested, 2 µL of lipofectamine and 10 nM of siRNA led to the greatest depletion of GAPDH protein, indicating that this ratio is sufficient to deliver the siRNAs into the target cells.

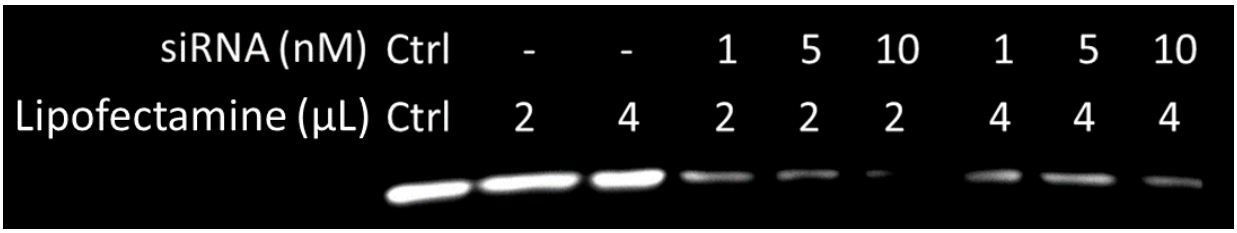



**Figure 15. Optimization of transfection protocol using GAPDH.**

1HAEo- cells were grown in 24-well culture plates and tested with varying amounts of lipofectamine and GAPDH siRNA per well. Western blotting was conducted to visualize the remaining protein levels of GAPDH after siRNA knockdown. Samples were collected at 72 hours post siRNA transfection.

Media from the Figure 15 experiment was also tested using an LDH assay to measure cytotoxicity. There appears to be no significant difference between the different test conditions (Figure 16). Furthermore, the lack of a cytotoxic response to the lipofectamine and siRNA indicates there is no notable toxicity associated with their use. It is not uncommon for addition of siRNA to trigger innate immune responses that lead to altered cytokine release and/or cell death [114] that would confound the results of our IL-8 quantification.

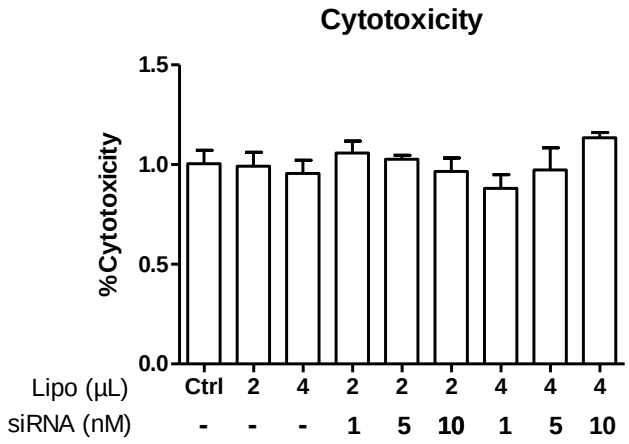



**Figure 16. The cytotoxicity of varying concentrations of lipofectamine and Silencer Select siRNA**.

1HAEo- were tested with varying amounts of lipofectamine RNAiMax and GAPDH siRNA.

After determining a functional protocol for the delivery of the siRNA, we attempted to optimize using the β2AR siRNAs (s1121, s1122, s1123, s531994) either individually or as an equi-molar pool. Literature has shown that pooled samples of multiple interfering RNA sequences may be more effective by targeting different regions of the mRNA transcript [115, 116]. Numerous experiments testing different parameters were unsuccessful in eliciting knockdown of this receptor in the 1HAEo- cell line despite consistent knockdown of GAPDH at both the protein and mRNA level (data not shown). This was determined to be cell-line dependent as gene silencing of β2AR was successful using NCI-H292 (Figure 17). The lack of gene silencing was therefore due to inherent characteristics of the 1HAEo- cell line and may be a result of its high proliferative potential. In contrast, the NCI-H292 cells proliferate at a significantly slower rate.

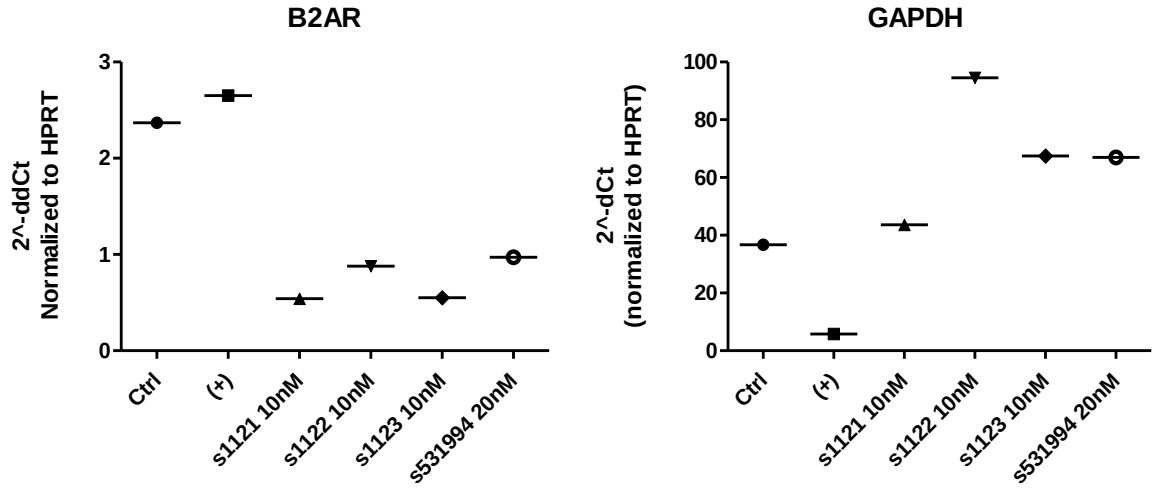
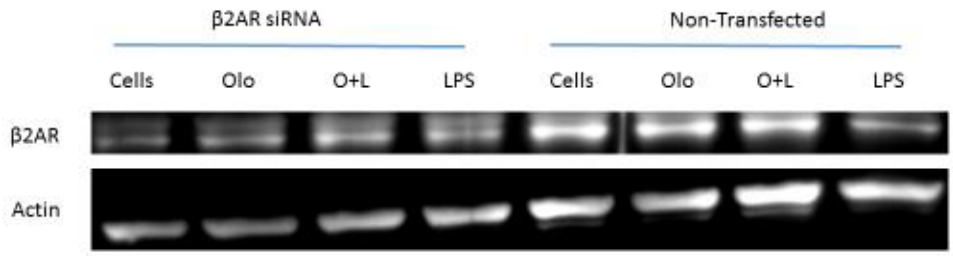



**Figure 17. Quantification of β2AR gene-silencing in NCI-H292 cells using Silencer Select siRNA (s1121, s1122, s1123, s531994).**

Samples were collected 48 hours after transfection for RT-qPCR. GAPDH-targeting siRNA ((+)) was also used as a positive control to confirm transfection efficiency.

As the goal of gene-silencing was the depletion of the β2AR protein, the knockdown efficiency was also visualized at the protein level. As shown in Figure 18A, use of β2AR-targeting siRNA caused a decrease in β2AR chemiluminescence in NCI-H292 cells compared to non-transfected cells. Densitometry analysis showed that use of siRNA notably inhibited protein expression (Figure 18B).

**(A)**




**(B)**

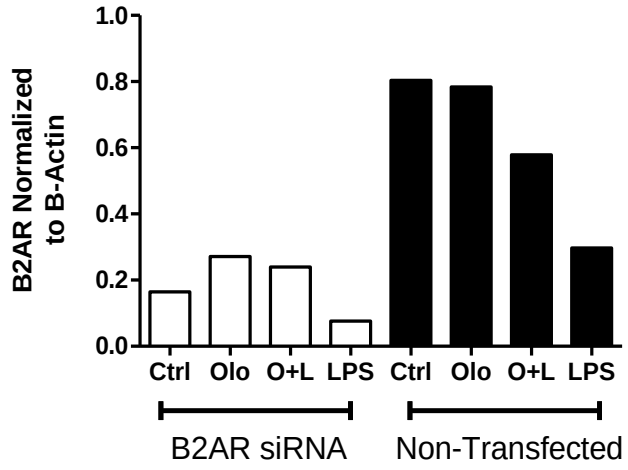



**Figure 18. B2AR protein expression post-gene silencing.**

(A) Western blot chemiluminescence visualization of the β2AR protein in NCI-H292 cells transfected with β2AR-targeting siRNA pool and non-transfected cells. (B) Densitometry analysis of β2AR protein expression as normalized to the housekeeping gene β-actin.

### 5.2.2 The Effect of β2AR Gene Silencing on Inflammation

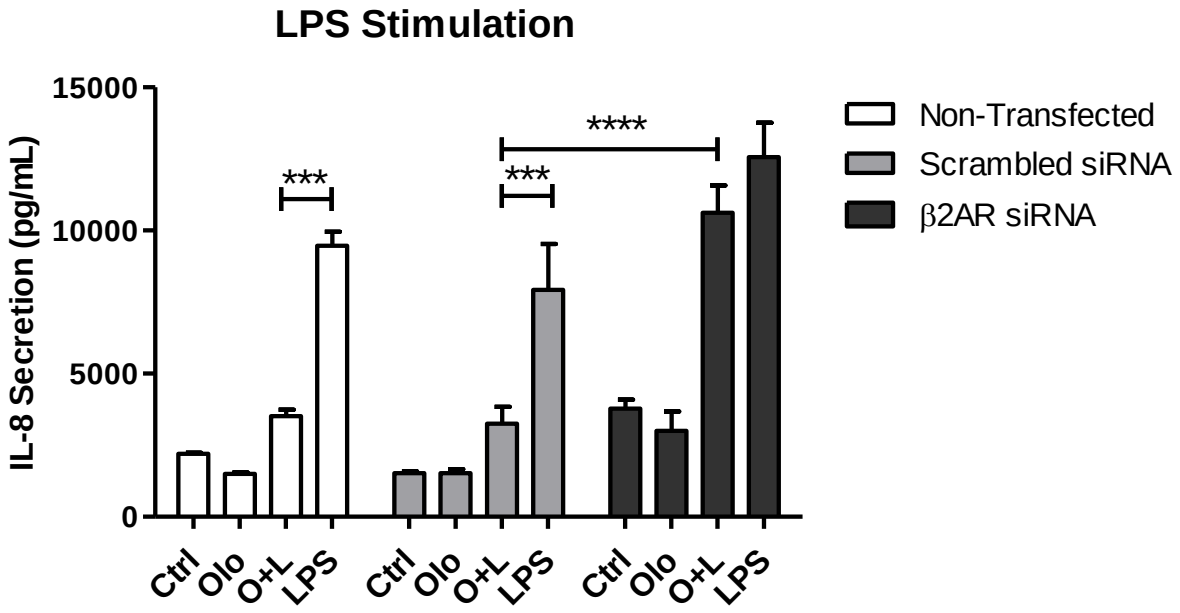
The maximum effectiveness of β2AR gene silencing using the NCI-H292 cell line appears to be approximately 80% efficacy at the mRNA level. Following the successful establishment of a working transfection protocol, we investigated the effect of β2AR gene

silencing on olodaterol-mediated inhibition of IL-8 secretion to determine whether the effects observed proceed through this receptor or if it is an off-target effect.

NCI-H292 cells were divided into three groups and transfected with scrambled non-targeting siRNA, β2AR-targeting siRNA pool (s1121, s1122, s1123, s531994) or a non-transfected control group. All three groups were treated with 10 μM olodaterol, 2 μg/mL LPS, O+L or no treatment. From Figure 19A, in the non-transfected and the scrambled siRNA-transfected NCI-H292 groups, treatment with olodaterol inhibited LPS-mediated IL-8 secretion significantly ($P < 0.001$ and $P < 0.001$ respectively). However, when β2AR was silenced via siRNA, olodaterol was no longer able to significantly attenuate LPS-mediated IL-8 secretion (Figure 19A). A comparison between the O+L treatment group of scrambled-siRNA transfected cells and β2AR-siRNA transfected cells also showed that IL-8 secretion was significantly higher when β2AR was silenced ($P < 0.0001$).

The experiment was repeated with RSV as the inflammatory stimulus and resulted in the same effect (Figure 19B). In non-transfected and scrambled siRNA-transfected cell groups, olodaterol significantly suppressed RSV-driven IL-8 secretion ($P < 0.01$ and $P < 0.0001$, respectively) while transfection with β2AR-targeting siRNA abolished this significant difference.

**(A)**




**(B)**

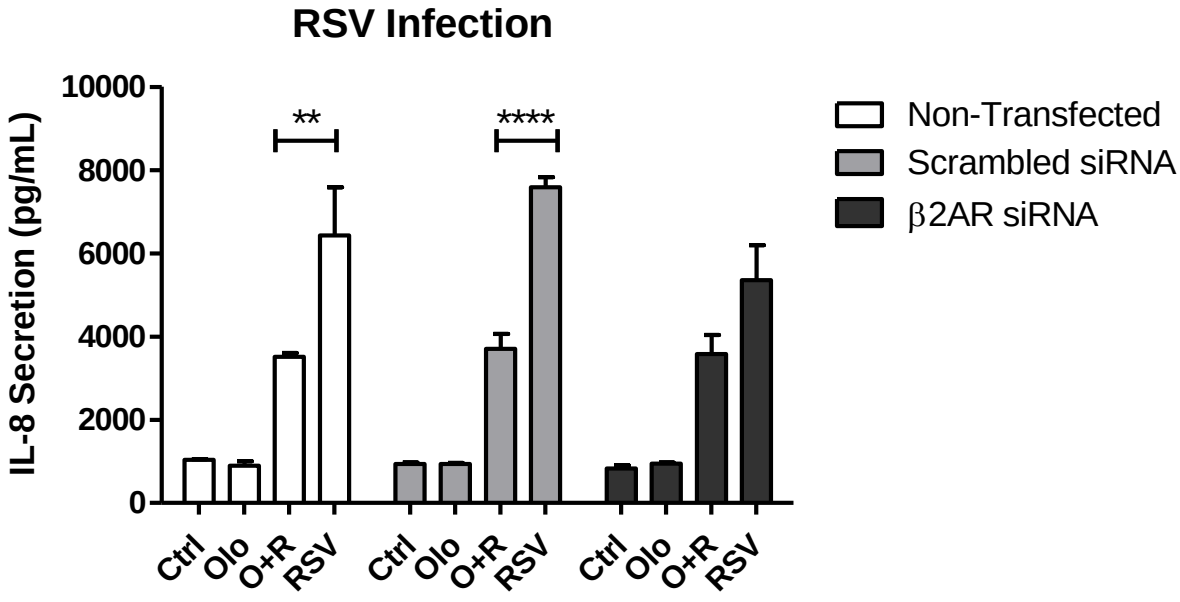



**Figure 19. Olodaterol-mediated IL-8 secretion post-β2AR silencing with LPS or RSV stimulation.**

NCI-H292 cells were transfected with scrambled non-targeting siRNA, a pool of β2AR-targeting siRNA (s1121, s1122, s1123, s531994) or non-transfected. Cells were treated with olodaterol (Olo), olodaterol and LPS or RSV (O+L, O+R) or LPS or RSV only. (A) In non-transfected cells and scrambled siRNA-transfected cells, O+L showed significantly inhibited IL-8 levels compared to the LPS-only group ($P < 0.001$, $P < 0.001$), but not in the β2AR-siRNA transfected cells. The IL-8 secretion of the O+L treatment condition was significantly lower in the scrambled siRNA-treated group than the β2AR-siRNA treated group ($P < 0.0001$). (B) IL-8 secretion was significantly lower in the O+R treatment compared to the RSV-only condition in both non-transfected and scrambled siRNA-transfected cells ($P < 0.01$ and $P < 0.0001$, respectively). However, this was not seen in cells transfected with β2AR-targeting siRNA.

## 5.3 Discussion

Following the creation of a functional cell line model of airway inflammation using olodaterol and LPS, the next step was to optimize a gene silencing protocol for β2AR, the binding receptor of LABAs. Knockdown of the mRNA could not be achieved using the 1HAEo- cell lines despite varying siRNA sequence, concentration and transfection strategies due to inherent properties of this cell line. However, gene silencing in NCI-H292 was possible up to approximately 80% of β2AR mRNA transcripts and approximately 50% at the protein level. While the knockdown is lower than what is seen with GAPDH (>90%), it can be assumed that the gene silencing would still induce an observable effect on IL-8 secretion as stimulated by olodaterol and LPS.

The results indicate that in non-transfected NCI-H292 cells, olodaterol suppressed RSV and LPS mediated IL-8 secretions. Similarly, transfection with scrambled non-targeting siRNA did not alter this response either. However, silencing of the canonical binding receptor, β2AR, negated this significant inhibition, indicating that β2AR is a key mediator of this effect. The loss

of β2AR therefore signals that the observed anti-inflammatory effect of olodaterol is a highly specific event, rather than an off-target effect. The loss of IL-8 inhibition also indicates that the effects are inherent properties of olodaterol. Nonetheless, β2AR is a GPCR that activates a host of downstream pathways through protein kinase A and thus it is still unknown which pathways are directly involved in the effects observed.

LABAs were initially theorized to non-specifically increase the efficacy of ICS' anti-inflammatory effect but this project has demonstrated that they can have their own inherent anti-inflammatory properties. While the current literature on LABA's synergy with ICS remains contentious, there was already published data suggesting that LABA monotherapy can elicit anti-inflammatory effects such as increasing transcription of anti-inflammatory genes and decreasing cytokine production [97-99]. The exact mechanism behind this is still unknown but as demonstrated in this chapter, the effect on IL-8 is specifically mediated through the canonical binding receptor, β2AR and independent of any specific inflammatory stimuli. Both of the stimuli used, LPS and RSV, induce IL-8 secretion via different pathways but overlap in their role of releasing NF-κB inhibition. Thus, olodaterol may potentially act upon NF-κB to inhibit its translocation into the nucleus.

The effect of LABAs as characterized in this study may be clinically significant as the experiments outlined in this thesis employed a concentration (10 μM) roughly equivalent to that of a single inhaler dose as prescribed to patients. The results therefore suggest that patients may benefit from the additional anti-inflammatory effects of this drug but *in vivo* studies are needed to be conclusive. Furthermore, 10 μM of olodaterol inhibited IL-8 to a similar degree as 1 μM of the ICS dexamethasone (approximately 50%), a well-established anti-inflammatory agent used in patient therapy [117]. The 1 μM dexamethasone concentration was also derived from the

estimated concentration of one inhalation of the drug dissolved into the average periciliary fluid volume of a patient assuming a 10-30% deposition [104]. As olodaterol was also able to elicit approximately 50% inhibition of IL-8 secretion on an airway epithelial cell line, the findings suggest that olodaterol may hold clinical significance at its current usage concentration on par with available ICS treatment options.

The limitations of the experiments in this chapter include the use of just one type of LABA, olodaterol. It would be worthwhile to observe whether the effects documented with olodaterol is also mediated through the β2AR in other LABAs (eg. Formoterol, Salmeterol) and to the same degree of effectiveness since all LABAs activate the β2AR. The mechanistic data of β2AR-mediated IL-8 suppression was obtained through use of a bronchial epithelial cancer cell line. As gene silencing using primary tissue culture presents great difficulty due to the heterogeneity and fragility of the cells, use of a cell line was the only viable option given time constraints but may not be fully representative of the *in vivo* cellular mechanisms.

Future directions of this study would benefit from a closer look into the downstream pathway of β2AR activation. It would be of interest to interrogate other key proteins in the cascade to determine the effects, if any, they have on known inflammatory pathways to better understand the phenomenon we have witnessed. Additional cytokine panels and inflammatory mediator assays can be employed to visualize the effect of LABAs on other aspects of the inflammatory cascade other than IL-8. Nonetheless, the data presented in this chapter documents compelling evidence that LABAs have a strong and specific anti-inflammatory that warrants further study.

## Chapter 6: Conclusion

The research presented in this thesis represents a closer investigation into how LABAs elicit an anti-inflammatory effect in the airway. We have shown that the LABA olodaterol has a potently anti-inflammatory ability on our model cell lines in the form of a significant IL-8 reduction resulting from an inflammatory stimulus, either RSV or LPS. In our primary pseudo-stratified airway cultures, olodaterol inhibited both IL-8 and mucus secretion resulting from RSV infection. The effect is intrinsic to the LABA and is not cell line dependent, or stimulus dependent. Importantly, the anti-inflammatory effects of olodaterol are a highly specific effect that is mediated by its canonical binding receptor, the β2AR, thereby suggesting that LABAs can be inherently anti-inflammatory.

In the past, an increased anti-inflammatory effect was observed with LABA/ICS combination therapy, leading to the speculation that LABAs were increasing the efficacy of ICS via an off-target effect. While there has been research demonstrating the increased effectiveness of LABA/ICS treatment to curb airway inflammation than either treatment alone, few have examined the inherent anti-inflammatory ability of LABAs or the mechanism by which it happens. Early contemporaries suggested that LABAs only exhibit anti-inflammatory effects in the presence of ICS. However, we have demonstrated that this is untrue and that while synergy with ICS may improve airway inflammation than either individual treatment, LABAs have the clear ability to attenuate airway inflammation independent of any ICS treatment in an *in vitro* setting.

However, the overall project is not without limitations. The focus of the study was on airway epithelial cells as they are the primary site of disease in COPD patients and responsible for the initial cytokine response. However, LABAs are known to facilitate their established

bronchodilation effects via the airway smooth muscle. Therefore, more research is needed to examine if the airway smooth muscle plays any significant role in the anti-inflammatory response. Additionally, this study only focused on olodaterol. It would be interesting to investigate whether the effects witnessed can be replicated using other LABAs. Only one cytokine, IL-8, was investigated in this study. It is well established that while a large component of COPD inflammation is neutrophilic, other cytokines and immune cells also play significant roles. Additional experiments would benefit from looking at an inflammatory cytokine panel. Finally, this study was conducted *in vitro* using both primary and cell line tissue culture. While the results present more evidence that LABAs can be inherently inflammatory and that the effects are significant enough to be worthy of closer study, the clinical translation is still uncertain. *In vitro* models are not entirely representative of the cellular mechanisms *in vivo* and thus it is too early to say if these findings have any definitive impact on patient pharmacotherapy. Nonetheless, the research documented in this thesis is a novel and important stepping stone towards better understanding of an important aspect of COPD treatment and may in the future lead to improved therapies for patients.

Future directions for this study can focus on elucidating the *in vivo* implications of LABAs exhibiting anti-inflammatory effects. The next potential step may be to conduct randomized clinical trials using olodaterol monotherapy to see if the results observed *in vitro* hold in a COPD and non-COPD patient population and whether the effects are significant. The data would establish clear clinical translatability and provide evidence on whether certain subsets of patients may be eligible for LABA monotherapy to control airway inflammation and bronchodilation. A simplified regimen would also increase patient adherence. Additional clinical trials employing both LABAs and ICS can explore whether LABAs' effects are great enough to

replace or lower current ICS dosing regimens. ICS treatment is accompanied by a host of unpleasant side effects and patients would benefit from potentially lowered doses of ICS and the subsequent decrease in side effects prevalence.

To our knowledge, this is the first study to look at the anti-inflammatory effects of LABAs in a primary pseudo-stratified model of the airway epithelium from patient-derived samples and to determine that the effect is specifically mediated through the canonical binding receptor. The specific pathways that are activated by β2AR to generate this effect is still unclear and more research is necessary to better understand the mechanisms involved. At present, the clinical implications are still uncertain but the results are hopeful that LABAs can offer additional protection against airway inflammation in tandem with bronchodilation. Finally, more research is needed into this field to elucidate the downstream pathways, as understanding how LABAs generate an anti-inflammatory effect can be a stepping-stone towards creating better therapeutic drugs for COPD patients.

## Bibliography


1. Bagdonas, E., et al., *Novel aspects of pathogenesis and regeneration mechanisms in COPD.* Int J Chron Obstruct Pulmon Dis, 2015. **10**: p. 995-1013.
2. Pauwels, R.A., et al., *Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary.* Am J Respir Crit Care Med, 2001. **163**(5): p. 1256-76.
3. Barnes, P.J., *Small airways in COPD.* N Engl J Med, 2004. **350**(26): p. 2635-7.
4. Angelis, N., et al., *Airway inflammation in chronic obstructive pulmonary disease.* J Thorac Dis, 2014. **6 Suppl 1**: p. S167-72.
5. Kim, V. and G.J. Criner, *Chronic bronchitis and chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2013. **187**(3): p. 228-37.
6. Stockley, J.A., et al., *Small airways disease: time for a revisit?* Int J Chron Obstruct Pulmon Dis, 2017. **12**: p. 2343-2353.
7. Burgel, P.R., *The role of small airways in obstructive airway diseases.* Eur Respir Rev, 2011. **20**(119): p. 23-33.
8. Vestbo, J., et al., *Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary.* Am J Respir Crit Care Med, 2013. **187**(4): p. 347-65.
9. Barnes, P.J., *Inflammatory mechanisms in patients with chronic obstructive pulmonary disease.* J Allergy Clin Immunol, 2016. **138**(1): p. 16-27.
10. MacNee, W., *Pathology, pathogenesis, and pathophysiology.* BMJ : British Medical Journal, 2006. **332**(7551): p. 1202-1204.
11. Turato, G., R. Zuin, and M. Saetta, *Pathogenesis and pathology of COPD.* Respiration, 2001. **68**(2): p. 117-28.
12. Szilasi, M., et al., *Pathology of chronic obstructive pulmonary disease.* Pathol Oncol Res, 2006. **12**(1): p. 52-60.
13. Pirina, P., et al., *Small airway inflammation and extrafine inhaled corticosteroids plus long-acting beta2-agonists formulations in chronic obstructive pulmonary disease.* Respir Med, 2018. **143**: p. 74-81.
14. Baraldo, S., G. Turato, and M. Saetta, *Pathophysiology of the small airways in chronic obstructive pulmonary disease.* Respiration, 2012. **84**(2): p. 89-97.
15. Rabe, K.F. and H. Watz, *Chronic obstructive pulmonary disease.* Lancet, 2017. **389**(10082): p. 1931-1940.
16. Higham, A., et al., *The pathology of small airways disease in COPD: historical aspects and future directions.* Respir Res, 2019. **20**(1): p. 49.
17. Barnes, P.J., et al., *Chronic obstructive pulmonary disease.* Nat Rev Dis Primers, 2015. **1**: p. 15076.
18. Hogg, J.C., *Pathophysiology of airflow limitation in chronic obstructive pulmonary disease.* Lancet, 2004. **364**(9435): p. 709-21.
19. De Rose, V., et al., *Airway Epithelium Dysfunction in Cystic Fibrosis and COPD.* Mediators of inflammation, 2018. **2018**: p. 1309746-1309746.

20. Gao, W., et al., *Bronchial epithelial cells: The key effector cells in the pathogenesis of chronic obstructive pulmonary disease?* Respirology, 2015. **20**(5): p. 722-9.
21. Tam, A., et al., *The airway epithelium: more than just a structural barrier.* Ther Adv Respir Dis, 2011. **5**(4): p. 255-73.
22. Pouwels, S.D., et al., *Susceptibility for cigarette smoke-induced DAMP release and DAMP-induced inflammation in COPD.* Am J Physiol Lung Cell Mol Physiol, 2016. **311**(5): p. L881-L892.
23. Perotin, J.-M., et al., *Delay of airway epithelial wound repair in COPD is associated with airflow obstruction severity.* Respiratory research, 2014. **15**(1): p. 151-151.
24. Puchelle, E., et al., *Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease.* Proc Am Thorac Soc, 2006. **3**(8): p. 726-33.
25. Ganesan, S. and U.S. Sajjan, *Repair and Remodeling of airway epithelium after injury in Chronic Obstructive Pulmonary Disease.* Curr Respir Care Rep, 2013. **2**(3).
26. Mannino, D.M., *Smoking and Emphysema: Looking Beyond the Cigarette.* Chest, 2015. **148**(5): p. 1126-1127.
27. Siafakas, N.M. and E.G. Tzortzaki, *Few smokers develop COPD. Why?* Respir Med, 2002. **96**(8): p. 615-24.
28. Huang, G., et al., *Neutrophilic Inflammation in the Immune Responses of Chronic Obstructive Pulmonary Disease: Lessons from Animal Models.* J Immunol Res, 2017. **2017**: p. 7915975.
29. Mukaida, N., *Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2003. **284**(4): p. L566-L577.
30. Quint, J.K. and J.A. Wedzicha, *The neutrophil in chronic obstructive pulmonary disease.* Journal of Allergy and Clinical Immunology, 2007. **119**(5): p. 1065-1071.
31. Dixit, N. and S.I. Simon, *Chemokines, selectins and intracellular calcium flux: temporal and spatial cues for leukocyte arrest.* Frontiers in immunology, 2012. **3**: p. 188-188.
32. Wang, Y., et al., *Role of inflammatory cells in airway remodeling in COPD.* Int J Chron Obstruct Pulmon Dis, 2018. **13**: p. 3341-3348.
33. Panettieri, R.A., Jr., *The Role of Neutrophils in Asthma.* Immunol Allergy Clin North Am, 2018. **38**(4): p. 629-638.
34. Ravin, K.A. and M. Loy, *The Eosinophil in Infection.* Clinical Reviews in Allergy & Immunology, 2016. **50**(2): p. 214-227.
35. Tworek, D. and A. Antczak, *Eosinophilic COPD - a distinct phenotype of the disease.* Adv Respir Med, 2017. **85**(5): p. 271-276.
36. King, P.T., *Inflammation in chronic obstructive pulmonary disease and its role in cardiovascular disease and lung cancer.* Clinical and translational medicine, 2015. **4**(1): p. 68-68.
37. Chung, K.F., *Cytokines in chronic obstructive pulmonary disease.* European Respiratory Journal, 2001. **18**(34 suppl): p. 50s.
38. Lomas, D.A., *Does Protease-Antiprotease Imbalance Explain Chronic Obstructive Pulmonary Disease?* Ann Am Thorac Soc, 2016. **13 Suppl 2**: p. S130-7.
39. Abboud, R.T. and S. Vimalanathan, *Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema.* Int J Tuberc Lung Dis, 2008. **12**(4): p. 361-7.

40. Gadek, J.E., et al., *Antielastases of the human alveolar structures. Implications for the protease-antiprotease theory of emphysema.* J Clin Invest, 1981. **68**(4): p. 889-98.
41. O'Donnell, R., et al., *Inflammatory cells in the airways in COPD.* Thorax, 2006. **61**(5): p. 448-54.
42. Meyer, M. and I. Jaspers, *Respiratory protease/antiprotease balance determines susceptibility to viral infection and can be modified by nutritional antioxidants.* Am J Physiol Lung Cell Mol Physiol, 2015. **308**(12): p. L1189-201.
43. Pandey, K.C., S. De, and P.K. Mishra, *Role of Proteases in Chronic Obstructive Pulmonary Disease.* Front Pharmacol, 2017. **8**: p. 512.
44. Cosio, M.G., et al., *Alpha-1 Antitrypsin Deficiency: Beyond the Protease/Antiprotease Paradigm.* Ann Am Thorac Soc, 2016. **13 Suppl 4**: p. S305-10.
45. Pouwels, S.D., et al., *DAMPs activating innate and adaptive immune responses in COPD.* Mucosal Immunol, 2014. **7**(2): p. 215-26.
46. Barnes, P.J., *Cellular and molecular mechanisms of asthma and COPD.* Clin Sci (Lond), 2017. **131**(13): p. 1541-1558.
47. Stewart, J.I. and G.J. Criner, *The small airways in chronic obstructive pulmonary disease: pathology and effects on disease progression and survival.* Curr Opin Pulm Med, 2013. **19**(2): p. 109-15.
48. Sethi, S. and T.F. Murphy, *Infection in the pathogenesis and course of chronic obstructive pulmonary disease.* N Engl J Med, 2008. **359**(22): p. 2355-65.
49. Linden, D., et al., *Respiratory viral infection: a potential "missing link" in the pathogenesis of COPD.* European Respiratory Review, 2019. **28**(151): p. 180063.
50. Walsh, E.E., *Respiratory Syncytial Virus Infection: An Illness for All Ages.* Clinics in chest medicine, 2017. **38**(1): p. 29-36.
51. Liu, D., et al., *Association of Respiratory Syncytial Virus Toll-Like Receptor 3-Mediated Immune Response with COPD Exacerbation Frequency.* Inflammation, 2018. **41**(2): p. 654-666.
52. Hosseini, S.S., et al., *Association between respiratory viruses and exacerbation of COPD: a case-control study.* Infectious Diseases, 2015. **47**(8): p. 523-529.
53. Park, B.S. and J.-O. Lee, *Recognition of lipopolysaccharide pattern by TLR4 complexes.* Experimental & molecular medicine, 2013. **45**(12): p. e66-e66.
54. Ghorani, V., et al., *Experimental animal models for COPD: a methodological review.* Tobacco induced diseases, 2017. **15**: p. 25-25.
55. Wedzicha, J.A., R. Singh, and A.J. Mackay, *Acute COPD exacerbations.* Clin Chest Med, 2014. **35**(1): p. 157-63.
56. Di Marco, F., et al., *Symptom variability and control in COPD: Advantages of dual bronchodilation therapy.* Respir Med, 2017. **125**: p. 49-56.
57. Toy, E.L., et al., *Treatment of COPD: relationships between daily dosing frequency, adherence, resource use, and costs.* Respir Med, 2011. **105**(3): p. 435-41.
58. Celli, B.R., W. MacNee, and A.E.T. Force, *Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper.* Eur Respir J, 2004. **23**(6): p. 932-46.
59. Cazzola, M. and M.G. Matera, *Bronchodilators: current and future.* Clin Chest Med, 2014. **35**(1): p. 191-201.

60. Buels, K.S. and A.D. Fryer, *Muscarinic receptor antagonists: effects on pulmonary function.* Handb Exp Pharmacol, 2012(208): p. 317-41.
61. Viniol, C. and C.F. Vogelmeier, *Exacerbations of COPD.* Eur Respir Rev, 2018. **27**(147).
62. Matera, M.G. and M. Cazzola, *Muscarinic Receptor Antagonists*, in *Pharmacology and Therapeutics of Asthma and COPD*, C.P. Page and P.J. Barnes, Editors. 2017, Springer International Publishing: Cham. p. 41-62.
63. Busse, W.W., et al., *Long-acting muscarinic antagonists: a potential add-on therapy in the treatment of asthma?* European Respiratory Review, 2016. **25**(139): p. 54.
64. Bosmann, M., et al., *Anti-inflammatory effects of beta2 adrenergic receptor agonists in experimental acute lung injury.* FASEB J, 2012. **26**(5): p. 2137-44.
65. Singh, D., et al., *Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease: the GOLD science committee report 2019.* European Respiratory Journal, 2019. **53**(5): p. 1900164.
66. Johnson, M., *Molecular mechanisms of beta(2)-adrenergic receptor function, response, and regulation.* J Allergy Clin Immunol, 2006. **117**(1): p. 18-24; quiz 25.
67. Monaco, T.J. and N.A. Hanania, *Emerging inhaled long-acting beta-2 adrenoceptor agonists for the treatment of COPD.* Expert Opin Emerg Drugs, 2017. **22**(3): p. 285-299.
68. Lotvall, J., *Pharmacological similarities and differences between beta2-agonists.* Respir Med, 2001. **95 Suppl B**: p. S7-11.
69. Mutlu, G.M. and P. Factor, *Alveolar epithelial beta2-adrenergic receptors.* Am J Respir Cell Mol Biol, 2008. **38**(2): p. 127-34.
70. Reed, C.E., *Adrenergic bronchodilators: pharmacology and toxicology.* J Allergy Clin Immunol, 1985. **76**(2 Pt 2): p. 335-41.
71. Boulet, L.P., *Long- versus short-acting beta 2-agonists. Implications for drug therapy.* Drugs, 1994. **47**(2): p. 207-22.
72. Cook, D., et al., *Regular versus as-needed short-acting inhaled beta-agonist therapy for chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2001. **163**(1): p. 85-90.
73. Cazzola, M., et al., *Pharmacology and therapeutics of bronchodilators.* Pharmacol Rev, 2012. **64**(3): p. 450-504.
74. Fuso, L., et al., *Long-acting beta-agonists and their association with inhaled corticosteroids in COPD.* Curr Med Chem, 2013. **20**(12): p. 1477-95.
75. Cazzola, M., et al., *Assessing the clinical value of fast onset and sustained duration of action of long-acting bronchodilators for COPD.* Pulm Pharmacol Ther, 2015. **31**: p. 68-78.
76. Gibb, A. and L.P. Yang, *Olodaterol: first global approval.* Drugs, 2013. **73**(16): p. 1841-6.
77. Dhillon, S., *Tiotropium/Olodaterol: A Review in COPD.* Drugs, 2016. **76**(1): p. 135-46.
78. Anderson, G.P., *Current issues with beta2-adrenoceptor agonists: pharmacology and molecular and cellular mechanisms.* Clin Rev Allergy Immunol, 2006. **31**(2-3): p. 119-30.
79. Billington, C.K. and R.B. Penn, *Signaling and regulation of G protein-coupled receptors in airway smooth muscle.* Respiratory research, 2003. **4**(1): p. 2-2.
80. Lam, M., E. Lamanna, and J.E. Bourke, *Regulation of Airway Smooth Muscle Contraction in Health and Disease.* Adv Exp Med Biol, 2019. **1124**: p. 381-422.

81. Johnson, M., *Beta2-adrenoceptors: mechanisms of action of beta2-agonists.* Paediatr Respir Rev, 2001. **2**(1): p. 57-62.
82. Drummond, M.B., et al., *Inhaled corticosteroids in patients with stable chronic obstructive pulmonary disease: a systematic review and meta-analysis.* JAMA, 2008. **300**(20): p. 2407-16.
83. Eickelberg, O., et al., *Ligand-independent activation of the glucocorticoid receptor by beta2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells.* J Biol Chem, 1999. **274**(2): p. 1005-10.
84. Matera, M.G., et al., *Safety of inhaled corticosteroids for treating chronic obstructive pulmonary disease.* Expert Opin Drug Saf, 2015. **14**(4): p. 533-41.
85. Suissa, S., et al., *Inhaled corticosteroids in COPD and the risk of serious pneumonia.* Thorax, 2013. **68**(11): p. 1029-36.
86. Cazzola, M. and M.G. Matera, *The effective treatment of COPD: Anticholinergics and what else?* Drug Discovery Today: Therapeutic Strategies, 2006. **3**(3): p. 277-286.
87. Miller-Larsson, A. and O. Selroos, *Advances in asthma and COPD treatment: combination therapy with inhaled corticosteroids and long-acting beta 2-agonists.* Curr Pharm Des, 2006. **12**(25): p. 3261-79.
88. Donohue, J.F., *Combination therapy for chronic obstructive pulmonary disease: clinical aspects.* Proc Am Thorac Soc, 2005. **2**(4): p. 272-81; discussion 290-1.
89. Nadel, J.A. and P.J. Barnes, *Autonomic regulation of the airways.* Annu Rev Med, 1984. **35**: p. 451-67.
90. Gosens, R., et al., *Muscarinic receptor signaling in the pathophysiology of asthma and COPD.* Respir Res, 2006. **7**: p. 73.
91. Proskocil, B.J. and A.D. Fryer, *Beta2-agonist and anticholinergic drugs in the treatment of lung disease.* Proc Am Thorac Soc, 2005. **2**(4): p. 305-10; discussion 311-2.
92. Scott, G.D. and A.D. Fryer, *Role of parasympathetic nerves and muscarinic receptors in allergy and asthma.* Chem Immunol Allergy, 2012. **98**: p. 48-69.
93. Chung, K.F., G. Caramori, and I. Adcock, *Inhaled corticosteroids as combination therapy with β-adrenergic agonists in airways disease: present and future.* European Journal of Clinical Pharmacology, 2009. **65**(9): p. 853-871.
94. Barnes, P.J., *Scientific rationale for inhaled combination therapy with long-acting beta2-agonists and corticosteroids.* Eur Respir J, 2002. **19**(1): p. 182-91.
95. Usmani, O.S., et al., *Glucocorticoid receptor nuclear translocation in airway cells after inhaled combination therapy.* Am J Respir Crit Care Med, 2005. **172**(6): p. 704-12.
96. Haque, R., et al., *Inhaled long-acting beta2 agonists enhance glucocorticoid receptor nuclear translocation and efficacy in sputum macrophages in COPD.* J Allergy Clin Immunol, 2013. **132**(5): p. 1166-73.
97. Rider, C.F., et al., *Long-Acting beta2-Adrenoceptor Agonists Enhance Glucocorticoid Receptor (GR)-Mediated Transcription by Gene-Specific Mechanisms Rather Than Generic Effects via GR.* Mol Pharmacol, 2018. **94**(3): p. 1031-1046.
98. Altonsy, M.O., et al., *Long-acting beta2-agonists promote glucocorticoid-mediated repression of NF-kappaB by enhancing expression of the feedback regulator TNFAIP3.* Am J Physiol Lung Cell Mol Physiol, 2017. **312**(3): p. L358-L370.

99. Holden, N.S., et al., *Induction of regulator of G-protein signaling 2 expression by long-acting beta2-adrenoceptor agonists and glucocorticoids in human airway epithelial cells.* J Pharmacol Exp Ther, 2014. **348**(1): p. 12-24.
100. Wex, E., et al., *The long-acting beta2 -adrenoceptor agonist olodaterol attenuates pulmonary inflammation.* Br J Pharmacol, 2015. **172**(14): p. 3537-47.
101. Donnelly, L.E., et al., *Effects of formoterol and salmeterol on cytokine release from monocyte-derived macrophages.* Eur Respir J, 2010. **36**(1): p. 178-86.
102. Hegele, R.G., et al., *Production of acute bronchiolitis in guinea-pigs by human respiratory syncytial virus.* Eur Respir J, 1993. **6**(9): p. 1324-31.
103. Vogelmeier, C.F., et al., *Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease 2017 Report. GOLD Executive Summary.* Am J Respir Crit Care Med, 2017. **195**(5): p. 557-582.
104. Dorscheid, D.R., et al., *Apoptosis of airway epithelial cells induced by corticosteroids.* Am J Respir Crit Care Med, 2001. **164**(10 Pt 1): p. 1939-47.
105. Sharma, M., et al., *Salmeterol, a Long-Acting beta2-Adrenergic Receptor Agonist, Inhibits Macrophage Activation by Lipopolysaccharide From Porphyromonas gingivalis.* J Periodontol, 2017. **88**(7): p. 681-692.
106. Funda, D.P., et al., *CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited.* Infect Immun, 2001. **69**(6): p. 3772-81.
107. LeVan, T.D., et al., *Interaction of CD14 haplotypes and soluble CD14 on pulmonary function in agricultural workers.* Respir Res, 2017. **18**(1): p. 49.
108. Regueiro, V., et al., *Lipopolysaccharide-binding protein and CD14 are increased in the bronchoalveolar lavage fluid of smokers.* Eur Respir J, 2009. **33**(2): p. 273-81.
109. Schulz, C., et al., *Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549).* Scand J Immunol, 2002. **56**(3): p. 294-302.
110. Kolli, D., T.S. Velayutham, and A. Casola, *Host-Viral Interactions: Role of Pattern Recognition Receptors (PRRs) in Human Pneumovirus Infections.* Pathogens, 2013. **2**(2).
111. Lu, Y.C., W.C. Yeh, and P.S. Ohashi, *LPS/TLR4 signal transduction pathway.* Cytokine, 2008. **42**(2): p. 145-151.
112. Zanoni, I. and F. Granucci, *Role of CD14 in host protection against infections and in metabolism regulation.* Frontiers in cellular and infection microbiology, 2013. **3**: p. 32-32.
113. Knobloch, J., et al., *The T-Helper Cell Type 1 Immune Response to Gram-Negative Bacterial Infections Is Impaired in COPD.* American Journal of Respiratory and Critical Care Medicine, 2011. **183**(2): p. 204-214.
114. Judge, A. and I. MacLachlan, *Overcoming the innate immune response to small interfering RNA.* Hum Gene Ther, 2008. **19**(2): p. 111-24.
115. De Paula, D., M.V. Bentley, and R.I. Mahato, *Effect of iNOS and NF-kappaB gene silencing on beta-cell survival and function.* J Drug Target, 2007. **15**(5): p. 358-69.
116. Chen, Y. and R.I. Mahato, *siRNA pool targeting different sites of human hepatitis B surface antigen efficiently inhibits HBV infection.* J Drug Target, 2008. **16**(2): p. 140-8.
117. Rahman, M.M., et al., *Sphingosine 1-phosphate induces neutrophil chemoattractant IL-8: repression by steroids.* PLoS One, 2014. **9**(3): p. e92466.

# Appendices

## Appendix A : Additional BEC Subject Demographics Information

**Table 1**. Smoking designation and current medical regimen information of BEC bronchial brushing subjects.

| | Non-Smoker | Former Smoker | Current Smoker | Medications |
|---|---|---|---|---|
| **Control** | 1 | 2 | 2 | None |
| **COPD** | 1 | 2 | 2 | • LAMA/SABA/Prednisone<br>• LABA/SABA<br>• budesonide |

Three out of five COPD subjects were using respiratory medications as indicated. No control subject was currently using respiratory medications.

## Appendix B : qPCR Primer Sequences

Table 2. Forward and reverse primer sequences for qPCR.

| | Forward | Reverse |
|---|---|---|
| **β2AR** | 5'–ATGGGCACTTTCACCCTCTG–3' | 5'–GCTCCGGCAGTAGATAAG-GG–3' |
| **GAPDH** | 5'–AAGAAGGTGGTGAAGCA-GGC–3' | 5'–CGTCAAAGGTGGAGGAGTGG–3' |
| **HPRT** | 5'–TGACACTGGCAAAACAATGCA–3' | 5'–GGTCCTTTTCACCAGCAAGCT–3' |