INFLAMMATORY PROFILES OF ASTHMA AND COPD PRIMARY HUMAN BRONCHIAL EPITHELIAL CELLS IN RESPONSE TO LPS AND AN INFLAMMATORY INHIBITOR

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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Date: Fall Semester 2017
George Mason University
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Inflammatory Profiles of Asthma and COPD Primary Human Bronchial Epithelial Cells in Response to LPS and an Inflammatory Inhibitor

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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DEDICATION

This thesis is dedicated to Drs. Sharon Babcock and Carol Hurney, who first introduced me to the study of biology in 2002 when I was a freshman at James Madison University and served as role models to me throughout my undergraduate experience. They not only equipped me with the scientific skillsets necessary to excel in my professional career, but also provided me with the confidence that I could successfully pursue graduate education. Equally important, I also dedicate this to my parents for allowing me the freedom to follow my academic pursuits and for providing endless encouragement.
ACKNOWLEDGEMENTS

I would like to express my appreciation to the members of my graduate committee and to the members of ATCC senior management, especially Dr. Stephen Arold and Dr. James Kramer, who kindly granted the approval and provided the financial and logistical support for the experiments outlined in this thesis. I would like to also thank the leadership of the Cell Biology Manufacturing Science and Technology department for providing the lab space and equipment for me to perform this work. To my MSAT colleagues and team members, I thank you for reviewing my protocols and providing your expertise when needed. Above all, I am extremely grateful for the encouragement and mentorship provided by Dr. Fatah Kashanchi, who expertly guided me through the final months of my graduate career at George Mason University.
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LIST OF ABBREVIATIONS

Human Bronchial Epithelial Cells ................................................................. HBECs
Chronic Obstructive Pulmonary Disease ....................................................... COPD
Airway epithelial cells .................................................................................. AECs
Lipopolysaccharide ....................................................................................... LPS
Toll-like receptor 4 ......................................................................................... TLR4
Lipopolysaccharide binding protein ............................................................... LBP
Pseudomonas aeruginosa ............................................................................... P. aeruginosa
Escherichia coli ............................................................................................. E. coli
Enzyme-linked Immunosorbent Assay ........................................................... ELISA
Interleukin 6 ................................................................................................... IL-6
Interleukin 8 ................................................................................................... IL-8
Tumor Necrosis Factor-α .............................................................................. TNF-α
Glucocorticoids ............................................................................................. GCs
Dulbecco’s Phosphate Buffered Saline ......................................................... DPBS
Optical density ............................................................................................... OD
ABSTRACT

INFLAMMATORY PROFILES OF ASTHMA AND COPD PRIMARY HUMAN BRONCHIAL EPITHELIAL CELLS IN RESPONSE TO LPS AND AN INFLAMMATORY INHIBITOR

Heather Branscome, M.S.

George Mason University, 2017

Thesis Director: Dr. Stephen Arold

Chronic obstructive pulmonary disease (COPD) and asthma are two of the most common inflammatory diseases of the respiratory system. Collectively, they affect hundreds of millions of individuals worldwide and are associated with high levels of mortality. Treatment options are currently limited to the use of long-acting bronchodilators and glucocorticoid steroids, which act only to reduce symptoms rather than to reverse disease progression. While airway inflammation is a hallmark feature of asthma and COPD, there are inherent differences in the types of cells and molecular mechanisms involved. Previous research has highlighted the importance of the innate immune system in orchestrating the inflammatory response and unique inflammatory patterns have been identified for each of these diseases. The current thesis examined the inflammatory profiles of ATCC human primary bronchial epithelial cells (HBECs) obtained from a variety of normal and diseased donors. We have demonstrated by ELISA that these cell types can be activated by an
exogenous stimulus (LPS) to produce increased levels of IL-8 cytokine when grown in two-dimensional culture. The relative gene expression of other critical pro-inflammatory cytokines such as IL-6 and TNF-α was also examined and found to be dysregulated after exposure to LPS. Taken together, these factors may contribute to disease pathogenesis. Lastly, the anti-inflammatory effects of a glucocorticoid steroid, budesonide, were evaluated in LPS-challenged cells. Budesonide was effective in reducing IL-8 cytokine levels in five out of the six cell types tested. Ongoing research will focus on repeating these studies in three-dimensional cultures to better recreate the conditions in vivo.
INTRODUCTION

Chronic inflammation is a serious health consequence associated with many disorders and diseases of the lung. Among the most common of these diseases are asthma and chronic pulmonary obstructive disease (COPD). In 2015 an estimated 6.7% of all deaths were caused by chronic respiratory diseases, and COPD has been ranked as the fourth-leading cause of death worldwide (Barnes 2016; Dwyer-Lindgren et al. 2017). Likewise, the number of individuals affected by asthma is projected to exceed 400 million by the year 2020 (Pelaia et al. 2016). In the United States the medical costs associated with COPD are estimated to be in excess of $50 billion while the total costs associated with asthma are reported to exceed $18 billion annually (Caramori et al. 2014; Lambrecht et al. 2015). Undoubtedly, these factors place an extreme burden on the healthcare industry. To address this burden, there is an immense need to face the challenges associated with diagnosis and management of these diseases. Current treatments for asthma and COPD are mainly directed towards alleviating symptoms rather than reversing disease progression and this is compounded by the often overlapping symptoms and heterogeneous nature of each disease (Gras et al. 2013). Therefore, it is essential for researchers to more thoroughly characterize the molecular mechanisms contributing to these pathologies so that novel therapeutics can be developed.
COPD and asthma share certain clinical features and are both characterized by inflammation, hyper responsiveness, obstruction, and remodeling of the airways (Barnes 2011). In fact, it was proposed in 1961 that all airway diseases share a common genetic origin and could be jointly classified as a single disease, “chronic nonspecific lung disease” (CNSLD). This so called “Dutch hypothesis” was originally met with strong opposition and continues to cause much debate amongst clinicians and scientists who argue that common features are not indicative of common pathogenesis (Barnes 2006; Vermeire et al. 1991).

For reference, the 2016 Global Initiative for Asthma defines asthma as “a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation” (Global Initiative for Asthma 2016). According to the most recent definition provided by the Global Initiative for Chronic Obstructive Lung Disease, COPD is “a common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients” (Global Initiative for Chronic Obstructive Lung Disease 2017).

Cytokines are small molecules that act locally to regulate a variety of cellular functions. Even at low concentrations cytokines are extremely potent due to the numerous cascading events that take place to amplify their signals (Stadnyk 1994). It is well known
that cytokines play a critical role in the inflammatory process; this is achieved primarily through the recruitment and activation of a variety of inflammatory cells (Barnes 2008).

Over fifty cytokines (including lymphokines, chemokines, pro- and anti-inflammatory cytokines, and growth factors) have been identified for playing a role in the pathogenesis of chronic respiratory diseases (Barnes 2008, Barnes 2011). While it is not possible to completely elucidate their mechanisms, certain patterns of expression have been identified and attributed to certain pathologies. For example, although inflammation is a characteristic shared by COPD and asthma, there are distinct differences in the type of inflammatory cells involved and the types of mediators released in each disease (Barnes 2011). Notably, increases in the numbers of eosinophils, mast cells, and CD4$^+$ T-helper cells are mainly associated with asthma, while increases in the numbers of neutrophils, macrophages, and CD8$^+$ T-cells are commonly associated with COPD (Caramori 2003; Barnes 2004; Barnes 2011). In addition to this there are differences in the histopathology of the airways of asthmatic and COPD patients. Increases in collagen deposition, thickening of airway smooth muscle cells, and mucus hyperplasia are commonly associated with asthma while COPD is usually characterized by peribronchiolar fibrosis, destruction of alveolar walls, and increased production of elastolytic enzymes (Barnes 2008; Barnes 2011).

Among some of the most widely reported cytokines involved in the amplification of inflammation in chronic respiratory diseases are tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) (Barnes 2008). TNF-α is an extremely potent cytokine of the tumor necrosis superfamily of proteins. As most cell types express its
receptor, TNF-α exerts a wide range of effects. Within the lung a number of cell types (epithelial, monocytes, and macrophages) are able to produce TNF-α in response to inflammatory stimuli (Lundblad et al. 2005). *In vitro* studies, as well as mouse models, have indicated that prolonged production of TNF-α has a deleterious effect on various pulmonary tissues. This, in turn, may contribute to respiratory disease pathogenesis (Mukhopadhyay et al. 2006; El Shimy et al. 2014).

Along with TNF-α, IL-6 is considered to be a general marker for inflammation. IL-6 is secreted by a number of cell types, including immune cells (macrophages, neutrophils, B-cells), fibroblasts, and epithelial cells (Rincon 2012). Due to the pleiotropic inflammatory effects exerted on its target cells, IL-6 is highly regarded as a multifunction cytokine (Mills et al. 1999). Accordingly, focus has shifted away from viewing IL-6 solely as a byproduct of inflammation and more towards evaluating its role as an active contributor to asthma and COPD pathogenesis. In support this viewpoint, elevated levels of IL-6 have been detected in serum samples, induced sputum, and bronchoalveolar lavage fluid (BALF) collected from asthma and COPD patients (Rincon 2012; El Shimy et al. 2014). In addition, increases in IL-6 have been tied to acute exacerbations associated with COPD (Wedzicha et al. 2000).

Chemotactic cytokines, or chemokines, are also heavily involved in the orchestration of an inflammatory response. The chemokine IL-8 (CXCL8) is a member of the α-subgroup within the chemokine super family and is recognized as one of the most potent chemotactic cytokines released by airway epithelial cells (AECs). *In vivo*, IL-8 is synthesized in response to external stimuli and is responsible for the attraction of
neutrophils to the site of inflammation (Mills et al. 1999; Barnes 2004). Due to the purported role of neutrophils in perpetuating lung inflammation, IL-8 is of high interest in the study of chronic respiratory diseases. Analysis of cytokine expression in clinical samples of induced sputum and BALF have repeatedly shown that increased levels of IL-8 are found in cases of COPD and asthma (Pease 2002; Schultz et al. 2003). It is also worth noting that many pulmonary cell-based assays measure IL-8 as a reliable indicator of an inflammatory response.

Another key difference among COPD and asthma lies in their response to therapeutic agents. As highlighted above, there are notable differences in the nature of inflammatory cells involved in each disease, and this subsequently impacts responsiveness to treatment (Buist 2003). The current leading pharmacological agents are inhaled glucocorticoid (GC) steroids, which have demonstrated a high efficiency in reducing inflammation associated with asthma and COPD (Haghi et al 2015). GCs have long been known to inhibit many cell types (neutrophils, macrophages, lymphocytes, and eosinophils) and inflammatory mediators such as chemokines and cytokines (van der Velden 1998). Through the binding of GCs to their cytoplasmic glucocorticoid receptors (GR), the bound complex is able to translocate into the nucleus where it can exert its effect on gene transcription through the processes of both trans-activation and trans-repression (Szefler 1999; Jaffuel et al. 2000). Thus, GCs have wide-ranging effects on the inflammatory process.

Budesonide has emerged as one of the most popular and widely prescribed GCs in recent years. As compared to other inhaled GCs, budesonide has demonstrated superior
potency and, from a clinical standpoint, budesonide has been recognized for its efficacy, tolerability, and safety (Brodgen 1992; Pelaia et al. 2016). Several recent studies have reported the effectiveness of budesonide in reducing pro-inflammatory cytokine expression, specifically IL-6 and IL-8, in human bronchial epithelial cells challenged by a variety of different stimuli (Strandberg et al. 2008; Haghi et al. 2015; Heijink et al. 2014; Heijink et al. 2016). From a clinical standpoint, the patient greatly benefits from these actions and can experience an improved quality of life.

The actions of the adaptive immune system have traditionally been viewed as the main driver for the long-term inflammation associated with chronic respiratory diseases. In addition, there is mounting evidence that the innate immune system is intricately involved in this phenomenon and epithelial cells have been identified as key mediators (Holtzman et al. 2014; Lambrect 2012). Epithelial cells were originally believed to serve solely as a barrier against the environment. Indeed, the epithelial cells lining the respiratory tract serve as a physiochemical barrier from the external environment, protecting the host from a variety of allergens, bacteria, viruses, and air pollutants (Stadnyk 1994; Mills et al. 1999). However, it is now well known that AECs have the ability to secrete a wide range of cytokines, chemokines, and other pro-inflammatory mediators. Determination of the roles that these cell types play in the initiation and progression of chronic lung inflammation in the context of asthma and COPD is an active area of research that continues to expand as researchers investigate potential targets for anti-inflammatory therapeutics.
Many studies employ the use of various external stimuli, such as endotoxin, to elicit an inflammatory response in target cells. While the potential for endotoxins to induce pathophysiological reactions was pioneered in the late 1800’s, they continue to be routinely used in a wide variety of applications (Schletter et al. 1995). The endotoxin lipopolysaccharide (LPS) is present in the outer membrane of gram-negative bacteria and is known for its ability to evoke an immune response in humans. While exposure to low doses of LPS may confer immunological benefits to the host, severe infections that result in large quantities of LPS in the bloodstream can lead to septic shock (Schletter et al. 1995).

LPS can be structurally defined by its three main components: a core oligosaccharide, an O-specific polysaccharide, and a lipid A region. The toxicity associated with LPS is directly associated to the lipid A component, which is conserved among many gram-negative bacteria (Galanos 1993). In humans, the pattern recognition receptor for LPS is the Toll-like receptor 4 (TLR4). MD-2 is a small protein that associates with TLR4 and is responsible for directly binding to LPS (Ranoa et al. 2013). The sensitivity of cells expressing TLR4 is furthermore enhanced by the presence of the LPS-binding protein (LBP) and CD14. Through its association with LBP, LPS is transferred to its TLR4/MD-2 receptor complex via CD14, resulting in the initiation of a signaling cascade that results in the secretion of many pro-inflammatory molecules (Knapp et al. 2006; Regueiro et al. 2009; Ranoa et al 2013). Dentener et al. were the first to report the ability of human respiratory epithelial cells to produce LBP, demonstrating the potential for respiratory cells
to be involved in a highly specific and local response to bacterial endotoxin (Dentener et al. 2000).

The purpose of this study was to characterize, for what is believed to be the first time, the inflammatory profiles of ATCC primary human bronchial epithelial cells (HBECs) in two-dimensional cell cultures. To achieve this, both normal and diseased HBECs were challenged with LPS from different sources (*Escherichia coli* and *Pseudomonas aeruginosa*) to evaluate their relative potency and the dose-response relationship. Additionally, to demonstrate their responsiveness to a broad-spectrum anti-inflammatory agent, the same cell types were pre-treated with budesonide and then immediately challenged with LPS. IL-8 cytokine expression was chosen for evaluation in these experiments due to its significance in the inflammatory process. To take into account possible inter-patient variability, HBECs from a total of six donors, two of normal health, two with COPD, and two with asthma were utilized.

The use of human primary cells, as opposed to transformed or immortalized cell lines, is highly preferred in the context of studying complex disease pathways. Cultures of primary cells most accurately represent the phenotype and physiology of the cells *in vivo* and, thus, have the potential to yield extremely relevant scientific data. The use of diseased primary cell types in the present study also provides a unique advantage, as these cell types are extremely difficult to obtain and are still poorly understood at the molecular level. Data generated from this study will provide a direct comparison of the ability of normal and diseased HBECs to respond to pro- and anti-inflammatory agents. Additionally, it will assess the variability among different donors of the same cell type. Taken together, these
results can be used to support the relevance and applicability of ATCC primary HBECs. We ultimately hope to demonstrate the potential of these cell types to serve as high quality models that can be utilized in more advanced studies surrounding pulmonary research.
MATERIALS AND METHODS

Cell Culture and Reagents

A549

The human lung carcinoma cell line A549 (CCL-185) from the American Type Culture Collection (ATCC, Manassas, VA) was initiated and maintained in F-12K medium supplemented with 10% Fetal Bovine Serum (ATCC). Confluent cultures were washed 1× with Dulbecco’s Phosphate Buffered Saline (DPBS) and trypsinized with 0.25% Trypsin/0.53 mM EDTA solution (ATCC). Cell counts were performed using the Vi-Cell XR automated cell counting system (Beckman Coulter, Indianapolis, IN). Cells were then seeded into 12-well plates at a seeding density of 100,000 viable cells/cm² and were grown to 100% confluence for the LPS activation assay. Cultures were incubated at 37°C with 5% CO₂.

Primary Human Bronchial Epithelial Cells

Normal, asthma, and COPD primary HBECs (ATCC catalog # PCS-300-010, PCS-300-011, and PCS-300-013, respectively) were previously isolated from donor tissue and cryopreserved by the Cell Derivation Unit of the ATCC. All primary cell types referenced in this study underwent routine characterization and quality control testing to authenticate their identity prior to use. Donor specifications (age/sex) are as follows: normal donor 1: 14/male, normal donor 2: 71/female; asthma donor 1: 41/female, asthma donor 2: 34/female; COPD donor 1: 55/male, COPD donor 2: 46/female.
Primary cell cultures were initiated and maintained in Airway Epithelial Cell Basal Medium (ATCC catalog # PCS-300-030) supplemented with Bronchial Epithelial Cell Growth Kit (ATCC catalog # PCS-300-040). Subcultures were performed when cells reached approximately 80-90% confluence. Cells were washed 1× with DPBS, trypsinized with 0.05% trypsin-EDTA for primary cells, and neutralized with trypsin neutralizing solution (ATCC). Cells were spun at 200 × g for 8 minutes and resuspended in complete culture medium. Cell counts were performed using the Vi-Cell XR automated cell counting system. Cells were seeded into 12-well plates at a seeding density of 100,000 viable cells/cm² and were grown to 100% confluence for subsequent assays. All cultures were incubated at 37°C with 5% CO₂.

LPS Activation

LPS from *E. coli* O55:B5 (catalog # L5418, Sigma-Aldrich, St. Louis, MO), supplied as a 1 mg/mL ready-made sterile solution, was prepared in working solutions of 10 and 100 µg/mL) in either basal F-12K medium (for A549 cells) or non-supplemented Airway Epithelial Cell Basal Medium (for HBECs). LPS from *P. aeruginosa* 10 (catalog # L7018, Sigma-Aldrich) was solubilized in DPBS to make a master stock solution of 1 mg/mL. Aliquots were prepared and stored at -20°C. Working solutions were prepared by serially diluting the master solution in either basal F-12K medium (for A549 cells) or non-supplemented Airway Epithelial Cell Basal Medium (for primary HBECs). Each solution was sterile filtered using a 0.2 µm supor polyethersulfone (PES) membrane disc filter (Pall Corporation, Port Washington, NY).
Culture media was removed from confluent cell cultures and the cells were gently washed 1× with DPBS. Cells were then treated with the appropriate solution by adding the LPS directly to the monolayer. Non-treated control cells received basal F-12K (for A459 cells) or non-supplemented Airway Epithelial Cell Basal Medium (for HBECs). Cells incubated for 24 hours at 37°C and 5% CO₂. After 24 hours, supernatant samples (100 µL) were collected from each well and stored at -20°C.

**Budesonide Treatment**

Budesonide (catalog # 2671, R&D systems, Minneapolis, MN) was solubilized in DMSO to make a master stock solution of 20 mM. Aliquots were prepared and stored at -20°C. Working solutions of 10 µM were prepared fresh prior to use by diluting the master solution in non-supplemented Airway Epithelial Cell Basal Medium. Working solutions were sterile filtered using a 0.2 µm supor polyethersulfone (PES) membrane disc filter.

Culture media was removed from confluent cell cultures and the cells were gently washed 1× with DPBS. Cells were treated with budesonide by adding the solution directly to the monolayer. Non-treated control cells received non-supplemented Airway Epithelial Cell Basal Medium. Cultures were incubated for 2 hours at 37°C with 5% CO₂. After treatment, the budesonide solution was aspirated from the cultures and 100 µg/mL LPS solution was added to the monolayer. Budesonide control cells received non-supplemented Airway Epithelial Cell Basal Medium. Cells incubated for 24 hours at 37°C and 5%
After 24 hours supernatant samples (100 \( \mu \)L) were collected from each well and stored at -20\(^\circ\)C.

**ELISA**

**IL-8 ELISA**

Supernatants were assayed for IL-8 via Quantikine Elisa (R&D Systems, Minneapolis, MN). All reagents, buffers, and standards were prepared as outlined in the instruction handbook. Calibrator diluent RD5P (for cell culture supernates) was prepared in a 1:5 dilution by adding 20 mL of Calibrator diluent RD5P concentrate to 80 mL of deionized water. Human IL-8 standard was reconstituted with 5 mL of Calibrator Diluent RD5P (diluted 1:5). Cell culture supernatants were thawed on ice and then spun at 200 \( \times \) g for 5 minutes to pellet any residual cells. No samples were diluted for this assay and all experimental samples were run in triplicate. One hundred microliters of assay diluent and 50 \( \mu \)L of either standard or undiluted sample were added to each well and allowed to incubate at room temperature for 2 hours. All wells were washed 4\( \times \) with wash buffer. One hundred microliters of human IL-8 conjugate were added to each well and incubated at room temperature for 1 hour. All wells were washed 4\( \times \) with wash buffer again. Two hundred microliters substrate solution were added to each well and incubated at room temperature, protected from light, for 1 hour. Fifty microliters of stop solution were added to each well. Optical density (OD) was measured at 450 nm by the Victor X5 light plate reader (Perkin Elmer, Waltham, MA).

The average zero standard OD was subtracted from each sample to obtain the corrected absorbance values. A standard curve was created by plotting the mean
absorbance of each standard against the known protein concentration using Microsoft Excel software. The concentration of IL-8 for each experimental samples was then calculated.

*Multi-Analyte ELISA*

Supernatants were simultaneously assayed for 12 pro-inflammatory cytokines via Multi-Analyte ELISArray (Qiagen, Germantown, MD). All reagents and buffers were prepared as outlined in the manual. Sample Dilution Buffer 1 was prepared by diluting 2 mL of 10% BSA into 18 mL of Sample Dilution Buffer Stock. The Antigen Standard Cocktail was generated immediately prior to use by preparing a Concentrated Antigen Standard Cocktail containing 10 μL of each of the 12 Antigen Standards in 880 μL of Sample Dilution Buffer. Two hundred microliters of the Concentrated Antigen Standard Cocktail was further diluted into 800 μL of Sample Dilution Buffer to yield the final Antigen Standard Cocktail. Cell culture supernatants were thawed on ice and spun at 200 × g for 5 minutes to pellet any residual cells. No samples were diluted prior to this assay. Fifty microliters of Assay Buffer were added to each well. Fifty microliters of Sample Dilution Buffer 1 (negative control), 50 μL of test sample, and 50 μL of the final Antigen Standard Cocktail (positive control) were added to appropriate wells and the plate was incubated at room temperature for 2 hours. Detection Antibodies were thawed on ice for 30 minutes and diluted by adding 855 μL Assay Buffer to each tube. The plate was washed 3× with wash buffer. One hundred microliters of diluted Detection Antibodies were added to the appropriate wells and incubated at room temperature for 1 hour. The Avidin-HRP
Conjugate was thawed on ice for 20 minutes. Eleven milliliters of Avidin-HRP Conjugate was added to 11 mL of Assay Buffer. The plate was washed 3× with wash buffer. One hundred microliters of dilute Avidin-HRP were added to each well and the plate was incubated at room temperature, protected from light, for 30 minutes. The plate was washed 4× with wash buffer. One hundred microliters of Development Solution were added to each well and the plate was incubated at room temperature, protected from light, for 15 minutes. One hundred microliters of Stop Solution were added to each well. OD was read at 450 nm by the Victor X5 plate reader. The average OD of the negative control was subtracted from each experimental sample to obtain the corrected absorbance values.

RNA Extraction, cDNA synthesis, and Quantitative Real-Time PCR

RNA extraction was performed using the Aurum Total RNA Mini Kit (BioRad catalog # 7326820; Hercules, CA). Prior to extraction, 500 μL β-mercaptoethanol (Gibco catalog #21985023; Waltham, MA) were added to the lysis buffer stock solution to achieve a final concentration of 1%. All centrifugation steps were performed at 12,000 × g. Cells were lysed by adding 350 μL of lysis buffer (guanidinium isothiocyanate + β-mercaptoethanol) to each tube and gently mixing. An equal volume of 70% EtOH was added and each sample was transferred to a RNA binding column (within a 2 mL centrifuge tube) and spun for 30 seconds. The filtrates were discarded and 700 μL of low stringency wash solution was added to each tube and spun for 30 seconds. Filtrates were discarded and 80 μL of DNase solution was added per tube and allowed to incubate at room temperature for 15 minutes. Tubes were spun for 30 seconds, filtrates were discarded, 700
µL high stringency wash solution was added per tube, followed by another 30 second spin. Filtrates were discarded, and 700 µL low stringency solution was added per tube and spun for 1 minute. Filtrates were discarded and tubes were spun for an additional 2 minutes. The RNA binding columns were transferred to 1.5 mL centrifuge tubes, then 40 µL of elution buffer were added to each membrane and allowed to sit for 1 minute. Tubes were spun for 2 minutes to elute total RNA. Samples were kept on ice and immediately measured with the Nanodrop 2000 (Thermo Scientific; Rockville, MD) to assess concentration and purity. Total RNA was stored at -80°C.

For cDNA synthesis, the High-Capacity cDNA Reverse Transcription Kit was used (Thermo Scientific catalog # 4368814). Reverse transcription master mix was prepared following the manufacturer’s instructions and 10 µL master mix was added per tube. RNA input was normalized to 1 µg total RNA per reaction. Ten microliters of normalized RNA (diluted with the appropriate volume of molecular grade water) was added per tube and reverse transcription was performed on a Veriti thermal cycler (Thermo Scientific). The conditions of the thermal cycling program were as follows: samples were incubated at 25°C for 10 minutes, heated to 37°C for 120 minutes, then heated to 85°C for 5 minutes followed by a hold at 4°C. The cDNA was stored at -20°C.

For quantitative analysis of gene expression, commercially available TaqMan Fast Array Plates (96-well) were customized (Thermo Scientific catalog # 4413263). These plates were configured and shipped with pre-designed primer/probe sets specific for 8 target human genes: 18s rRNA, CXCL-8, IL-6, IL-1α, IL-1β, TNFα, TLR-4, and IL-2. A plate layout is shown in Table 1.
The specific assay IDs and catalog #’s for the primer/probe sets are listed in Table 2.

Table 2: Vendor information for custom qPCR primer/probe sets.

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s rRNA</td>
<td>4331182 Hs99999901_s1</td>
</tr>
<tr>
<td>CXCL8</td>
<td>4331182 Hs00174103_m1</td>
</tr>
<tr>
<td>IL-6</td>
<td>4331182 Hs00174131_m1</td>
</tr>
<tr>
<td>IL1-β</td>
<td>4331182 Hs01555410_m1</td>
</tr>
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<td>IL1-α</td>
<td>4331182 Hs00174092_m1</td>
</tr>
<tr>
<td>TLR4</td>
<td>4331182 Hs00152939_m1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4331182 Hs00174128_m1</td>
</tr>
<tr>
<td>IL-2</td>
<td>4331182 Hs00174114_m1</td>
</tr>
</tbody>
</table>

Primer efficiencies, as determined by the vendor, were specified to be 100% ± 10%.

Additionally, all probes for target genes were designed to span exon-exon junctions to ensure that fluorescence was only generated from properly spliced templates.

cDNA inputs were 50 ng per reaction. Five microliters of TaqMan Gene Expression Master Mix (Thermo Scientific catalog # 4369016), 1 µL of cDNA, and 4 µL of molecular grade water was added to each well for a final reaction volume of 10 µL.
Each sample was run in triplicate. qPCR reactions were performed using the BioRad CFX 96 Real-Time System. The conditions of the thermal cycling program were as follows: 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The ramp rate between each step of the protocol was adjusted to 1.6°C per second, as recommended by the vendor. Relative gene expression among non-treated control cells and treated cells was determined using the $2^{-\Delta\Delta CT}$ (Livak) method. Use of this method was justified based on the previously referenced amplification efficiencies of the commercially purchased primers.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism software. Levels of cytokines were calculated from 3 replicates per each condition tested. One-way analysis of variance (ANOVA) with multiple comparisons was used to analyze all experimental data. Use of one-way ANOVA was justified under the assumption that the experimental data approximated a normal distribution. $P$ values of less than 0.05 were considered statistically significant. Results are presented as mean plus or minus standard deviation.
RESULTS

Dose-response Expression of IL-8 Cytokine Expression in A549 Cells

Preliminary experiments to examine IL-8 cytokine expression were performed using the human lung carcinoma cell line A549. The purpose of these experiments was to establish proof of concept for the LPS activation assay prior to initiating experiments in primary HBECs, since it is well-documented that A549 cells can be activated by LPS to produce IL-8 (Shulz et al. 2002; Guillot et al. 2004; Grandel et al. 2009). To achieve this, increasing concentrations of LPS from both *E. coli* and *P. aeruginosa* were added to confluent cultures and supernatant samples were collected 24 hours post-treatment. As shown in Figure 1, IL-8 was detected at low levels in non-treated control cells and there was a dose-dependent increase in IL-8 production after exposure to LPS from each source. **Fig. 1A** shows the levels of IL-8 released in response to LPS from *E. coli* and **Fig. 1B** shows the levels of IL-8 released in response to LPS from *P. aeruginosa*. Each stock of LPS, at a concentration of 100 µg/mL was able to induce a significant increase in IL-8 production as compared to non-treated control cells. Overall, A549 cells demonstrated the strongest response to LPS from *P. aeruginosa*. At concentrations of 10 µg/mL and 100 µg/mL, the levels of IL-8 produced in response to LPS from *P. aeruginosa* were approximately 3.7 and 3.2 times higher, respectively, when compared to LPS from *E. coli*. 
Figure 1. The effect of LPS on IL-8 cytokine secretion in A549 cells. Cells were seeded into 12-well plates and grown to confluence. Media was removed and cells were treated with increasing concentrations of LPS from both *E. coli* and *P. aeruginosa*. Non-treated control cells only received basal media. Supernatant samples were collected after the 24 hour incubation and assayed for IL-8 by ELISA. Levels of IL-8 released in response to LPS from *E. coli* (A) and levels of IL-8 released in A549 cells in response to LPS from *P. aeruginosa* (B) are shown. Data represents mean ± standard deviation of each condition performed in triplicate. **P < 0.01 as compared to non-treated, ***P < 0.001 as compared to non-treated.

The gross appearance of the cells was microscopically examined post-treatment to assess whether or not there were any visible toxicities associated with exposure to LPS. Representative photographs of each treatment condition were taken to serve as a visual reference. As seen in Figure 2A and 2B, A549 cells maintained their adherent properties and there was no obvious change to the characteristic epithelial-like morphology of LPS treated cells as compared to non-treated control cells after 24 hours.
Figure 2. Images of A549 cells after 24 hour treatment with LPS. Cells were observed and photographed (100×) with a Nikon Eclipse TS100 inverted microscope after 24 hours of incubation in the presence of LPS from either E. coli (A) or P. aeruginosa (B). Photographs are representative of each treatment condition.

Dose-response Expression of IL-8 Cytokine Expression in Primary HBECs

The next set of experiments aimed to reproduce the A549 LPS activation assay using ATCC primary HBECs (normal and COPD). Concentrations of 10 and 100 µg/mL of LPS from both E. coli and P. aeruginosa were chosen based on their performance in the A549 assay. Low-passage normal and COPD HBECs were grown to confluence and challenged by the addition of LPS to the monolayers. Supernatant samples were collected after 24 hours of incubation. As shown in Figure 3, low levels of IL-8 were detected in non-treated control cells and the amount of IL-8 increased as LPS concentration increased in each cell type. Data in Fig. 3A shows the amount of IL-8 secreted by normal HBECs and Fig. 3B indicates the amount of IL-8 secreted by COPD HBECs. As compared to each cell type, a concentration of 100 µg/mL LPS from P. aeruginosa resulted in the highest levels of IL-8 production. There was approximately a 35-fold increase and a 22-fold
increase in IL-8 produced by donor 1 and 2, respectively, when compared to non-treated control cells. With respect to the source of LPS, exposure to LPS from \textit{P. aeruginosa} resulted in higher production of IL-8 in each cell type. The amount of IL-8 released from COPD cells exposed to 100 µg/mL LPS from \textit{P. aeruginosa} was 4.9 times greater than the amount of IL-8 released from the same concentration of LPS from \textit{E. coli}. In normal cells, there was a 3.9 times more IL-8 released in response to 100 µg/mL \textit{P. aeruginosa} when compared to the same concentration of LPS from \textit{E. coli}.

Figure 3. The effect of LPS on IL-8 cytokine secretion in primary HBECs. Cells were seeded into 12-well plates and grown to confluence. Media was removed and cells were treated with either 10 or 100 µg/mL LPS from both \textit{E. coli} and \textit{P. aeruginosa}. Non-treated control cells received basal media only. Supernatant samples were collected after the 24 hour incubation and assayed for IL-8 by ELISA. Levels of IL-8 released in response to LPS from both sources in normal cells (A) and COPD cells (B) are shown. Data represents mean ± standard deviation of each condition performed in triplicate. *p < 0.05 as compared to non-treated, **p < 0.01 as compared to non-treated, ***p <0.001 as compared to non-treated.
Again, the gross appearance of the cells was microscopically examined post-treatment to assess whether or not there were any visible toxicities associated with exposure to LPS. Representative photographs of each treatment condition were taken to serve as a visual reference. Both normal (Figure 4) and COPD (Figure 5) cells maintained their adherent properties and there was no obvious change to the characteristic epithelial-like morphology of LPS treated cells as compared to non-treated control cells after 24 hours.

Figure 4. Images of normal HBECs after 24 hour treatment with LPS. Cells were observed and photographed (100×) with a Nikon Eclipse TS100 inverted microscope after 24 hours of incubation in the presence of LPS from either E. coli (A) or P. aeruginosa (B). Photographs are representative of each treatment condition.
Figure 5. Images of COPD HBECs after 24 hour treatment with LPS. Cells were observed and photographed (100×) with a Zeiss inverted microscope after 24 hours of incubation in the presence of LPS from either *E. coli* (A) or *P. aeruginosa* (B). Photographs are representative of each treatment condition.

The Effect of Budesonide on IL-8 Levels in LPS-challenged Normal HBECs

The effect of an anti-inflammatory agent on IL-8 cytokine levels was next examined in primary HBECs. For these experiments, the glucocorticoid budesonide was selected for use based on its wide-ranging abilities to inhibit inflammation in a variety of cell types (Szefler et al. 1999). A fixed concentration of 10 µM budesonide was used because it was comparable to concentrations that have been used in similar experiments (Sabatini et al. 2002; Strandberg et al. 2008; von Scheele et al. 2010; Haghi et al. 2015). Cells were pre-treated with budesonide and immediately challenged by the addition of LPS.
from *P. aeruginosa* at a fixed concentration of 100 µg/mL. The source and concentration of LPS were selected according to the results of previous experiments.

Low passage normal HBECs from 2 different donors were grown to confluence and pre-treated with budesonide for two hours. After budesonide treatment cells were then challenged with LPS for a period of 24 hours. Non-treated control cells received basal medium only; budesonide control cells received basal medium (no LPS) after pre-treatment with drug. Supernatant samples were collected after 24 hours and assayed for IL-8 by ELISA. Data in Figure 6 shows the levels of IL-8 cytokine detected in normal cells from donor 1 (Fig. 6A) and donor 2 (Fig. 6B). Low levels of IL-8 were detected in non-treated control cells (donor 1 = 170 pg/mL, donor 2 = 210 pg/mL). Treatment with LPS alone induced IL-8 secretion with fold increases of 3.9 and 4.8 in donor 1 and 2, respectively, compared to non-treated control cells. Cells that received budesonide only had no detectable levels of IL-8 after the 24 hour incubation. In donor 1, pre-treatment with budesonide followed by LPS challenge resulted in 3.8-fold decrease in IL-8 production as compared to cells that received only LPS. In donor 2, a 2.4-fold decrease was observed between these conditions.
The Effect of Budesonide on IL-8 Levels in LPS-challenged COPD HBECs

Low passage COPD HBECs from 2 different donors were grown to confluence and pre-treated with budesonide for two hours. After budesonide treatment cells were then challenged with LPS for a period of 24 hours. Non-treated control cells received basal medium only; budesonide control cells received basal medium (no LPS) after pre-treatment with drug. Supernatant samples were collected after 24 hours and assayed for IL-8 by ELISA. Figure 7 shows the levels of IL-8 cytokine detected in COPD cells from donor 1 (Fig. 7A) and donor 2 (Fig. 7B). Low levels of IL-8 were detected in non-treated control cells (donor 1 = 156 pg/mL, donor 2 = 105 pg/mL). Treatment with LPS alone induced IL-8 secretion with fold increases of 2.3 and 4.8 in donor 1 and 2, respectively, compared to non-treated control cells. Cells that received budesonide only had no detectable levels
of IL-8 after the 24 hour incubation. In donor 1, pre-treatment with budesonide followed by LPS challenge resulted in 4.0-fold decrease in IL-8 production as compared to cells that received only LPS. In donor 2, a 2.4-fold decrease was observed between these conditions.

Figure 7. The effect of budesonide on IL-8 secretion in LPS-challenged COPD HBECs. Cells were seeded into 12-well plates and grown to confluence. Media was removed and cells were pre-treated with budesonide for 2 hours. After treatment, cells were challenged by the addition of LPS from *P. aeruginosa* for 24 hours. Non-treated cells received only basal media. Budesonide control cells received basal media after budesonide treatment. Supernatant samples were collected after the 24 hour incubation and assayed for IL-8 by ELISA. Levels of IL-8 cytokine from donor 1 (A) and donor 2 (B) are shown. Data represents mean ± standard deviation of each condition performed in triplicate. * P < 0.05 as compared to non-treated (for LPS 100 µg/mL) and as compared to LPS 100 µg/mL (for budesonide + LPS), **P < 0.01 as compared to non-treated, ***P < 0.001 as compared to LPS 100 µg/mL.

The Effect of Budesonide on IL-8 Levels in LPS-challenged Asthma HBECs

Low passage asthma HBECs from 2 different donors were grown to confluence and pre-treated with budesonide for two hours. After budesonide treatment cells were then challenged with LPS for a period of 24 hours. Non-treated control cells received basal medium only; budesonide control cells received basal medium (no LPS) after pre-treatment.
with drug. Supernatant samples were collected after 24 hours and assayed for IL-8 by ELISA. Figure 8 shows the levels of IL-8 cytokine detected in COPD cells from donor 1 (Fig. 8A) and donor 2 (Fig. 8B). Low levels of IL-8 were detected in non-treated control cells (donor 1 = 78 pg/mL, donor 2 = 36 pg/mL). Treatment with LPS alone induced a significant increase in IL-8 secretion in donor 1; there was a 26.8-fold increase as compared to non-treated control cells. In donor 2, there was a 7.8-fold increase of IL-8 in response to LPS as compared to non-treated control cells. Cells that received budesonide only had no detectable levels of IL-8 after the 24 hour incubation. In donor 1, pre-treatment with budesonide followed by LPS challenge resulted in a slight decrease of IL-8 production as compared to LPS treated cells. In donor 2, there were no detectable levels of IL-8 after budesonide pretreatment followed by LPS challenge.

Figure 8. The effect of budesonide on IL-8 secretion in LPS-challenged asthma HBECs. Cells were seeded into 12-well plates and grown to confluence. Media was removed and cells were pre-treated with budesonide for 2 hours. After treatment, cells were challenged by the addition of LPS from P. aeruginosa for 24 hours. Non-treated control cells received only basal media. Budesonide control cells received basal media after budesonide treatment. Supernatant samples were collected after the 24 hour incubation and assayed for IL-8 by ELISA. Levels of IL-8 cytokine from donor
1 (A) and donor 2 (B) are shown. ***P <0.001 as compared to non-treated. Data represents mean + standard deviation of each condition performed in triplicate.

The Effect of Budesonide on Cell Attachment and Morphology

The budesonide used in these experiments was reconstituted in DMSO to formulate a master stock solution at 20 mM. Working solutions were further diluted in basal media to achieve the desired final concentration of 10 µm; the amount of residual DMSO in the solutions used to pre-treat cells was 0.05%. The gross appearance of the cells was microscopically examined post-treatment to assess whether or not there were any visible toxicities associated with budesonide treatment. Photographs of each cell type were taken after treatment to serve as a visual reference. Images in Figure 9 represent the general appearance of primary HBECs from normal (Fig. 9A), COPD (Fig. 9B), and asthma (Fig. 9C) donors after 24 hours of incubation. Images included are from non-treated and budesonide treated cells. As shown, all HBECs maintained their adherent properties, with no obvious change to the characteristic epithelial-like morphology detected in budesonide-treated cells as compared to non-treated control cells.
B)

<table>
<thead>
<tr>
<th>COPD Untreated</th>
<th>COPD Bud 10 µM</th>
<th>COPD Bud 10 µM + LPS 100 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>Donor 2</td>
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The Effect of LPS on the Expression of Multiple Pro-inflammatory Cytokines in Primary HBECs

As a follow-up to the single-analyte IL-8 ELISA, a multi-analyte ELISA assays were performed using the remaining supernatant samples collected from non-treated and
LPS-challenged (P. aeruginosa 100 µg/mL) normal and COPD HBECs. These samples were simultaneously screened to analyze the levels of six different pro-inflammatory cytokines. For each analyte, the resulting OD values were compared among samples to assess the relative changes in protein level. Data in Figure 10 collectively displays the OD values generated from the multi-analyte ELISA; higher OD values corresponding to IL-8 and IL-1α are shown in Figures 10A and 10B, respectively, and lower OD values corresponding to IL-1β, IL-2, IL-6, and GMCSF are shown in Fig. 10C. In normal cells there was a visible increase in the level of each cytokine in response to LPS and this was the most apparent with IL-8 and GMSCF. In COPD cells the level of each cytokine, with the exception of IL-2, also increased in response to LPS and this was the most apparent with IL-8 and IL-1β. Overall, IL-8 displayed the most extreme increase in protein levels when comparing non-treated control cells to LPS treated cells of each cell type. With respect to non-treated cells, IL-2 displayed a distinctive increase in COPD cells relative to normal cells.

Figure 10. The effect of LPS on secretion of multiple pro-inflammatory cytokines by primary HBECs. Normal and COPD HBECs were seeded into 12-well plates and grown to confluence. Media was removed and cells were treated
with 100 µg/mL LPS from *P. aeruginosa*. Non-treated cells received only basal media. Supernatant samples were collected after the 24 hour treatment and assayed by multi-analyte ELISA. Levels of IL-8 (A), IL-1α (B) and IL-1β, IL-2, IL-6, and GMCSF (C) are displayed. Data represents single values for non-treated (normal and COPD) cells and an average of 2 independent measurements for LPS treated (normal and COPD) cells.

The Effect of LPS and Budesonide on Gene Expression in primary HBECs

To assess levels of TLR4 gene and cytokine-encoding gene expression, all cell types were harvested immediately following the 24 hour treatment period. RNA was extracted and then converted to cDNA for subsequent qPCR. Pre-validated, commercially available TaqMan gene expression arrays were customized for these assays. The average Cq values for each set of samples were normalized to the corresponding average Cq values of the housekeeping gene (18s rRNA) to yield the ΔCq. Then the ΔΔCq values were calculated by normalizing each treatment sample to the non-treated sample for each target gene. The relative fold change of each treatment compared to non-treated sample was next determined by the following equation: \(2^{-\Delta\Delta Cq}\). Figure 11 shows the relative fold change in TLR4 and cytokine-encoding gene expression observed in Normal (donor 1) HBECs.
Figure 11. The effect of LPS and budesonide on select gene expression in normal HBECs. Cells were seeded into 12-well plates and grown to confluence. Media was removed and cells were pre-treated with budesonide for 2 hours. After treatment, cells were challenged by the addition of LPS from *P. aeruginosa* for 24 hours. Non-treated control cells received only basal media. Budesonide control cells received basal media after budesonide treatment. Cells were harvested after treatment and qPCR was performed. Relative gene expression of six target genes was determined by the Livak method.

The expression of TLR4 does not appear to be affected by any of the treatment conditions in normal HBECs. LPS exposure appears to increase the expression of all pro-inflammatory genes (IL-8, IL-6, IL-1β, IL-1α, and TNF-α). This is the most visible in TNF-α which displays a 5.6-fold increase as compared to non-treated control cells. The fold change in IL-8 expression is approximately 2.5 as compared to non-treated control cells, and the fold changes of IL-6, IL-1α, and IL-1β are each less than 2. The effect of budesonide on reduction of gene expression is the most apparent in TNF-α, which decreases from 7.0 in LPS-challenged cells to 2.7 in LPS-challenged cells that were pre-treated with budesonide. The relative fold changes for the rest of the pro-inflammatory genes do not appear to change drastically in response to budesonide. Normal donor 2
samples are not shown due to a physical defect in the custom qPCR plate that was to be used to assay those samples. The plate had to be returned to the vendor and a replacement plate is to be provided. At the time of publication of this thesis, the replacement plate has not yet been received.

COPD cells were next assayed for gene expression. Figure 12 shows the relative fold changes in gene expression observed in donor 1 (Fig. 12A) and donor 2 (Fig. 12B).

![Figure 12. The effect of LPS and budesonide on select gene expression in COPD HBECs.](image)

Cells were seeded into 12-well plates and grown to confluence. Media was removed and cells were pre-treated with budesonide for 2 hours. After treatment, cells were challenged by the addition of LPS from *P. aeruginosa* for 24 hours. Non-treated control cells received only basal media. Budesonide control cells received basal media after budesonide treatment. Cells were harvested after treatment and qPCR was performed. Relative gene expression of six target genes was determined by the Livak method.

The relative fold change in gene expression in LPS-treated cells as compared to non-treated control cells is less than 2 for each gene assayed in COPD donor 1. Compared to all other genes, TNF-α displays the greatest relative increase in expression in response to LPS and this is observed in both COPD donors, but to a lesser extent in donor 1 (1.8-
fold change) than in donor 2 (7.9-fold change). In donor 2, there is a 6.1-fold increase in IL-6 in response to LPS, and the relative expression of the other 5 genes also increase in smaller degrees in response to LPS. In each COPD donor, pre-treatment of LPS-challenged cells with budesonide appears to reduce the levels of expression for each gene assayed. In donor 1, the levels of gene expression are reduced to levels lower than those in non-treated control cells, with the exception of TNF-α. In donor 2, the reduction in gene expression in cells pre-treated with budesonide is the most apparent with IL-6 and TNF-α.

The relative levels of gene expression in asthma HBECs are displayed in Figure 13. Donor 1 is shown in Fig. 13A and donor 2 is shown in Fig. 13B.

**Figure 13. The effect of LPS and budesonide on select gene expression in asthma HBECs.** Cells were seeded into 12-well plates and grown to confluence. Media was removed and cells were pre-treated with budesonide for 2 hours. After treatment, cells were challenged by the addition of LPS from *P. aeruginosa* for 24 hours. Non-treated control cells received only basal media. Budesonide control cells received basal media after budesonide treatment. Cells were harvested after treatment and qPCR was performed. Relative gene expression of six target genes was determined by the Livak method.
In donor 1, both IL-8 and TNF-α display fold change values greater than 25 in response to LPS as compared to non-treated control cells. The expression of IL-6 in response to LPS is also approximately 8.4 times greater than non-treated control cells in donor 1. In donor 2, the genes with the largest relative fold change in response to LPS are IL-6 (approximately 3.5) and TNF-α (approximately 2.9). Pre-treatment of LPS-challenged cells with budesonide appears to lower the relative expression of each gene assayed in donor 1. However, the relative levels of IL-8 and TNF-α expression after pre-treatment are still 15-fold higher than in non-treated control cells. In donor 2, the relative levels of expression for each gene are increased after pre-treatment with budesonide.

For reference, IL-2 levels were not reported for any of the qPCR assays as none of the 5 independent assays yielded Cq values for IL-2.
DISCUSSION

COPD and asthma are two of the most prevalent chronic respiratory diseases; collectively, they affect hundreds of millions of individuals worldwide (Barnes 2016; Pelaia et al. 2016). While this number is already quite staggering, it is possible that it is underestimated due to under diagnoses or misdiagnoses as many COPD and asthma patients exhibit similar clinical symptoms and overlapping features (Barnes 2006; Prosser 2017). While there are treatments that can alleviate and partially reverse the symptoms associated with these diseases there are no known cures. Even after long-term treatment, many patients experience a reoccurrence of symptoms (Caramori 2003). As the mortality rate associated with chronic respiratory diseases has increased by approximately 30% in recent decades, it is imperative to more thoroughly investigate the mechanisms that are involved with their associated pathologies (Dwyer-Lindgren et al. 2017).

Inflammation plays a central role in the development and progression of COPD and asthma. The inflammatory process involves a milieu of chemokines and cytokines which are, in turn, regulated by complex signaling pathways and other molecular mediators (Barnes 2011). Both COPD and asthma are characterized by an upregulation of mediators involved in the inflammatory cascade due to the enhanced transcription of genes involved in regulating inflammation (Caramori et al. 2003; Pelaia et al. 2016). IL-8 is one of the most potent cytokines released during the inflammatory response and serves to activate and recruit neutrophils to the site of inflammation (Baggili 1992, Barnes 2011). Airway epithelial cells have long been recognized as an important source of IL-8 and other pro-
inflammatory cytokines (Standiford et al. 1990; Koyama et al. 2000; Strandberg et al. 2008). Thus, in addition to lining the airways and acting as a structural barrier against environmental allergens and pathogens, AECs are poised to play a critical role in the initiation of the inflammatory response. A proper balance and homeostasis must exist between efficient clearance of inhaled pathogens and maintaining appropriate lung function (Gomez et al 2008). Disruption of this balance can put the host at risk for developing infections and incurring damages to the epithelial cells lining the lung.

LPS is routinely used in cell-based assays due to its well-established ability to induce the expression of pro-inflammatory cytokines. In the lung, the LPS-induced inflammatory response is moderated by CD14, TLR4, and MD-2. Previous research has confirmed that AECs possess these required factors and it has been suggested that primary cells may be more susceptible to LPS activation due to up-regulation of their receptor complex (Knapp et al. 2006; Wondwossen et al. 2010). The gram-negative bacteria *P. aeruginosa* is a ubiquitous and opportunistic pathogen (Lyczak et al. 2000). Infections resulting from *P. aeruginosa* usually arise when the host’s natural defense mechanisms are compromised, such as in the case of chronic illness or injury (Yum 2014). In patients with COPD, *P. aeruginosa* has repeatedly been linked to occurrences of acute exacerbations, and the prevalence of infection increases with disease severity. Additionally, infection may persist long-term in some COPD patients and this further compromises their prognosis (Soler et al. 1998; Martinez-Solano et al. 2008; Millares et al. 2014).

For this study, LPS from *P. aeruginosa* was deemed a relevant stimulatory agent for use in the HBEC assay based upon the reasons described above. Our first aim was to
assess the potential of primary HBECs to mimic an inflammatory response that would be comparable to the *in vivo* response. IL-8 was chosen as an endpoint marker of inflammation due to its well-documented role in inflammation. Data from all donors of normal, COPD, and asthma primary HBECs showed a significant increase in IL-8 cytokine production in response to exogenous stimulation by LPS. It is worth noting that levels of IL-8 in all non-treated cell types were relatively low and there were no significant differences in basal IL-8 when comparing normal and diseased cells. qPCR data shows increases in IL-8 mRNA in response to LPS in each of the cell types and donors assayed with the exception of COPD donor 1. With respect to other pro-inflammatory cytokines, IL-6 and TNF-α also displayed the largest increases in relative gene expression in response to LPS. These results are in accordance to previous research that has linked high levels of IL-6 and TNF-α to asthma and COPD (Schultz et al. 2003; Barnes 2008; El Shimy et al. 2014s). To confirm that these cytokines are being released by the cells, supernatant samples could be analyzed via ELISA. Expression of the TLR4-encoding gene was demonstrated via qPCR in each cell type. Due to the role that TLR4 plays in recognizing pathogenic stimuli and activating an immune response in AECs, it was important to evaluate the expression of this receptor in the primary HBECs used in these studies. Although relative expression of TLR4 did not appear to drastically change in response to LPS, the overall responsiveness of the HBECs to LPS is expected to be mediated through TLR4 signaling pathways.

Collectively, the results from the LPS assay were consistent with the expected outcome and support the rationale that ATCC primary HBECs can be activated to express
pro-inflammatory cytokines. We next sought to evaluate the effect of budesonide on levels of IL-8 cytokine levels in LPS-stimulated primary HBECs. Budesonide is one of the most reliable and most potent GCs used in the management and control of chronic lung inflammation. GCs are able to suppress inflammation through several different mechanisms. At the transcriptional level, budesonide acts to increase the transcription of genes that encode anti-inflammatory proteins through the interactions of the activated GR with glucocorticoid response elements (GRE) in GC-inducible genes (Pelaia et al. 2016). Additionally, through protein-protein interactions, budesonide can act to suppress the actions of NF-κB. Since NF-κB is activated in response to inflammation, the suppression of its actions by budesonide inhibits the expression of numerous pro-inflammatory cytokines and chemokines (Jaffuel et al. 2000; Pelaia et al. 2016).

Data from both donors of normal and COPD primary HBECs and 1 donor of asthma primary HBECs (donor 2) showed a significant decrease in IL-8 cytokine production when comparing levels between LPS challenged cells and LPS challenged cells that were pre-treated with budesonide. Interestingly, budesonide was ineffective in reducing LPS-induced IL-8 cytokine expression in primary HBECs from one of the asthma donors (donor 1).

At the level of mRNA, a relative reduction in expression of several pro-inflammatory genes, including IL-8, IL-6, and TNF-α, was observed in two separate donors of LPS-challenged COPD HBECs that were pre-treated with budesonide. The relative expression of pro-inflammatory genes in LPS-challenged asthma HBECs displayed variable response when pre-treated with budesonide. One donor (asthma donor 1)
displayed a hyper-responsiveness to LPS as indicated by the significant increase in IL-8 cytokine production and the extremely high fold-increase of both IL-8 and TNF-α gene expression compared to non-treated control. Although pre-treatment with budesonide appears to slightly lower these levels, the resulting levels of cytokine and levels of gene expression are still substantially higher than the levels in non-treated control cells. Furthermore, in the second asthma donor (donor 2) an increase in pro-inflammatory gene expression was observed for five different cytokines in LPS-challenged cells that were pre-treated with budesonide. The IL-8 ELISA, however, detected no levels of IL-8 in cell culture supernatants from the same sample. One possibility to explain this result could be through an alternative mechanisms of action of budesonide in which the drug is acting at a post-transcriptional level to repress pro-inflammatory mRNA. More thorough studies are needed to evaluate this phenomenon.

These results partially confirm our expectations that budesonide can suppress inflammation by reducing the expression of pro-inflammatory cytokines in primary HBECs. With respect to the observed phenomenon in asthma donor 1, one explanation may be that the donor had developed GC-insensitivity and was therefore resistant to budesonide treatment. Although it is outside of the scope of the current study to reach this conclusion, future studies using the same donor material could be performed to further evaluate this phenomenon. A repeat qPCR on the asthma donor 2 samples could also be performed to confirm the unusual results.

Overall, we have demonstrated that LPS from P. aeruginosa amplifies the release of IL-8 from normal and diseased primary HBECs in submerged culture. This data is in
consensus with the concept that AECs can directly participate in the initiation of an immune response. Furthermore, the ability of budesonide to reverse LPS-induced IL-8 production in these cell types has also been assessed. We have found that pre-treatment with budesonide can decrease LPS-activated IL-8 production in certain cell types, lowering the levels to those comparable in non-treated cells.

While these experiments have exhibited scientific merit, additional studies are needed to confirm and further expand upon our findings. The sample size should be increased for each cell type being studied and the evaluation of multiple pro-inflammatory cytokines should also be included in future research. Due to the observed relative increases in gene expression, IL-6 and TNF-α cytokines should be evaluated via ELISA to evaluate the correlation between protein level and mRNA. Samples could also be collected at extended time points (48 hours, 72 hours) to evaluate the long term effect of LPS exposure and to determine the dose/time-response of budesonide on cytokine expression. At the conclusion of these studies, a cell-based assay, such CellTiter-Glo, could be used to assess the overall cell health and viability. For long term studies, these experiments should be repeated at the air-liquid interface. The use of three dimensional models such as these are expected to more closely resemble the in vivo phenotype and may provide more relevant data in biomedical research.

As previously stated, a better understanding of the underlying inflammatory mechanisms contributing to chronic respiratory diseases is urgently needed. Not only will this knowledge aid in improving diagnoses, but it will also help to identify which pathways could potentially be targeted by novel therapeutics. While many researchers are interested
in characterizing these mechanisms in the laboratory, one of the challenges associated with this is the difficulty of obtaining pure populations of primary cells. For this reason, many studies are performed using immortalized or transformed cell lines. These cell types are often easily accessible and do not have passage limitations, making them ideal candidates for routine laboratory use. However, the use of primary cells offers several benefits. Since primary cells are isolated directly from donor material they are expected to retain the key characteristics and functionality of their tissue of origin. Therefore, they are more physiologically relevant and have the potential to yield more reliable data that is expected to more closely resemble the *in vivo* phenotype.

ATCC has recently emerged into the market of primary cells and the experiments outlined in the current thesis were conducted on high quality, low-passage primary HBECs isolated from multiple donors of normal and diseased tissue. A search of the literature suggests that these studies are the first to characterize the inflammatory patterns of ATCC HBECs. Thus, this thesis demonstrates the potential for ATCC HBECs to be used in cell-based assays and, furthermore, to serve as models for advanced studies of chronic respiratory diseases.
REFERENCES


BIOGRAPHY

Heather Branscome earned a Bachelor of Science degree from James Madison University in 2006. She spent a year working with elementary school students before beginning her career at the American Type Culture Collection (ATCC). Throughout her career at ATCC she has worked in various roles supporting bio manufacturing and quality control testing in both cell biology and molecular biology laboratories. She is currently a Lead Biologist in the Cell Biology Manufacturing Science and Technology department where she is responsible for accessioning new cell lines into the ATCC portfolio and leading cross functional teams to support new product development and technology transfer activities. Her favorite pastime is listening to Stevie Nicks, who is her biggest musical inspiration.