The effect of cigarette smoke exposure on the expression and regulation of Toll-like receptors in chronic obstructive pulmonary disease

Jessica Nadigel

Department of Pathology
Faculty of Medicine
McGill University, Montreal
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For both my grandfathers, Ted Goldman and Nat Nadigel

I would also like to dedicate this thesis to everyone who has helped me along my journey throughout the past 5 years. I would not have been able to accomplish this without your love and support. A special thank you goes out to my parents, Lynn and Harvey and my sister, Robyn

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a slow, progressive disorder which results in a gradual loss of lung function. Cigarette smoke is a major risk factor for COPD, although biomass fuel, outdoor air pollution, bacteria and childhood respiratory viruses have all been proposed to play a role in the disease. The sustained inflammation typical of COPD is characterized by inflammatory cytokine production and the recruitment of several cell types to the lungs, most specifically $CD8^+$ T cells. These cytotoxic cells are increased in both the central and peripheral airways of COPD patients, and are normally located within the submucosa and invading the epithelium. Toll-like receptors (TLR) are transmembrane receptors which respond to foreign microbes through specific pathogen-associated molecular patterns. Once a pathogen breaches the epithelium, the first line of defense of the airways, these receptors are activated and elicit an appropriate inflammatory response. Consequently, there has been growing interest in the role of TLRs in COPD as a site for potential therapy. We hypothesized that TLR expression is increased in COPD patients compared to control subjects and that this increase is mediated by cigarette smoke exposure.

Bronchial biopsies, peripheral blood, and primary cells were used to investigate the expression and regulation of TLRs in response to cigarette smoke exposure. The bronchial biopsies from COPD patients and healthy control subjects had similar TLR4 and TLR9 expression in the submucosa as well as in the epithelial layer. Interestingly, patients with COPD had 90% of lung $CD8^+$ T cells express TLR4 and TLR9 compared to less than 20% in control subjects. In

contrast, peripheral blood $CD8^+$ T cells lacked significant TLR expression in both groups. This suggests that cigarette smoke may be causing the increased TLR expression locally in the lungs of COPD patients, as this was not observed in control subjects or peripheral blood. $CD8^+$ T cells isolated from COPD patients and exposed to cigarette smoke increased the protein expression of TLR4 and TLR9, as well as several inflammatory cytokines including, IL-1β, IL-6, TNF-α, and IFN-γ. Normal human bronchial epithelial cells (NHBE) exposed to cigarette smoke were able to produce more IL-8 than COPD-diseased human bronchial epithelial cells (DHBE) indicating a potential failure in immune function in the diseased cells. Finally, with the use of signaling pathway inhibitors, it was demonstrated that the IL-8 induction was both TLR4- and extracellular regulated kinase (ERK) 1/2- dependent in epithelial cells.

Collectively, the data of this thesis demonstrates the complex role of TLRs in COPD pathogenesis. Our study reveals that there are cell-specific mechanisms by which cigarette smoke induces TLR-dependent inflammatory cytokine production. Furthermore, it seems that TLRs play a dual role in COPD, by initiating inflammation following cigarette smoke exposure, as was seen with the epithelial cells, as well as perpetuating the inflammation in diseased patients, as was demonstrated with the $CD8^+$ T cells. This research is a first step towards understanding the complex relationship between specific lung cell types in COPD pathogenesis in response to cigarette smoking and suggests that the use of anti-TLR therapy may be beneficial in alleviating inflammation.

ABRÉGÉ

La maladie pulmonaire obstructive chronique (MPOC) est un trouble évoluant lentement et progressivement qui est caractérisé par une perte graduelle de la fonction pulmonaire. La fumée de cigarette est un facteur de risque majeur de la MPOC, mais il a été proposé que les combustibles à base de biomasse, la pollution de l'air, les bactéries, et les virus respiratoires infantiles jouent aussi un rôle dans la maladie. L'inflammation typique de la MPOC est caractérisée par la production de cytokines inflammatoires et le recrutement pulmonaire de plusieurs types de cellules, mais principalement de cellules T $CD8^+$. Ces cellules cytotoxiques augmentent en nombre dans les voies respiratoires centrales et périphériques, et sont généralement situées à l'intérieur des sous-muqueuses et de l'épithélium. Les récepteurs de type Toll (TLR) sont des récepteurs transmembranaires qui utilisent des voies moléculaires spécifiques pour répondre à des microbes. Lorsqu'un pathogène passe à travers la première ligne de défense des voies respiratoires, soit l'épithélium, ces récepteurs sont activés et provoquent une réponse inflammatoire. Le rôle des TLRs dans la MPOC suscite un intérêt grandissant, vu leur utilisation potentielle dans le traitement de la maladie. Notre hypothèse est que l'expression des TLR est plus élevée chez les patients souffrant de la MPOC que chez les sujets contrôles, et que l'exposition à la fumée de cigarette joue un rôle dans cette augmentation.

Nous avons utilisé des biopsies de bronches, du sang périphérique, et des cellules primaires pour étudier l'expression des TLRs suite à une exposition à la fumée de cigarette. Les biopsies bronchiales des patients souffrant de MPOC et

les sujets contrôles avaient une expression similaire de TLR4 and TLR9 dans les sous-muqueuses, ainsi que dans la couche épithéliale. Dans les poumons, 90% des cellules T $CD8^+$ des patients souffrant de MPOC expriment TLR4 et TLR9, comparé à 20% chez les contrôles. Par contre, dans le sang périphérique, les cellules T $CD8^+$ n'expriment pas les TLRs de façon significative, et ce dans les deux groupes. Ceci suggère que la fumée de cigarette pourrait être la cause de l'augmentation des TLR spécifiquement dans les poumons de patients souffrant de la MPOC, puisque cette augmentation n'a pas été observée chez les contrôles, ni dans le sang périphérique. Les cellules T $CD8^+$ isolées de patients souffrant de la MPOC et exposées à de la fumée de cigarette augmentent non seulement l'expression de TLR4 et TLR9, mais aussi l'expression de diverses cytokines inflammatoires telles que IL-1β, IL-6, TNF-α, et IFN-γ. Suite à une exposition à la fumée de cigarette, les cellules épithéliales bronchiales humaines normales (EBHN) ont été capable de produire plus d'IL-8 que les cellules épithéliales bronchiales humaines malades de MPOC (EBHM), ce qui indique un échec potentiel de la fonction immunitaire des cellules malades. En dernier lieu, avec l'aide d'inhibiteurs de voies métaboliques, nous avons montré que l'induction d'IL-8 est dépendante de TLR4 et de la kinase régulée de façon extracellulaire (ERK) 1/2 dans les cellules épithéliales.

Mises ensemble, les données de cette thèse montrent un rôle complexe des TLRs dans la MPOC. Notre étude révèle qu'il existe des mécanismes spécifiques à certaines cellules par lesquels la fumée de cigarette induit une production de cytokines inflammatoires en passant par les TLRs. Il semble d'ailleurs que les TLRs jouent un double rôle dans la MPOC, en initiant l'inflammation suite à

l'exposition à la fumée de cigarette, tel que montré avec les cellules EBHN, et en perpétuant l'inflammation chez les patients malades, tel que montré avec les cellules T $CD8^+$. Cette recherche est un premier pas vers la compréhension de la relation entre les différents types cellulaires pulmonaires dans la MPOC en réponse à la fumée de cigarette, et suggère que l'utilisation d'un traitement anti-TLR pourrait être bénéfique pour diminuer l'inflammation.

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CONTRIBUTIONS OF AUTHORS

Chapter 2: Cigarette smoke increases TLR4 and TLR9 expression and induces cytokine production from $CD8^+$ T cells in chronic obstructive pulmonary disease

Jessica Nadigel, David Préfontaine, Carolyn J. Baglole, Jean Bourbeau, François Maltais, David H. Eidelman, and Qutayba Hamid

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J. Nadigel carried out almost all of the experiments and drafted the manuscript. D. Préfontaine helped with the experiments and participated in the design of the study. J. Bourbeau and F. Maltais participated in the sample collection. C. Baglole and D. Eidelman participated in the design of the study and helped to draft the manuscript. Q. Hamid conceived the study. All authors read and approved the final manuscript.

Chapter 3: Cigarette smoke induces IL-8 production from epithelial cells without increasing TLR4 protein

Jessica Nadigel, Séverine Audusseau, Carolyn J. Baglole, David H. Eidelman, and Qutayba Hamid

J. Nadigel performed almost all of the experiments, carried out all of the analyses, as well as drafted the manuscript. S. Audusseau helped with some of the ELISA experiments. C. Baglole and D. Eidelman provided insight into the design of the project. Q. Hamid oversaw the development of the entire project.

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ABBREVIATIONS

A1AT	alpha-1 antitrypsin
APC	Antigen-presenting cell
BALF	Bronchoalveolar lavage fluid
BEBM	Bronchial epithelial basal medium
BEGM	Bronchial epithelial growth medium
cDNA	Complementary deoxyribonucleic acid
CIHR	Canadian Institutes of Health Research
COPD	Chronic obstructive pulmonary disease
CSC	Cigarette smoke condensate
DAMP	Danger-associated molecular patterns
DHBE	COPD-diseased human bronchial epithelial
DMSO	Dimethyl sulfoxide
dsRNA	Double-stranded ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EOMES	Eomesodermin
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FEV_1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate

FRSQ	Fonds de la recherche en santé du Québec
FVC	Forced vital capacity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOLD	Global initiative for chronic obstructive lung disease
GRO-α	Growth related oncogene alpha
H_2O_2	Hydrogen peroxide
hToll	Human homologue of Toll
IκB	Inhibitor of NF-κB
IFN-γ	Interferon gamma
IKK	IκB kinase
IL	Interleukin
IL-1R	Interleukin receptor 1
IRAK	IL-1R-associated kinase
IRF	Interferon-regulatory factor
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MKK6	MAPK kinase 6
MMP	Matrix metalloproteases
mRNA	Messenger ribonucleic acid
MSD	Meso Scale Discovery

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUHC	McGill University Health Centre
MyD88	Myeloid differentiation primary response gene 88
NHBE	Normal human bronchial epithelial
NF-κB	Nuclear factor kappa B
O_2^-	Superoxide
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
$PM_{2.5}$	Particles with a diameter of 2.5 microns
PM_{10}	Particles with a diameter of 10 microns
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SEM	Standard error of the mean
STAT4	Signal transducer and activator of transcription 4
TAB	TAK1-binding protein
TAK1	TGF-β-activated kinase 1
T-bet	T-box expressing T cells
Tc1	T cytotoxic type 1 cell
TCR	αβ T-cell receptor

TEER	Trans-epithelial electric resistance
TGF-β	Transforming growth factor beta
Th	T helper
TIMP	Tissue inhibitors of matrix metalloproteases
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TPM	Total particulate matter
TRAF6	TNF receptor-associated factor 6
TRIF	TIR-domain-containing adaptor protein inducing IFN-β

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CHAPTER 1: INTRODUCTION

1.1 Chronic obstructive pulmonary disease (COPD)

1.1.1 COPD definition

Chronic obstructive disease (COPD) is defined by the American Thoracic Society to be a preventable disease state characterized by an airflow limitation that is only partially reversible [1]. The airflow limitation is progressive and is associated with an abnormal inflammatory response of the lung cells to noxious particles or gases, with cigarette smoke being the primary cause of the disease. The term COPD encompasses two main disease states; chronic bronchitis, which is characterized by the obstruction of the small airways, and emphysema, which is characterized by the destruction of the lung parenchyma and enlargement of air spaces due to loss of elasticity. Although COPD is considered a chronic inflammatory lung disease, systemic consequences have also been reported [1].

In 1977, Fletcher et al. published an 8-year prospective study looking at the difference in the forced expiratory volume in 1 second (FEV_1) of working men in London. They compared the FEV_1 of smokers to non-smokers [2]. It was revealed that the average decline in FEV_1 is faster in a subset of smokers (60 mL/year) compared to the non-smokers (30 mL/year). This groundbreaking study was the first to confirm the role of cigarette smoke in the development of COPD, as well as expose the need to test the FEV_1 to properly diagnose the disease.

COPD severity is based on a five-stage classification system using airflow measurements. The stages are determined by the FEV_1, as well as the ratio of FEV_1 to FVC (forced vital capacity) [3]. As the FEV_1 of a patient decreases, the

severity, (and stage), of COPD increases, reflecting a reduction in the capability to exhale air from the lung due to increased lung destruction.

1.1.2 COPD history

COPD was first used as an umbrella term and referred to both chronic bronchitis and emphysema. Over half a century ago, it was revealed that these lung diseases had similar characteristics to asthma, a well-understood chronic inflammatory disease. It was noted that in at least some patients, these entities appear to overlap, fuelling speculation that asthma, chronic bronchitis, and emphysema share a common foundation. In 1961, at the Bronchitis Symposium held in the Netherlands, the so-called Dutch hypothesis was born [4, 5]. Emphasizing the similarities, Orie and colleagues proposed that asthma, chronic bronchitis, and emphysema be considered as part of a single disease entity grouped under the umbrella term "chronic nonspecific lung disease" [4]. In response, a competing British hypothesis emerged which, argued that the mechanisms involved in asthma were different than those underlying both chronic bronchitis and emphysema, and they should therefore not be classified together under the same disease [5].

Despite the similarities in presentation, important differences between the two diseases become more apparent when delving further into their underlying mechanisms. Arguably the most important difference is that in asthma, airway obstruction is reversible and there is evidence of airway hyperresponsiveness, the tendency of the airways to respond to a broad array of stimuli by excessive airway narrowing. Asthma tends to begin early in life and can be triggered by numerous

factors depending on the phenotype of the disease [6], with allergens being the most common.

In contrast, in COPD, airflow obstruction is only partially reversible, and sometimes even fixed. Moreover, it takes many years before COPD can manifest itself, with the majority of patients being over 50 years of age at diagnosis [2]. It is therefore a progressive disorder that mainly affects both the peripheral airways and the parenchyma. Cigarette smoking is the primary risk factor for COPD with other potential factors being viral and bacterial respiratory infections, outdoor and indoor air pollution, and occupational exposure to dust and chemicals [1].

1.1.3 COPD epidemiology

The World Health Organization estimates that currently 200 million people suffer from moderate to severe COPD. In 2005, more than 3 million individuals died of this disease, which corresponds to approximately 5% of all deaths worldwide. As of 2001, COPD was the fifth leading cause of mortality worldwide and is thought to move into the third position by 2020. The reason for the increase in burden of this disease is thought to be due to the ageing of the world's population along with the continued use of tobacco products and biomass combustion fumes [7].

Determining the incidence rate of this disease has proven to be rather difficult. In industrialized countries, there are informative studies which can be used to determine the prevalence of COPD. The problem arises in developing countries, where it is difficult to estimate the prevalence due to the lack of knowledge and understanding of COPD. In addition, they are also missing the

necessary tools, such as spirometers, which are needed to properly diagnose the disease [8]. In order to correctly asses the global prevalence of COPD, an international study, The Burden of Obstructive Lung Disease (BOLD), was established. This study comprised 12 test sites from around the world and used a standardized questionnaire and lung function tests to try to properly diagnose COPD in adults aged 40 years or more [9]. It was revealed that on average, the prevalence of patients with mild to severe COPD is 10.1 ± 4.8%; with 11.8 ± 7.9% for males and 8.5 ± 5.8% for females. There was heterogeneity among the participating countries, with the Western Pacific region having the highest incidence of the disease (12.5%), while African and certain Asian countries had low incidence, approximately 6.3% [10].

COPD is known to occur mainly in adults as it takes many years to develop [2]. A study was performed in the United Kingdom to investigate whether or not COPD prevalence was increasing, and if this was occurring in both males and females. It was observed that while the prevalence among men had reached a plateau, the number of women being diagnosed with COPD was steadily on the rise [11]. Although the frequency of COPD is thought to occur in twice as many males as females, this study showed that the prevalence rates in women are now increasing and may become equal to men. This is most likely due to the fact that the 1980s and 1990s there was a rise in the prevalence of smoking among women in developed countries. Concurrently, in developing countries, there are longer life expectancies, resulting in more women being diagnosed with COPD [10].

1.2 Contributing factors of COPD

COPD is caused by an abnormal response of the lungs to inhaled toxins, particularly cigarette smoke [1]. Although, it should also be noted that genetics has been proposed to be involved as well as only certain smokers will eventually develop COPD [12]. Normally, the innate and adaptive immune systems can elicit an inflammatory response against said pathogens. However, for reasons that still remain unknown, certain individuals cannot properly rid these pathogens from their lungs, and the inflammation goes far beyond normal protection, and ultimately leads to the development of COPD. The pathological changes which occur in COPD result in an increased resistance to airflow within the airways.

1.2.1 Cigarette smoke exposure

Cigarette smoke is a complex substance made up of more than 4,500 components in its gaseous and particulate phases [13]. The various compounds which make up cigarette smoke can all act on the immune system [14]. Studies have shown that cigarette smoke can cause inflammation within the central airways, peripheral airways, as well as the parenchyma [15]. Only 15-20% of smokers develop airway inflammation and damage which progresses to COPD [16], and unfortunately, it is still unknown as to why certain individuals develop the disease, while the majority of smokers do not. Perhaps this discrepancy may relate to differences in TLR expression and subsequent effects of cytokine release.

The epithelium, which lines the airways, is the first line of defense against cigarette smoke. Cigarette smoke can cause direct damage to the epithelium by impairing mucociliary clearance and destroying the epithelial barrier by

increasing the permeability of the cells [17-19]. Epithelial cells produce inflammatory mediators in response to cigarette smoke, such as interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1 [20, 21]. IL-8, a neutrophil chemoattractant, and MCP-1, a monocyte chemoattractant, can then induce the migration of neutrophils and monocytes to the lung. Cigarette smoke-induced inflammation can also lead to increased dendritic cells and T lymphocytes within the lungs. Together with other inflammatory and structural cells, these cells secrete proinflammatory mediators, reactive oxygen species (ROS), and proteolytic enzymes [14], which cause lung destruction and further propagate the inflammation.

Cigarette smoke-induced inflammation has also been linked to Toll-like receptor (TLR) activation, with most of the research focused on TLR4 and TLR9. In 2006, Karimi et al. demonstrated for the first time that inflammation resulting from cigarette smoke exposure was TLR4-dependent. They showed that macrophages exposed to cigarette smoke could produce high levels of IL-8, and that this production was abrogated when a TLR4 antibody was added prior to cigarette smoke exposure [22]. An additional study by the same group revealed that macrophages exposed to cigarette smoke could also increase ROS [23]. Other research has focused its efforts on investigating the expression of TLR4 in epithelial cells, given their position within the lungs. It was revealed that cigarette smoke could not only induce TLR4 expression, but also increase IL-8 secretion in a TLR4-dependent manner from bronchial epithelial cells [24, 25]. Furthermore, mice studies have also helped to understand the relationship between cigarette smoke and TLR4. Doz et al. showed that cigarette smoke-induced inflammation

in mice is dependent on TLR4 and myeloid differentiation primary response gene 88 (MyD88) signaling [26]. In addition, cigarette smoke exposure of wild-type mice resulted in increased TLR4 expression as well as tumor necrosis factor (TNF)-α production [27].

Several studies have also investigated the role of TLR9 in smoke-induced inflammation. It was demonstrated that cigarette smoke could induce IL-8 production from epithelial cells and neutrophils in a TLR9-dependent manner [25, 28]. In addition, when plasmacytoid dendritic cells were exposed to both cigarette smoke and a TLR9 ligand, there was an increase in the release of IL-8 when compared to either given alone [29].

1.2.2 Bacterial and viral infections

It is estimated that one third of COPD patients have microbial colonization within their respiratory tract [30] and these infections can contribute to the pathogenesis of COPD in two ways. First, bacterial and viral infections are the primary cause of acute exacerbations [31]. Second, infections within the respiratory tract can intensify and perpetuate inflammation resulting in a continual inflammatory state that cannot be resolved. Studies have shown that the percentage of COPD patients colonized with microbial infections increases with disease severity and the rate of FEV_1 decline in these patients is positively correlated with the extent of colonization [32, 33].

Current reports estimate that bacterial infections are involved in 50% of COPD exacerbations [34]. The two most predominant bacteria found within the lungs of COPD patients are *Haemophilus influenzae* and *Pseudomonas*

aeruginosa, which affect nearly 29% of patients with stable COPD and 54% of exacerbated patients [35]. Bacterial infections that result in exacerbation lead to increased lung inflammation, which is mainly neutrophilic in nature, and can also cause systemic inflammation [36, 37]. Even in asymptomatic smokers, and stable COPD patients, bacterial colonization can lead to increased inflammation and airflow obstruction [38, 39], suggesting that bacterial-induced inflammation contributes to the development of COPD.

Viral infections have been detected in at least one third of exacerbated COPD patients [34]. Various respiratory viruses have been shown to induce exacerbations, with rhinovirus being the one most studied. Rhinovirus infects airway epithelium and elicits an inflammatory response [40]. Interestingly, viral infections which lead to exacerbations can induce both neutrophil and eosinophil recruitment to the lungs [41].

Childhood respiratory infection is also a risk factor for the development of COPD [42]. Studies have suggested that latent adenoviral infection may be associated with the disease. The E1A region of the adenoviral genome has be studied, and it was observed that the E1A protein is more commonly found in the lungs of COPD patients than smokers without COPD [43] and these proteins can bind DNA to enhance inflammation by upregulating IL-8 [44].

Cigarette smoke can also complicate bacterial and viral infections. Smokers tend to have an increased risk of infection, however it is unclear if this is due to the increased susceptibility to microbes or the inability to effectively clear these microbes [14]. Interestingly, cigarette smoke has been shown to contain

lipopolysaccharide (LPS) in both main stream and side stream smoke [45], and like other bacterial ligands, can activate TLRs to induce an inflammatory response.

Dr. Sanjay Sethi has proposed the vicious circle hypothesis (Figure 1), which attempts to connect bacterial and viral infections, along with cigarette smoke, to the pathogenesis of COPD [34]. The basis of this hypothesis is that cigarette smoke, together with chronic infections, can disrupt normal innate lung defense and lead to airway epithelial injury, mucus secretion, and impair mucociliary clearance. These injuries cause a favourable environment for microbes and can therefore lead to further colonization, which can then cause acute exacerbations, as well as increased damage of the epithelium. The exacerbations can lead to the release of inflammatory mediators, which can perpetuate the inflammation and the development of COPD. Consequently, this persistent inflammatory environment remains a good breeding ground for infection, and the entire process can continue in a cyclical manner [34].

1.2.3 Alpha-1 antitrypsin deficiency

Alpha-1 Antitrypsin (A1AT) is a serine protease inhibitor that can be synthesized by peripheral blood monocytes, alveolar macrophages, as well as bronchial epithelial cells and is involved in the inhibition of neutrophil elastase [46, 47]. Individuals who lack A1AT have the genetic disorder α1-antitrypsin deficiency, which predisposes them to COPD. A study carried out on 965 COPD patients, found that 1.9% had a severe deficiency of A1AT, enabling the progression of the disease [48].

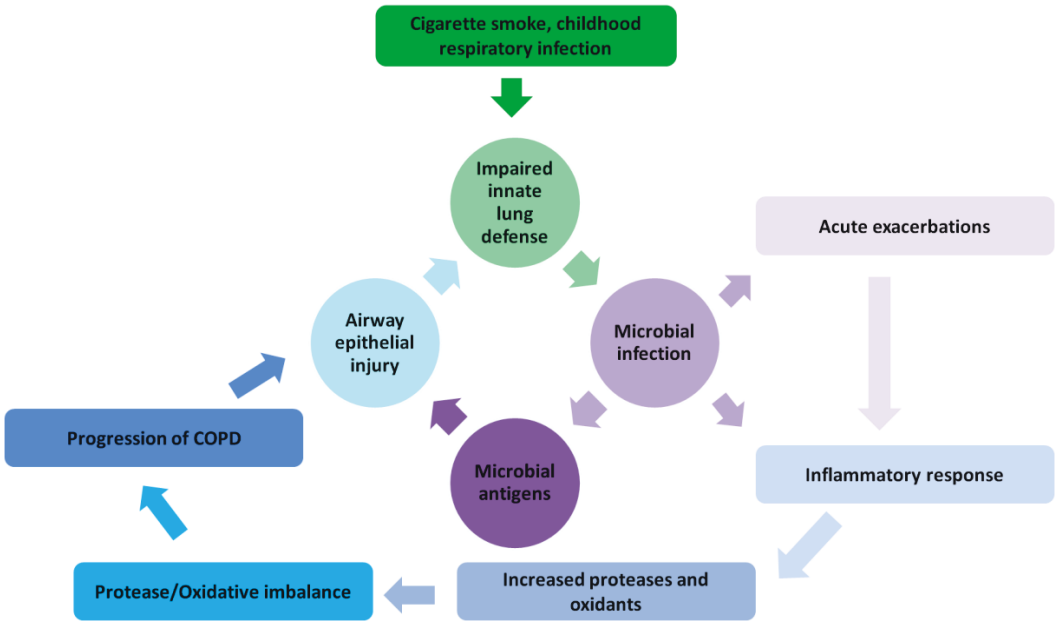

Figure 1.
Vicious circle hypothesis. Factors such as cigarette smoke and childhood respiratory diseases cause impaired innate lung defense, which leads to a favorable environment for bacteria and viruses to cause infection. These microbes can also cause exacerbations and airway epithelial injury. Under exacerbated states, COPD patients have increased inflammation, consequently advancing the development of COPD, which in turn results in more destruction of the epithelium. Once the epithelium is injured, pathogens can readily infect the airways, and the entire process commences again.

Neutrophil elastase is a protease produced by neutrophils in the respiratory tract that has been implicated in the pathogenesis of COPD [49]. It is released during inflammation once neutrophils become activated, and it causes damage to the lungs by hydrolyzing several proteins, including elastin [50]. A1AT is an essential player in the defense against neutrophil elastase degradation and individuals who lack A1AT have increased proteolytic activity, which leads to COPD. This leads to a protease-antiprotease imbalance, which favors destruction of the lung by the decrease in anti-elastin defense. Therefore, even individuals with A1AT deficiency who do not smoke can develop COPD, although it has been shown that cigarette smoke can accelerate the disease in these individuals. An early study done in 1983 on patients with A1AT deficiency found that 90% of those who were smokers had COPD, however 65% of the non-smokers were diagnosed with the disease as well [51].

Patients with COPD that also have A1AT deficiency normally undergo standard COPD therapy; however promising new treatments have emerged to help with their lack of A1AT. New therapies include, a gene therapy treatment in which a virus carrying the A1AT gene is given to patients in the hope that there will be an increase in the production of the A1AT protein [52], as well as inhibiting neutrophil elastase through small inhibitory molecules [53].

1.2.4 Biomass fuel and air pollution

It is estimated that 50% of households worldwide use a form of biomass fuel for cooking and heating, and it has been shown that these types of fuels are associated with obstructive lung disease. These biomass fuels can include coal,

dung, and crop residues, with wood fuel being the most popular [54]. The majority of households which use biomass fuels are found in developing countries around Asia and Africa, and due to the role of women in these societies, they tend to be the ones most affected by the fuel pollution as they are the ones who cook [55]. Several types of particles emitted by the burning fuel can damage the lungs, including carbon monoxide, sulphur oxides, nitrous oxides, formaldehyde and benzo[a]pyrene [56]. Particles with a diameter of 10 microns (PM_{10}), and especially those with a diameter of 2.5 microns ($PM_{2.5}$), can infiltrate deep within the lungs. When the indoor concentrations of particles was measured in several of these households, it was observed that the PM_{10} levels ranged from 300-3000 $\mu g/m^3$, much higher than the standards set by the United States Environmental Protection Agency, which is 150 $\mu g/m^3$ [57]. Most of the cooking tends to be done inside the home and therefore these particles are continuously breathed into the lung.

1.3 Pathogenesis of COPD

COPD is considered a small airways disease with most of the inflammation and destruction located within the peripheral airways and lung parenchyma. Neutrophils, macrophages, and T lymphocytes are activated and recruited to the lungs where they release various inflammatory mediators, including cytokines, chemokines, proteases, and oxidants. These mediators perpetuate the inflammation by recruiting more inflammatory cells to the lungs, causing structural changes within the airways, destruction of lung parenchyma, and the release of more inflammatory mediators. As the severity of the disease

becomes greater, the inflammation is amplified, and the degree of airflow obstruction worsens. There can also be periods of exacerbation, normally a result of a bacterial or viral infection, in which there is increased airflow obstruction. Lastly, COPD is also associated with certain systemic effects, such as generalized weakness and increased likelihood of developing cardiovascular disease [58-60] (Figure 2).

1.3.1 Airway inflammation

Airway inflammation begins early in the development of COPD, as it has been reported that even smokers without COPD have inflammatory infiltrates within the lung [61]. It was also observed that in asymptomatic smokers, the number of alveolar wall cells correlated with the extent of alveolar wall destruction [62], although there is evidence that this damage may be reversible [61, 63]. The inflammation in smokers with normal lung function is due to cigarette smoke and is nonspecific in nature [61]. It is still unknown as to why certain individuals develop structural abnormalities that eventually lead to COPD, while others continue to exhibit mild inflammation with normal airflow measurements and never go on to develop the disease.

Individuals who develop COPD have enhanced inflammation as well as remodeling within their lungs. In COPD, the major sites affected by remodeling are the small airways, (bronchioles < 2mm in diameter), and the lung parenchyma. One of the most common remodeling changes which occurs in COPD patients compared to healthy controls is goblet cell hyperplasia within the peripheral airways [64]. Fibrosis has also been observed in the lungs of COPD patients,

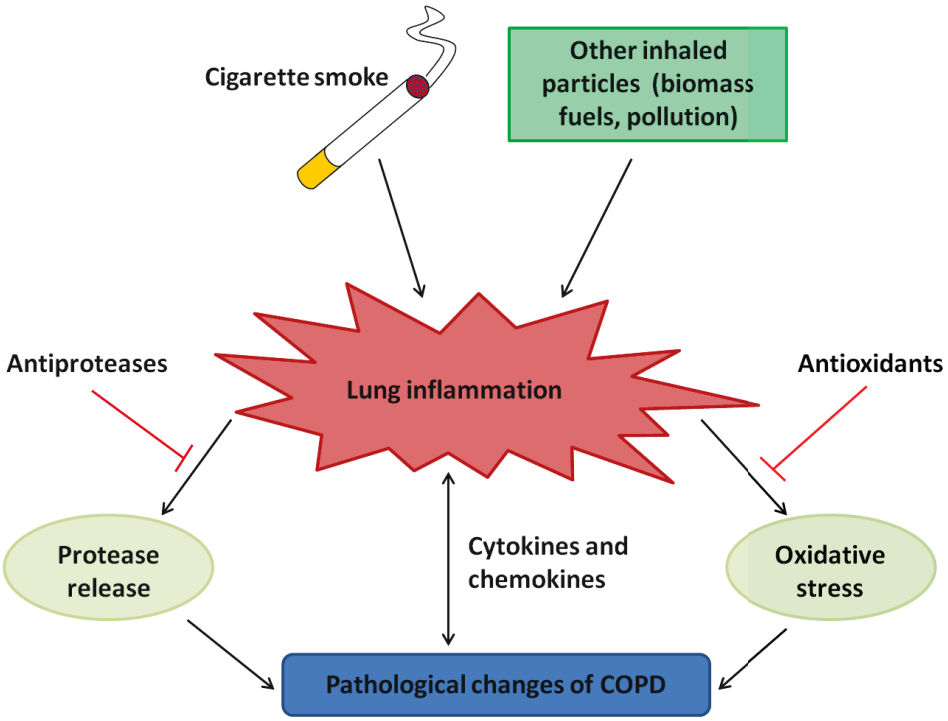

Figure 2.
Pathogenesis of COPD. COPD is an abnormal response of the lungs to inhaled toxins or particles, particularly cigarette smoke. This results in inflammation within the central airways, peripheral airways, and parenchyma. Neutrophils, macrophages and T lymphocytes are recruited to the lungs and release various types of inflammatory mediators including, cytokines, chemokines, proteases and oxidants. These mediators cause pathological changes within the lungs of COPD patients, which consequently results in further inflammation.

although the thickening of the basement membrane occurs to a lesser degree than in asthmatics [65]. In general, smokers with COPD also have increased smooth muscle area in their peripheral airways when compared to smokers with normal lung function [66], and this increase is highly responsible for the increased airflow resistance in COPD [67]. Remodeling of the peripheral airways also involves vascular changes, which are not observed within the central airways [68]. As the arterial wall thickness increases in these patients, the number of $CD8^+$ T cells is augmented [69], suggesting that the inflammatory response in COPD may be directly linked to the vascular changes observed within the lungs.

There is an increase in the numbers of neutrophils, macrophages, and T lymphocytes, predominantly $CD8^+$ T cells, within the lungs of patients with COPD [66, 70, 71]. Once activated, these cells produce many inflammatory mediators which perpetuate the inflammation. Several cytokines and chemokines are associated with COPD and have been repeatedly shown to be increased. These include, IL-8, TNF-α, MCP-1, interferon (IFN)-γ, and growth related oncogene (GRO)-α [72-75], with many of these being chemotactic factors, which continually induce the migration of inflammatory cells into the lung (Figure 3).

1.3.2 Protease-antiprotease and oxidative-antioxidative imbalance

There are two other factors which cause inflammation as well as destruction within COPD lungs – the protease-antiprotease imbalance and the oxidative-antioxidative imbalance. Proteases are a type of protein which can cause the breakdown of the extracellular matrix. The most notorious protease is elastase, which as mentioned previously, is a neutrophil-derived serine protease which

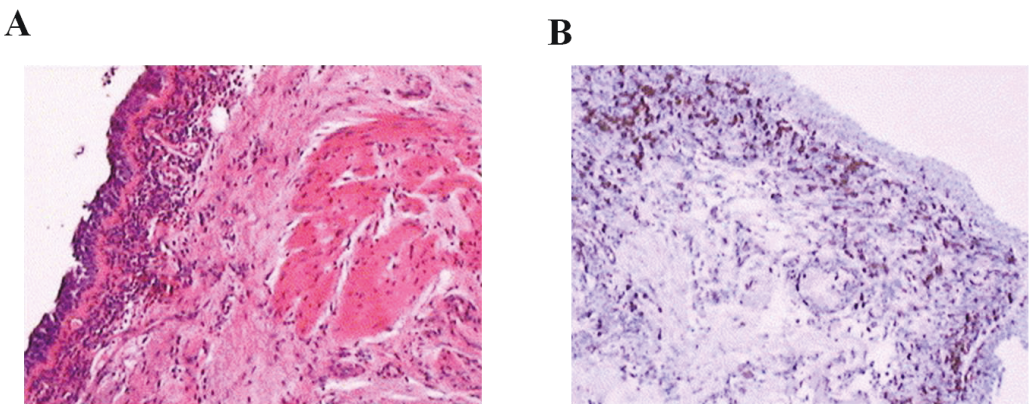

Figure 3.
Histopathology of COPD. Biopsy specimen from a COPD patient stained with (A) hematoxylin and eosin, which makes evident the large smooth muscle bundle and the inflammatory cells and (B) antibodies to CD8, indicating that these cells are found in the submucosa as well as invading the epithelium. Reprinted from Journal of Allergy and Clinical Immunology, 114/6, Hamid Q., Cosio M., Lim S. Inflammation and remodeling in chronic obstructive pulmonary disease, 1479-81. Copyright 2004 with permission from Elsevier.

breaks down elastin. Due to its increased expression and persistent activation in COPD, elastase can cause destruction of the lung parenchyma [76]. There is also a class of proteases called matrix metalloproteases (MMP), which are derived from macrophages and neutrophils. Several of these MMPs are increased in COPD, including MMP-1, MMP-2, and MMP-9 [77-80]. In a mouse model of COPD, it was observed that by knocking out MMP-12, cigarette smoke-induced inflammation can be inhibited [81], indicating a potential major role for MMP-12 in the pathogenesis of COPD. Under normal circumstances, protease activity is counterbalanced by antiproteases, with A1AT being the major inhibitor of elastase, and tissue inhibitors of matrix metalloproteases (TIMP) inhibiting the activity of MMPs. It seems that in smokers with normal lung function, the antiproteases are able to counteract the effects of the proteases, but in COPD, there is an imbalance which is skewed towards the proteases. Therefore, there are not enough antiproteases to neutralize the breakdown of tissue in a timely manner.

There is also an oxidative imbalance with increased ROS activity in COPD. Cigarette smoke itself contains free radicals and other oxidants, and the inflammatory process can produce certain oxidants as well. There seems to be an imbalance in the amount of oxidants compared to antioxidants, which leads to oxidative stress in COPD [82]. Increased hydrogen peroxide (H_2O_2) can be found in the exhaled breath of COPD patients [83] and alveolar macrophages from patients with COPD can produce increased amounts of superoxide (O_2^-) and H_2O_2 [84]. A major antioxidant is glutathione, which is produced by epithelial cells and is elevated in the bronchoalveolar lavage fluid (BALF) of smokers [85] and may attempt to dampen the effect of the oxidants. However, similar to antiproteases,

there does not seem to be an adequate increase of antioxidants to completely balance the amount of oxidative stress. In addition, these oxidative species can not only cause damage to cells, but they can destroy lung matrix adding to the damage of the proteases and causing increased lung destruction [86] (Figure 4).

1.3.3 Neutrophils

Elevated neutrophil numbers are found in stable and exacerbated COPD patients when compared to healthy controls. Neutrophils infiltrate the BALF [87, 88], sputum [89], and within the bronchial mucosa [71, 90] and their numbers increase as disease severity worsens [72]. Early on it was observed that cigarette smoke could not only increase the number of circulating neutrophils, but cause them to remain within the lung for longer period of time than normal [91]. This is most likely due to the release neutrophil chemoattractants, such as IL-8 and GRO-α, which help to promote the infiltration of neutrophils into the lungs. These cytokines are also increased in COPD [75, 92]. The number of neutrophils in the lungs of COPD patients has also been shown to be related to the rate of decline of lung function [71, 89].

1.3.4 Eosinophils

Eosinophils are not an essential cell type in COPD. However, there is evidence that a subset of COPD patients who have partial reversibility of their airflow limitation have a more asthma-like phenotype, which also includes increased eosinophilia in their sputum [93]. Eosinophils seem to play more of a

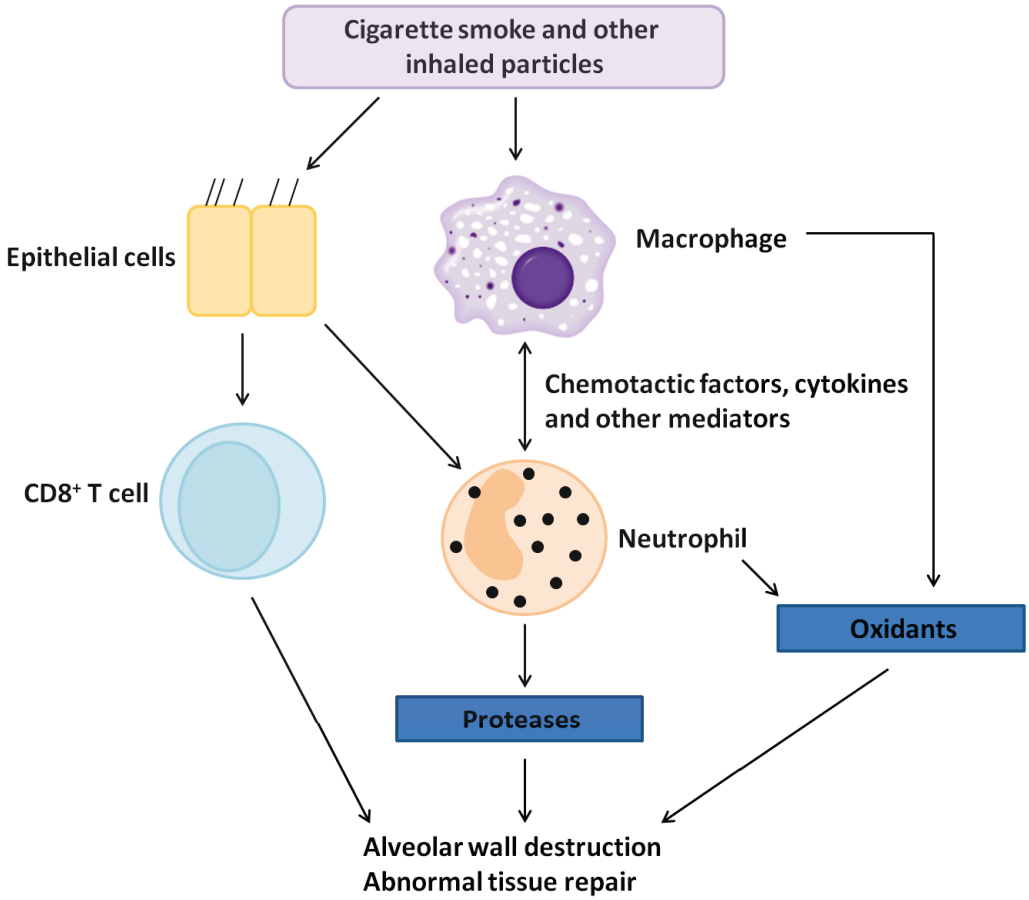

Figure 4.
Inflammatory process of COPD. Cigarette smoke and other inhaled particles or gases can cause epithelial injury and macrophage activation. These two types of cells can release inflammatory mediators which recruit neutrophils and T lymphocytes, (predominantly $CD8^+$ T cells), to the airways. Together, these cells can release cytokines, oxidants, and proteases which cause further cell migration to the lungs, as well as destruction of the parenchyma. Due to the fact that there are not enough antioxidants and antiproteases to counterbalance the extent of oxidants and proteases released, the damage continues and cannot be repaired.

role in exacerbated patients, as the numbers of these cells increase during periods of viral exacerbation [94]. Unlike in asthma, this increase does not correspond to an increase of IL-5, suggesting that eosinophils in COPD do not act the same way as they do in asthma.

1.3.5 Monocytes

Monocytes circulate throughout the body in the peripheral blood. Under inflammatory conditions they are recruited to the lung and become macrophages. MCP-1 and GRO-α are two of the monocyte chemoattractants, which are significantly increased in COPD and trigger the migration of monocytes from circulation into the lungs of patients [74, 95].

1.3.6 Macrophages

Macrophages are increased within the airways, parenchyma, as well as the BALF of COPD patients [60]. Additionally, the extent of macrophage accumulation in the peripheral airways may be indicative of disease severity as the peripheral airways of smokers with severe COPD had a greater infiltration of macrophages compared to smokers with mild or no COPD [96]. Macrophages play an important role in the inflammation of the disease as they are highly responsible for releasing various chemokines that can attract T lymphocytes, neutrophils, and more monocytes to the airways [97]. Cigarette smoke can induce macrophages to release several inflammatory cytokines, including TNF-α and IL-8, as well as MMPs and ROS, which can cause further destruction to the lungs [61]. MMP-9 and MMP-12 have been linked to COPD, as macrophages from

smokers secrete more of these potent extracellular matrix-degrading enzymes than macrophages from non-smokers [98, 99]. In addition, circulating monocytes from COPD patients were also able to secrete MMP-9 to a greater extent than control subjects [100].

1.3.7 Structural cells as inflammatory cells

Airway epithelial cells have been well studied in COPD and cigarette smoke-induced inflammation. These cells can produce several types of inflammatory mediators in response to cigarette smoke including, IL-8, IL-1β, and TNF-α, although they can produce antiproteases and antioxidants as well [61]. Transforming growth factor-beta (TGF-β) 1 is also highly expressed from the epithelium of COPD patients and its levels have been to be correlated to the smoking history of patients [101] (please see section 1.5 for more information on epithelial cells).

Most studies on the role of airway smooth muscle in COPD focus on the remodeling aspect and less on the inflammation itself. As mentioned previously, smooth muscle thickening can be observed in the peripheral airways and is correlated to airflow limitation [66], but the same was not observed within the central airways. However, airway smooth muscle cells can also produce cytokines and inflammatory mediators. Under inflammatory conditions, smooth muscle cells produce IL-8, GRO-α, MCP-1, eotaxin, along with certain extracellular matrix proteins [102-104]. Trying to mimic a COPD environment, an *in vitro* stimulation of smooth muscle cells with IFN-γ and TNF-α caused the upregulation of CXCL10, which, has been implicated in the pathogenesis of

COPD as it is a potent chemoattractant for many cells, including neutrophils and T lymphocytes [105]. Lastly, in COPD, neutrophils and $CD8^+$ T cells can evenbe found to accumulate within the airway smooth muscle bundles more so than in non-smokers [106], suggesting that these structural cells play a role in the inflammatory process of COPD.

1.3.8 Lymphocytes

Lymphocytes are mononuclear leukocytes that are found within the blood and lymphoid tissues. All lymphocytes stem from a common precursor cell and can then become B cells, which develop in the bone marrow and T cells, which develop in the thymus (Figure 5). T cells can then be further subdivided into more subtypes, with the two most common being $CD4^+$ or $CD8^+$ (for more information on the development of T cells please see section 1.4.1).

In 1995, Finkelstein and colleagues made the association between COPD severity and the extent of T cell infiltrates. Using immunohistochemistry staining, they discovered that T lymphocytes and macrophages were the predominant inflammatory cells within the COPD lung [70]. This work was followed by a comprehensive study done by O'Shaughnessy et al. to characterize the T cell subsets in COPD. They demonstrated that $CD8^+$ T cells were significantly increased in the central airways of COPD patients compared to control subjects, while $CD4^+$ T cells were not. Furthermore, they revealed that the $CD8^+$ T cells were also negatively correlated with the FEV_1 of COPD patients [107]. Two similar studies were then carried out which investigated T lymphocytes within the peripheral airways as well as the parenchyma. Similarly, it was observed that

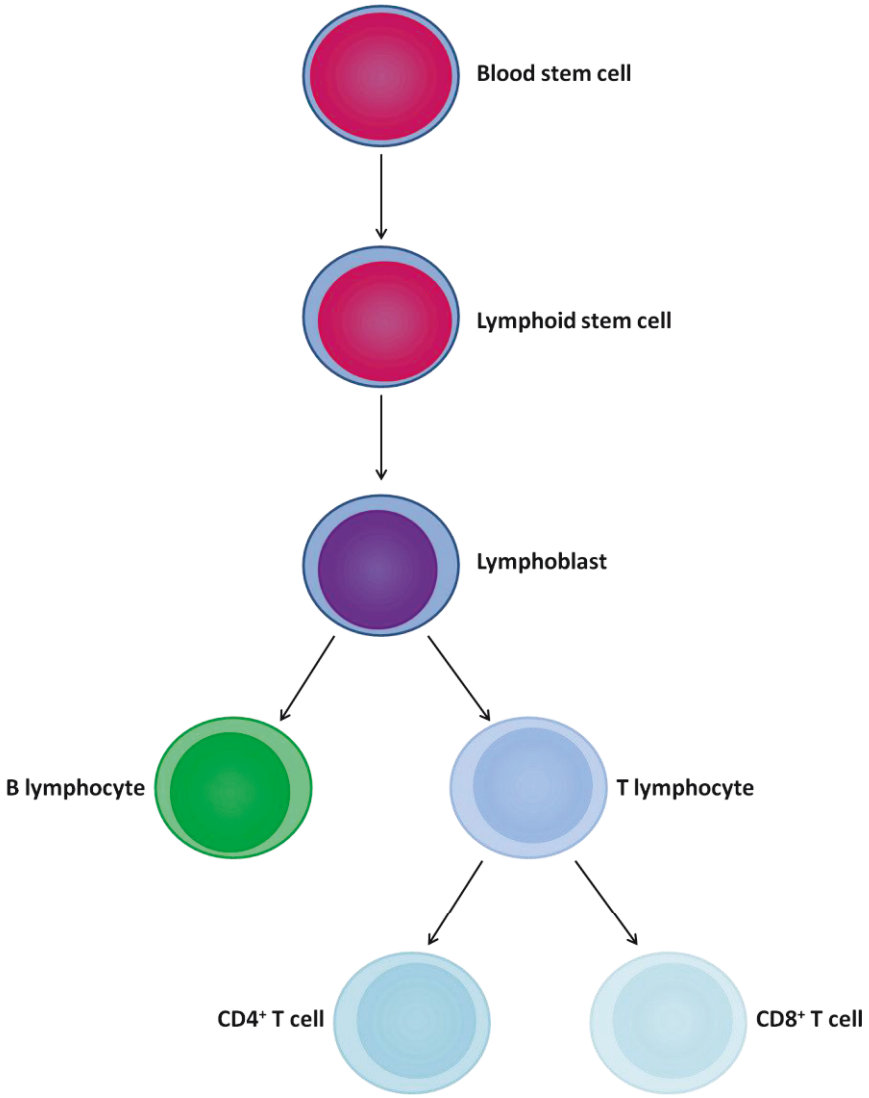

Figure 5.
The basic stages of lymphopoiesis. Blood stem cells can differentiate into various types of cells, including lymphoblasts, within the bone marrow. Lymphoblasts can then develop into B lymphocytes, which mature in the bone marrow, or T lymphocytes, which migrate and mature in the thymus. Next, T lymphocytes can develop into several types of T cells, which include $CD4^+$ T cells and $CD8^+$ T cells.

$CD8^+$ T cells were significantly increased in the peripheral airways and parenchyma, while there was no significant change in the number of $CD4^+$ T cells [66, 108]. Once again, there was also a negative correlation between the number of $CD8^+$ T cells within the peripheral airways and parenchyma compared to the FEV_1 of the COPD patients. Although $CD4^+$ T cells are not greatly increased in COPD, they are still needed to help regulate other cells; $CD4^+$ T cells are required for priming the cytotoxic responses of $CD8^+$ T cells, promoting their survival, and maintaining their memory [109, 110].

Hogg and colleagues have recently done a lot of work on understanding the role of B lymphocytes in COPD. They found that the number of B cells, as well as the number of lymphoid follicles, increase with disease severity, while the number of airways containing B lymphocytes and the absolute volume of these cells poorly associates with disease severity [111]. In addition, using immunostaining methods, they determined that the lymphoid follicles are formed of a core of B cells with $CD4^+$ T cells scattered around the edges. B lymphocytes also express certain chemokines, such as CXCL10, which help to promote the cytotoxic profile observed in COPD [112].

It has been proposed that COPD may be an autoimmune disease, triggered by antigens released from smoke-induced lung injury [113]. This body of evidence would suggest that B lymphocytes have been largely underestimated in COPD. However, there is not a lot of support for this hypothesis and further research into the role of B cells in the pathogenesis of COPD is needed to verify this concept.

1.4 $CD8^+$ T cells

T lymphocytes constitute 60-70% of lymphocytes, with $CD8^+$ T cells accounting for 30% of all T cells [114]. $CD8^+$ T cells are the most predominant cell to infiltrate the airways of patients with COPD however, the mechanisms and the rationale as to why these cells are so prominent in this disease still remains unknown. This section begins with investigating how T lymphocytes develop in the thymus, mature to become $CD8^+$ T cells, and then how they get recruited into the lung. Next, we examine how these cells function under general, healthy conditions and more specifically in COPD.

1.4.1 Development of $CD8^+$ T cells

The most common subset of T cells express αβ T-cell receptors (TCR). These cells can respond to antigens which bind the TCR as well as the major histocompatibility complex (MHC) class I or MHC class II molecules [115]. MHC class I activation results from intracellular infection and leads to the destruction of infected cells, while MHC class II becomes activated under extracellular infectious conditions. Therefore, αβ T cells are divided into two lineages depending on which types of antigens they recognize. In addition, αβ T cells can also express two other proteins, CD4 or CD8. Normally, MHC class II will bind the TCR from CD4 (helper or regulatory) T cells, while MHC class I will bind to the TCR from CD8 (cytotoxic) T cells [116, 117]. The CD4 and CD8 molecules are called co-receptors, as they co-recognize the antigens which bind the TCR [118]. There are two modes of thought as to how these αβ T cells develop into one of these distinct lineages; it is either through instruction or

selection. These cells originate from a common precursor, which expresses both the CD4 and CD8 co-receptors [119, 120]. However, only the cells which express the co-receptor and TCR that matches the proper MHC class, can correctly mature into T cells [119]. Under instruction, it is believed that the TCR/co-receptor/MHC class complex instructs the cell to stop the transcription of the incorrect co-receptor, while with the selection model, it is thought that the cell makes a random choice to stop transcribing either CD4 or CD8, and if the proper co-receptor is left, the cell will continue to mature properly [120].

Lymphoid progenitor cells start to develop in the bone marrow and then migrate to the thymus where they lose the potential to develop into B cells or natural killer cells [121, 122]. These T cell precursors, considered double-negative thymocytes, then go through four stages of differentiation before developing into αβ or γδ T cells [123, 124]. In order to become αβ T cells, these thymocytes must express the αβ TCR during this differentiation process [125]. During this time, the thymocytes also begin to express both the co-receptors allowing the cells to become double-positive αβ-TCR-expressing immature cells. Next, these cells undergo either instruction or selection to become CD4- or CD8-committed [120]. Once the thymocytes have matured to this stage, they can leave the thymus and head to peripheral lymphoid sites. Once in the lymphoid tissue, these naive T lymphocytes only become activated by antigen stimulation, which results in the proliferation and differentiation into effector cells. $CD8^+$ T cells become activated when an antigen-presenting cell (APC) presents an antigen bound to MHC class I. Activation of $CD8^+$ T cells is also based on the co-stimulation through the molecules CD28, CD40, as well as stimulation through the IL-12 and IFN

receptors found on these cells [126, 127]. Once primed, these effector cells can migrate to the site where the antigen is present and trigger functional responses to eliminate microbes.

1.4.2 Function of $CD8^+$ T cells

$CD8^+$ T cells are also known as cytotoxic cells, as their main function is to release cytotoxins, which directly induce the death of infected cells. However, another function of these cells is the production of cytokines. Due to their cytotoxic activity, $CD8^+$ T cells are known to function in the clearance of viral infections [128, 129]. There are two main methods by which $CD8^+$ T cells induce cell death. The first is through the release of cytotoxins, such as perforin and granzymes, which lead to the cytolysis of the infected cell. Perforin, a protein which disrupts the cell surface membrane by puncturing pores in it, is first released by $CD8^+$ T cells, followed by granzymes, serine proteases which can cause the cytolysis of the target cell [130]. The second method used by $CD8^+$ T cells is through FAS-ligand binding, which results in the apoptosis of the infected cell. This pathway requires the target cell to express FAS (CD95) and bind to the FAS ligand on the $CD8^+$ T cell, which leads to caspase-dependant apoptosis [131]. In addition, recent evidence has suggested that TNF-α-mediated apoptosis may be a third mechanism by which $CD8^+$ T cells induce cell death, particularly to epithelial cells [132]. It was revealed that TNF-α could be expressed on $CD8^+$ T cells as a membrane-bound molecule that could induce cytolysis. When cells were treated with a TNF-α antibody, there was a reduction in the number of epithelial cells which underwent cell death.

In order for these cells to function properly, naive $CD8^+$ T cells in the lymphoid tissue must undergo antigen-driven terminal differentiation into effector cells with the help of changes in their gene expression [133]. T-box expressed in T cells (T-bet) and eomesodermin (EOMES) are the two main transcription factors which are required for $CD8^+$ T cells to function properly and induce IFN-γ, perforin, and granzyme production [134, 135].

1.4.3 $CD8^+$ T cells and COPD

As mentioned previously, $CD8^+$ T cells are the hallmark cell of COPD. They are increased in the central and peripheral airways, as well as in the parenchyma of COPD patients [66, 107, 108]. $CD8^+$ T cells can also be found in the sputum [136, 137], bronchial glands [138], and interspersed within the epithelium and smooth muscle [106, 139, 140]. The activated $CD8^+$ T cells, which infiltrate into the lungs of these patients show a predominantly T cytotoxic 1 (Tc1) profile [99]. Interestingly, researchers have investigated the numbers of these cells in the peripheral blood, however the results are somewhat inconclusive, with certain reports claiming a decrease of these of cells within the blood, while others maintain that there is no change in the numbers of these cells [141-143]. Regardless, studies have revealed that the peripheral blood $CD8^+$ T cells still seem to maintain the same Tc1 phenotype that is observed in the lungs of patients with COPD [143, 144].

A characteristic of Tc1 cell subsets is that they express the chemokine receptors CXCR3 and CCR5 [145, 146]. Using flow cytometry it was demonstrated that $CD8^+$ T cells in the lungs of COPD patients express CXCR3

and CCR5 [99]. Increased CXCL10, the ligand for CXCR3, was also found in COPD patients [147]. The binding of CXCL10 to CXCR3 activates a signaling pathway which enhances Tc1 cell recruitment, while inhibiting Tc2 cell migration, and could therefore be the main factor causing $CD8^+$ T cell migration to the lungs. Furthermore, bronchial biopsies from COPD patients revealed that T lymphocytes also express the transcription factor signal transducer and activator of transcription 4 (STAT4), which is critical for the differentiation of $CD8^+$ T cells into Tc1 cells [148].

$CD8^+$ T cells also express IFN-γ and TNF-α, which are known mediators of lung injury and could therefore contribute towards the destruction of the parenchyma [149]. IFN-γ mRNA transcripts from lung $CD8^+$ T cells were also shown to correlate with COPD severity [150]. Furthermore, IL-18, a cytokine expressed in COPD patients [151], was able to increase the cytotoxicity of $CD8^+$ T cells by upregulating IFN-γ [152]. $CD8^+$ T cell-derived IFN-γ can activate macrophages to produce IL-12 resulting in a feedback mechanism to promote the Tc1 phenotype [110]. In addition, a murine model which overexpressed IFN-γ led to COPD-like destruction of the lungs, further corroborating the idea that COPD has a Tc1 profile and that IFN-γ can help cause tissue injury [153].

The numbers of $CD8^+$ T cells within the airways are significantly correlated with a decrease in FEV_1 as well as the number of pack years an individual has smoked [107, 154]. However, the precise role that these cells play in the pathogenesis of COPD is still relatively unknown. It is suspected that these cells play a part in the lung destruction through their cytotoxic capabilities. There are very few studies which investigate the cytotoxic ability of these cells in COPD,

however it was observed that $CD8^+$ T cells in the sputum of COPD patients have higher perforin expression than smokers without COPD and non-smokers [136]. In addition, $CD8^+$ T cells from the peripheral blood and BALF had increased perforin and granzyme-b in COPD patients compared to non-smokers. There was also a correlation between granzyme-b expression in the BALF and the extent of apoptosis of bronchial epithelial cells [155]. This suggests that in addition to the proteases and oxidants, these cytotoxic cells also cause damage to the lungs of patients with COPD.

1.5 Airway epithelial cells

The airway epithelium is the first line of defense against pathogens once they enter the airways. Under normal conditions, the epithelium can protect the airways against microbes by eliciting an immune response against them. However, in COPD, there is chronic inflammation and a breakdown in repair mechanisms. The airway epithelium can no longer efficiently protect the airways, allowing for cigarette smoke, viruses, and bacteria to enter the lungs and cause further damage. Here we will discuss various aspects of airway epithelial cells.

1.5.1 Structure of airway epithelium

There are various types of epithelial cells that can be found throughout the airways. Ciliated cells are the most common form of epithelial cell found within the lung, and these tend to be pseudostratified columnar in the central airways and then move towards simple columnar and cuboidal in the peripheral airways. The alveolar epithelium is made up of Type I and Type II cells [156].

Ciliated epithelial cells are terminally differentiated cells that account for 50% of all epithelial cells found within the airways [157, 158]. These ciliated cells tend to rest on basal epithelial cells in both the central and peripheral airways. Basal cells are considered the epithelial stem cell as they can proliferate to become whichever type of epithelial cell is necessary depending on the environment [159]. Basal cells are also extremely important as they anchor other forms of epithelial cells to the basement membrane. This is accomplished through hemidesmosomes which bind the basement membrane via integrins [160]. Interestingly, it has been demonstrated that the thickness of the epithelium is correlated to the number of basal cells attached to the basement membrane [161]. Mucous goblet cells are another type of epithelial cell, which secrete mucous into the airways as a means to trap pathogens [162]. Under diseased conditions, there is an increase in the numbers of these cells leading to excess mucous production [163]. Lastly, alveolar epithelial cells can be found in the alveolus and are responsible for sodium transport, fluid absorption, and gas exchange [164, 165].

1.5.2 Function of airway epithelium

The airway epithelium is a dynamic tissue that is constantly undergoing change and renewing itself [166]. Under normal conditions, the epithelium acts as a physical barrier, held together by tight junctions, to protect the lung. Other functions of the epithelium include, regulating lung fluid balance, defending against inhaled pathogens, and secreting various mediators [161].

Goblet cells produce mucus, which together with ciliated epithelial cells, trap and remove foreign matter from the airways using mucociliary transport

[167]. The most common form of mucin is MUC5AC, which is produced from goblet cells, while another form, MUC5B is mainly produced by submucosal glands [168, 169]. Epithelial cells can also secrete small microbial peptides including β-defensins and LL-37, which inhibit the growth of microbes until they can be properly removed from the airways, either through phagocytosis or mucociliary clearance [170].

Under normal conditions, the epithelium is an efficient barrier which inhibits the entry of environmental factors or toxins into the lung. If one of these factors triggers an immune response, the epithelium can activate several pathways which induce the production of cytokines. One of the most important family of receptors for maintaining proper immune functions, are the TLRs. These receptors have been heavily investigated on epithelial cells and are known to elicit an inflammatory response once activated [171].

1.5.3 Epithelial cells and COPD

Cigarette smoke, a key risk factor in COPD, can activate the epithelium to release many factors, as well as cause injury to the cells. Several studies have found that IL-8 expression from epithelial cells is significantly increased after cigarette smoke exposure [20, 24, 172]. This is not surprising considering that IL-8 is a neutrophil chemoattractant and it is known that that there is considerable infiltration of neutrophils within the lungs of COPD patients. IL-1β, TNF-α, MCP-1, and TGF-β were also upregulated after epithelial cells were exposed to cigarette smoke [173, 174]. Furthermore, both cigarette smoke, TNF-α, and IL-β were able to increase mucin production, specifically MUC5AC [175-177]. In

healthy individuals, 25-30% of the surface epithelial cells are ciliated, compared to only 10% in asymptomatic smokers and almost none in COPD patients [178]. This suggests that there is dysregulation in the mucociliary clearance as there are more goblet cells producing mucous and less cilia, allowing the mucous to build up in these patients. In addition, cigarette smoke can also breakdown the epithelial integrity which affects the defense mechanisms of the airway. This is done by decreasing the trans-epithelial electric resistance (TEER) and disassembling tight junctions, leading to impaired barrier function [179, 180]. There is also increased apoptosis of epithelial cells in COPD patients compared to controls. Cigarette smoke is able to activate the caspase signaling cascade to induce epithelial apoptosis via IL-18 receptor-dependent pathways [181] and this apoptosis persists even after smoking cessation [182, 183].

Remodeling of the epithelium also occurs in COPD, with one important feature being squamous metaplasia. Chronic injury to these cells in the form of cigarette smoke or other pathogens results in the loss of certain damaged cells and consequently, there are sequential and dynamic changes of the epithelial phenotypes during the process of cell regeneration. Normally, after mucus is released, the cilia are shed, and the cell becomes so damaged that it undergoes apoptosis, allowing the basal cells to begin their repair process [184]. The basal cells proliferate and begin to spread out and migrate towards the wounded area. However, under COPD conditions, with continual damage, the cells tend to undergo squamous metaplasia and therefore eventually change their phenotype.

Bacteria and viruses are another risk factor in the development of COPD. Respiratory viral infections tend to choose airway epithelial cells as their main

sites of infection. These viruses then cause the epithelium to secrete various types of inflammatory cytokines and chemokines, including IL-1β, IL-6, IL-8, TNF-α, and CXCL10 [185, 186]. Bacteria colonize along the epithelium and like to bind to cells which are damaged or undergoing repair. They can also cause the epithelium to release cytokines [187] and together with viruses can further damage barrier function [188].

Oxidative species from cigarette smoke or air pollution can also damage airway epithelial cells. However, epithelial cells are the primary source of antioxidants, with glutathione being the most common antioxidant produced by these cells after cigarette smoke or viral exposure [189-191]. Oxidative stress can also induce TNF-α production from epithelial cells, which can in turn upregulate other proinflammatory mediators and stress response genes, including heat shock proteins [192]. The cytokines released by epithelial cells under any of these conditions serve to create an inflammatory environment and recruit inflammatory cells in hopes of clearing the invading microbes. Conversely, in COPD, there is already chronic inflammation and this induction of cytokines and chemokines only serves to amplify the inflammation.

1.6 Toll-like receptors

Studies have shown that T lymphocytes as well as epithelial cells express innate immune receptors [193-195]. These receptors are necessary to promote inflammatory responses under diseased conditions. TLRs are one of the most commonly studied family of innate immune receptors on these cell types.

Throughout the last century many individuals have helped to uncover the intricacies of the innate immune system and unearth which mechanisms are responsible to help defend against infection by other organisms in a nonspecific manner. The receptors involved in this process are called pattern-recognition receptors (PRR). These receptors can be found on the cell surface, intracellularly, and can even be released into the blood. Once activated, these receptors activate several signaling cascades which can lead to apoptosis, phagocytosis, opsonisation, as well the induction of inflammatory pathways [196, 197]. There are specific factors which target the innate immune system and can therefore activate PRRs, and these are termed pathogen-associated molecular patterns (PAMP). PAMPs are factors that are only produced by pathogens and not by the host's cells, allowing for the clear distinction between self and non-self. All PAMPs are crucial for the microbe's survival and consequently, mutants tend to be lethal for the microorganism. Therefore, the PRRs have evolved to handle specific PAMPs and all other mutants will not survive [198]. The most common example of a PAMP is LPS. LPS is an endotoxin found on the outer membrane of Gram-negative bacteria and is composed of the O antigen, a variable polysaccharide portion, an oligosaccharide core, and an invariant lipid A domain [199]. The lipid A domain is conserved among all Gram-negative bacteria and is therefore the portion responsible for activating the innate immune system [200].

There are several types of PRRs, with TLRs being one family of PRRs that have been highly investigated. TLRs are a family of type I transmembrane receptors, which are characterized by an amino-terminal extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain. The

extracellular LRR was found to have at least 15 repeats of a 22-26 amino acid leucine-rich segment [201], while the TIR domain is a conserved structure which can also be found in the cytoplasmic region of other proteins, most specifically members of the IL-1 receptor (IL-1R) family [202].

1.6.1 TLR discovery

The protein Toll was first described in *Drosophila* in 1985. It was found to play a role in dorsal-ventral polarity during embryogenesis [201, 203, 204]. It was revealed that Toll could be activated by Spätzle, causing the induction of a signaling cascade that would result in the release of the transcription factor Dorsal into the nucleus to regulate specific genes [205]. This signaling pathway was revealed to be quite similar to the IL-1R pathway in mammals, which induces inflammatory responses through the activation of nuclear factor-κB (NF-κB) [206, 207]. It was therefore not surprising when it was revealed that the *Drosophila* Toll protein also functioned in innate immune responses to fungus and bacteria in adult fruit flies [208, 209].

In 1997, the first mammalian homologue of the *Drosophila* Toll protein (hToll) was discovered [210]. This groundbreaking study revealed that similar to *Drosophila* Toll, hToll had an extracellular LRR domain as well as an intracellular domain homologous to the IL-1R domain. In addition, Medzhitov et al. were able to show that constitutively active hToll was able to induce NF-κB activation resulting in the gene expression of IL-1, IL-6, and IL-8 [210]. Two years later, it was revealed that hToll, currently known as TLR4, was responsible for regulating LPS responses [211]. Once the first mammalian Toll homologue

was discovered, it did not take long for other structurally related receptors to be discovered. Together, these became the Toll-like receptor family [212-216]. Currently, there are 10 members of the TLR family (TLR1 - TLR10) which are each activated by specific PAMPs derived from microbes, as well as certain host-derived molecules called danger-associated molecular patterns (DAMP) [217].

1.6.2 TLR family and ligands

The 10 members of the TLR family are all transmembrane receptors, however they can be found in different cellular compartments (Figure 6). Most commonly, TLR1, TLR2, TLR4, TLR5, and TLR6 are located on the cell surface as they recognize lipid and protein ligands, while TLR3, TLR7, TLR8 and TLR9 are localized to intracellular vesicles as they recognize nucleic acids [218, 219]. Similar to other types of receptors, TLRs can form homodimers as well as heterodimers. Dimer formation allows for an increase in the number of potential ligands which can bind to each of these receptors (Table 1) and activate specific downstream signaling pathways [220-222]. Several studies have attempted to determine which cells predominantly express each TLR, however this has proven to be quite difficult. Due to the fact that these are innate immune receptors, TLRs are expressed in lymphoid and nonlymphoid tissues and their expression differs between cell types and if the tissue is in any sort of diseased state [223].

1.6.3 TLR signaling pathways

Once a ligand binds to its specific TLR, there is activation of signal transduction pathways which leads to the induction of inflammatory cytokines

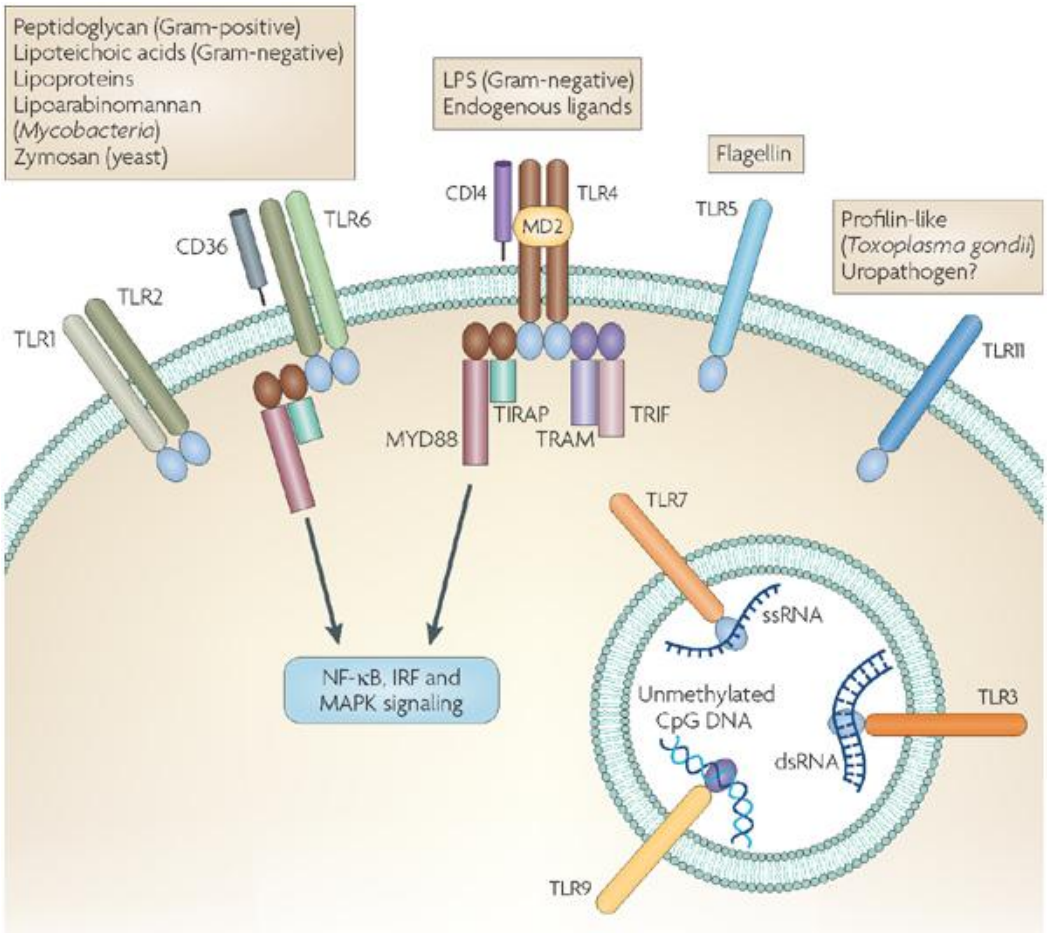

Figure 6.
Localization of Toll-like receptors. This diagram shows the location of each TLR within a cell, as well as their commonly associated ligands. TLR1, TLR2, TLR4, TLR5, and TLR6 can be found on the cell surface, while TLR3, TLR7, TLR8, and TLR9 are found within intracellular vesicles. It should be noted that the TLR11 protein is not found in humans. Once dimerization of the receptors occurs, TLRs can transmit signals down certain pathways including, NF-κB, IRF, and MAPK, to induce inflammatory responses. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. *Nat Rev Cancer.* 2009 Jan;9(1):57-63. Copyright 2009.

Table 1: Toll-like receptors and their ligands

TLR	Ligands	Ligand Origin
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
	Soluble factors	*Neisseria meningitides*
TLR2	Lipoprotein/lipopeptides	Various pathogens
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	*Staphylococcus epidermidis*
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Glycolipids	*Treponema maltophilum*
	Porins	*Neisseria*
	Zymosan	Fungi
	Heat-shock protein 70	Host
TLR3	Double-stranded RNA	Viruses
	Poly(I:C)	Synthetic compound
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Taxol	Plants
	Fusion protein	Respiratory syncytial virus
	Fibronectin A domain	Host
	Heat-shock protein 60	Host
	Heat-shock protein 70	Host
	Polysaccharide fragments of heparin sulphate	Host
	Hyaluronic acid	Host
	Fibrinogen	Host

Table 1 continued

TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	*Mycoplasma*
	Lipoteichoic acid	Gram-positive bacteria
TLR7	Imidazoquinoline	Synthetic compound
	Bropirimine	Synthetic compound
	Loxoribine	Synthetic compound
	Single-stranded RNA	Viruses
TLR8	Imidazoquinoline	Synthetic compound
	Single-stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	N.D.	N.D.
TLR11	N.D.	Uropathogenic bacteria

and molecules which help to destroy microbial pathogens. Several signaling pathways are involved in TLR activation, with some being specific to certain TLRs, while others are shared.

The most common TLR signaling pathway is the MyD88-dependent pathway (Figure 7). It was first described when MyD88-deficient mice were unable to properly respond to IL-1, LPS, or flagellin stimulation [224-226]. Furthermore, macrophages isolated from MyD88-defienct mice were unable to produce inflammatory cytokines after being stimulated with peptidoglycan, lipoproteins, dsRNA, imidazoquinolines, or CpG DNA [227-231], which recognize TLR2, TLR4, TLR3, TLR7, and TLR9 respectively. These studies all demonstrated the importance of the MyD88 protein in TLR activation.

MyD88 is composed of two domains, a TIR domain, which interacts with the TIR domain of the TLR, as well as a death domain which associates with the death domain of IL-1R-associated kinase (IRAK) 4 [232-234]. IRAK4 can then phosphorylate residues on IRAK1 causing the activation of IRAK1 [235], which then goes on to autophosphorylate itself enabling TNF receptor-associated factor 6 (TRAF6) to associate to the complex [236]. The IRAK1-TRAF6 complex then separates itself from the receptor and interacts with another complex composed of the serine/threonine kinase TGF-β-activated kinase 1 (TAK1) and the adaptor proteins TAK1-binding protein 1 (TAB1) and TAB2, (or sometimes TAB3), at the plasma membrane. This engagement causes the phosphorylation of TAK1 by TAB1, while TAB2 facilitates the interaction between TRAF6 and TAK1 [237-239]. Then, together with TRAF6, this complex can translocate to the cytoplasm. Once there, TRAF6 is polyubiquitinated, further activating TAK1 [240].

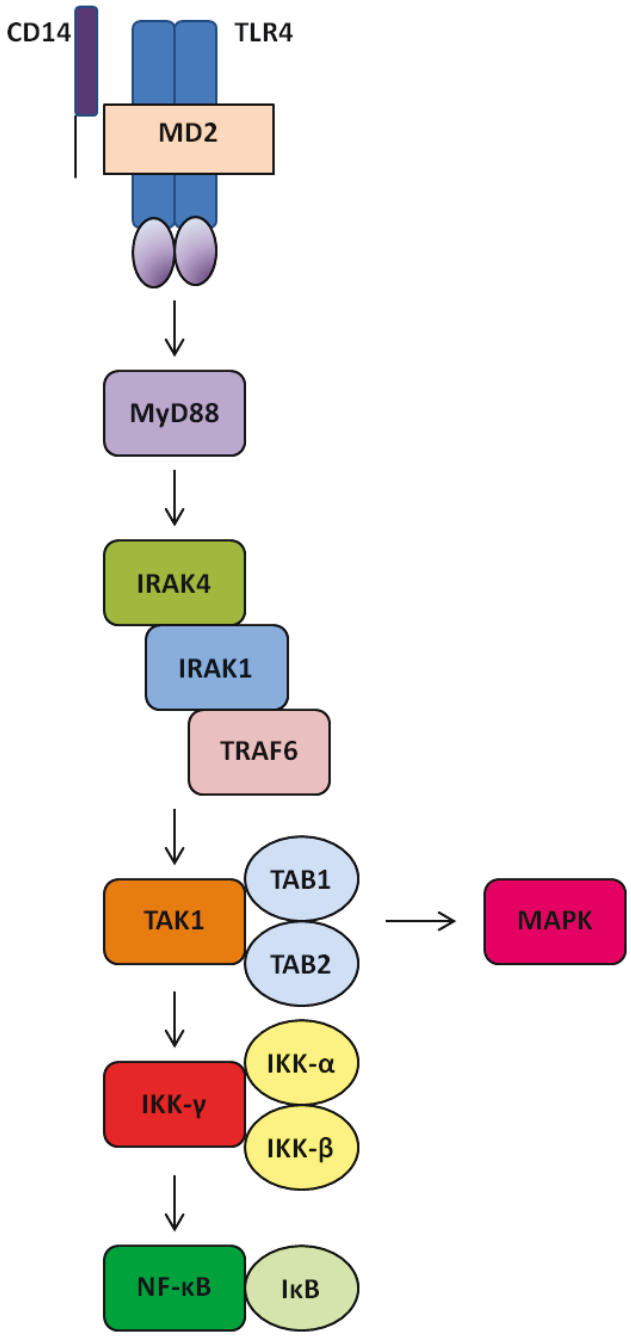

Figure 7.
Toll-like receptor 4 signaling pathway. This diagram depicts the MyD88-dependent signaling pathway for TLR4. Once activated, the intracellular domain of TLR4 binds to MyD88, which recruits IRAK4, IRAK1, and TRAF6 to the plasma membrane. The IRAK-TRAF6 complex then dissociates from the receptor and interacts with the TAK1/TAB1/TAB2 complex. Activation of TAK1 can result in MAPK signaling or the phosphorylation of the IKK complex (IKK-γ/IKKα/IKKβ), which can then cause IκB to disassociate from NF-κB, allowing the NF-κB transcription factor to enter the nucleus and induce inflammatory gene transcription.

Consequently, TAK1 can then phosphorylate both the mitogen-activated protein kinases (MAPK) and the inhibitor of nuclear factor-κB (IκB)-kinase (IKK) complex [241]. TAK1 was shown specifically to phosphorylate MAPK kinase 6 (MKK6) [241], activating the complex MAPK pathway which concludes in inflammatory cytokine release. The NF-κB pathway has been fully described, and it is known that once the IKK complex, (which is composed of IKK-α, IKK-β, and IKK-γ), is phosphorylated, it activates the inhibitor of NF-κB (IκB) [242]. The role of IκB is to sequester NF-κB in the cytoplasm. However, activation of IκB causes it to be ubiquinated and subsequently degraded, allowing the NF-κB transcription factor to enter the nucleus and promote gene expression of inflammatory cytokines and other mediators [243].

There is also the MyD88-independent pathway, which is the main pathway used by TLR3, but has also been shown to be involved in TLR4 signaling [243]. This pathway was elucidated after studies revealed MyD88-deficient mice were still able to activate NF-κB and MAPK family members including, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and p38 MAPK. However, it should be noted that there was a delay in the activation when compared to wild-type mice [225]. This pathway uses the adaptor molecule TIR-domain-containing adaptor protein inducing IFN-β (TRIF). TRIF acts similarly to MyD88, by binding TRAF6 and activating TAK1. It eventually leads to the activation of the NF-κB and interferon-regulatory factor 3 (IRF3) transcription factors, which consequently induce inflammatory and type I interferon cytokines [244].

1.6.4 TLR4 expression and regulation

TLR4 (also known as CD284) is expressed on various cell types, including monocytes [194, 245], macrophages [246], neutrophils [245], eosinophils [247], basophils [248], dendritic cells [245], smooth muscle [249], fibroblasts [250] mast cells [251], T lymphocytes [194], and B lymphocytes [194]. A lot of research has been done on the expression of TLR4 on epithelial cells [252] and it has been demonstrated that TLR4 can be found on the basolateral and subapical surfaces of these cells [252, 253]. TLR4 can be upregulated to the plasma membrane once the cell comes into contact with one of the ligands which activate the receptor [254]. The extent of TLR4 expression seems to depend on cell type and where in the body the cells are located, however it is most highly expressed on immune cells [198].

As mentioned previously, LPS is the main ligand for TLR4, however there are many other bacterial, viral, and host-derived molecules which can activate the receptor as well. TLR4 was first discovered to be the receptor for LPS when it was observed that both the C3H/HeJ and C57BL/10ScCr mouse strains, which have mutations in the *tlr4* gene, were hypo-responsive to LPS [255, 256]. In order for TLR4 to signal properly, two accessory proteins are required, MD-2 and CD14. MD-2 is a glycoprotein associated with the N-terminal of TLR4 and helps to confer LPS responsiveness [257]. MD-2 can be found in the endoplasmic reticulum or the *cis* Golgi, where it associates with TLR4, and together they move to the plasma membrane of the cell [258]. CD14 is a glycophospatidylinositol-anchored protein which helps to increase LPS [259], as well as other mycobacteria responses [260]. The CD14 protein binds the LPS or other microbe-

derived molecules and helps these molecules bind to the TLR4 receptor, thus activating the signaling cascade [261].

There are numerous ligands for TLR4 and each of these can modulate the expression of the receptor. At baseline levels, epithelial cells do not produce a lot of TLR4 mRNA. However, respiratory syncytial virus (RSV) was able to upregulate TLR4 mRNA after 24 hours of exposure. RSV was also able to increase TLR4 protein expression and membrane localization on epithelial cells [254]. Hepatitis C virus can induce TLR4 expression in hepatocytes as well as B cells [262]. Various endotoxins were shown to be able to induce TLR4 mRNA as well as TLR4 surface expression on monocytes [263], while macrophages exposed to ozone were able to increase the surface expression of TLR4 [264]. Recently, studies have shown that microRNA can modulate TLR4 expression. The microRNAs miR-let7e and miR-146a both cause downregulation of TLR4 in macrophages [265, 266], and miR-let7i was able to decrease protein expression of TLR4 in biliary epithelial cells [267].

The effect of LPS on TLR4 expression seems to be dependent on the cell type as well as duration of exposure. Macrophages stimulated with LPS for 2.5 hours reduced their TLR4 mRNA expression. Interestingly, after 20 hours of LPS stimulation, the mRNA expression returned to normal, although the protein expression was reduced [268]. In another study, LPS was able to induce TLR4 mRNA expression while concomitantly decreasing the surface expression on monocytes [269]. TLR4 expression was also decreased on $CD16^+$ monocytes after LPS stimulation for 2 hours [270]. LPS was able to induce TLR4 mRNA on both airway smooth muscle cells and vascular endothelial cells [271, 272] and TLR4

protein expression was increased on airway epithelial cells after 18 hours of LPS exposure [24]. This research has revealed that the modulation of TLR4 by LPS is quite specific.

Cytokines, which are released after TLRs are activated, have a feedback mechanism and can modulate TLR expression as well. IL-1β was able to induce TLR4 mRNA on airway smooth muscle cells [271]. The Th2 cytokines, IL-4 and IL-13 caused TLR4 mRNA and protein expression to decrease on intestinal epithelial cells, while IFN-γ increased TLR4 expression [273]. IFN-γ was also able to induce TLR4 mRNA and protein expression monocytes [269].

Cigarette smoke has also been shown to regulate TLR4 expression on various cell types, especially epithelial cells. Cigarette smoke extract was able to decrease TLR4 mRNA and protein expression on an epithelial cell line after 4 hours of exposure [274]. However, TLR4 protein expression was increased on epithelial cells after being exposed to cigarette smoke for 18 hours [24]. Using flow cytometry, it was observed that after a 3 hour smoke exposure, macrophages were able to decrease their TLR4 surface expression, while increasing their intracellular expression [23]. Similar results were observed by Mortaz et al. when they exposed epithelial cells to cigarette smoke for 2 hours, which resulted in decreased surface expression of TLR4, while the intracellular expression increased [25].

1.6.5 TLR9 expression and regulation

TLR9 (also known as CD289) is expressed on several cell types. The *TLR9* gene was found to be expressed in low levels in peripheral blood

mononuclear cells, but its mRNA expression was increased after IFN-γ treatment [275]. More specifically, TLR9 can be found on T lymphocytes [194, 195], neutrophils [276, 277], and platelets [278]. TLR9 is present on monocytes but its expression increases once they mature into alveolar macrophages [246]. It should also be noted that TLR9 is most predominately found on plasmacytoid dendritic cells and B cells [194, 195, 279], as well as airway epithelial cells [280]. Activated TLR9 on any cell type can induce several proinflammatory cytokines, with IL-6, IL-8, and TNF-α being the ones most widely studied [243, 280, 281].

As early as 1995, it was observed that CpG could activate cells to undergo proliferation [282]. It was a few years later when using *TLR9* knockout mice, it was revealed that CpG DNA and synthetic CpG motif-containing oligodeoxynucleotides (ODN) are recognized by TLR9 [279, 283]. Mammals do have their own CpG DNA within cells, however it is normally methylated. Bacterial CpG DNA is unmethylated and it is this distinction that allows only the microbial CpG DNA to target TLR9 [284]. In its inactive state, TLR9 is localized to the endoplasmic reticulum (ER) in association with UNC93B, an ER protein. Once the cell becomes active, TLR9 is proteolytically cleaved, and UNC93B helps to traffic the receptor to the endosome [285, 286]. In the absence of CpG DNA, TLR9 can be found inside endosomes localized near the plasma membrane [287]. In order for CpG motifs to bind TLR9, they too must be endocytozed into the vesicle before the receptor can be activated and once this happens, these vesicles tend to travel away from the plasma membrane [287, 288]. In addition, in order for proper TLR9 signaling to occur, endosomal maturation must occur. Chloroquine is a therapeutic agent which is able to inhibit endosomal acidification,

which is necessary for proper endosomal maturation. When chloroquine was added to cells in the presence of CpG motifs, the cells were unable to respond [288, 289] demonstrating the need for proper endosomal activity for TLR9 signaling.

Recently, studies have investigated the role of cigarette smoke on TLR9 activation. Mortaz and colleagues revealed that TLR9 mRNA expression was increased after 5 hours of cigarette smoke treatment. They also showed that cigarette smoke could induce TLR9 expression on neutrophils, although not to the same extent as CpG ODN stimulation. This increased TLR9 expression was also attenuated when inhibitors of nitrogen oxide or ROS were used [28].

Studies using TLR9-deficient mice have emphasized the role of this receptor in modulating immune responses against viral DNA [290-292]. These studies revealed that TLR9 activation does not only lead to the induction of inflammatory genes via NF-κB, but there is also an induction of IFN genes leading to a Th1 type immune response. Through this type of activation, CpG DNAs are active in several infectious diseases [292-294]. In addition, the strong induction of IFN genes after TLR9 activation can lead to the proliferation and activation of $CD8^+$ T cells [295]. Furthermore, due to the induction of IFNs which can consequently activate antigen presenting cells, CpG motifs can improve the immunogenicity of several antigens used in vaccines [296-298]. Studies have shown that the use of CpG ODN results in extremely strong Th1 responses when compared to other types of adjuvants [299, 300]. Additionally, the strong induction of a Th1 type immune response led researchers to believe that using CpG motifs as an adjuvant to asthma treatments may be beneficial as it will skew

the immune system towards a Th1 response while inhibiting the Th2 response normally observed in allergic diseases [301-303]. A mouse model of asthma revealed that CpG ODN can be given either with an antigen or post antigen sensitization to reduce allergen-induced airway inflammation [304]. Clinical trials have also demonstrated how the allergic Th2 response could be skewed towards a Th1 response with the use CpG ODN in conjunction with ragweed allergen [305, 306].

1.6.6 TLRs and COPD

TLRs have been shown to be expressed on various cell types within the lung, including airway epithelium, alveolar macrophages, monocytes, lymphocytes, smooth muscle, neutrophils, eosinophils and mast cells [217]. Although there has been no direct correlation between TLR expression and COPD [307], these receptors have been strategically placed on these cells so that they can play a vital role in pulmonary inflammatory diseases and therefore most probably contribute to the pathogenesis of COPD in some manner. In addition, bacteria and viruses are ligands for these receptors and the fact that both of these microbes have been shown to play a role in COPD development supports the fact that these receptors function in the disease. COPD is characterized by the breakdown of lung tissue, and studies have shown that TLR activation can lead to the release of matrix metalloproteases, along with the induction of cytokines [308, 309]. At the same time, activation of TLRs can also lead to the release of protective, antimicrobial peptides, including human β-defensin-2, which is upregulated in COPD patients [274, 310]. Furthermore, neutrophil recruitment to

the lungs of smoke-exposed mice was found to be TLR4-dependent [26]. Taken together, TLRs may play a very precise role in COPD development. While activation of these receptors may lead to the destruction of the lung, and therefore promote the disease; there is also a protective role these receptors may play in attempting to impede further damage through the release of certain mediators. In addition, throughout all of this, the activation of these receptors continually leads to inflammatory cytokine release, which itself can further promote inflammation while still protecting against further destruction. Therefore, these receptors must be delicately balanced in order for the best outcome to occur. It has been suggested that TLRs can be potentially used as therapeutic treatments for COPD [311], but it is clear that we need a better understanding of the interactions between the TLRs and immune cells before this can be achieved. In recent years, both TLR4 and TLR9 have been the most studied TLRs in terms of COPD [28, 29, 274, 312] and it is believed that these receptors may play a role in the development and sustained inflammation observed in COPD patients.

RATIONALE

In asthma, it was demonstrated that TLRs, particularly TLR4, can be activated on epithelial cells to produce cytokines. When these receptors were inhibited, it was found that airway allergic inflammation was abolished, demonstrating the role of TLRs in initiating inflammation in asthma [313]. Furthermore, our laboratory has previously shown that TLR4 is present on $CD3^+$ T cells in atopic individuals. In addition, it was also demonstrated that when nasal mucosa is stimulated with an allergen and LPS, there is an increase in the number of $CD4^+$ T cells, confirming that these cells are also playing a role in the inflammation observed in asthma [314].

In COPD, microbes and pathogens have been shown to induce the development of the disease. Epithelial cells in COPD have been shown to express TLRs, however $CD8^+$ T cells, the hallmark cell in COPD, have not been considered. I believe that TLRs may function in epithelial cells and $CD8^+$ T cells in COPD similarly to how they do in asthma. Furthermore, epithelial cells exposed to cigarette smoke can induce cytokine production and I think that cigarette smoke exposure of $CD8^+$ T cells may help drive the inflammation in COPD. I would like to investigate how cigarette smoke affects TLR expression and activation, using cytokines as a measure of this activation. Lastly, I will investigate how these cells function and regulate the inflammation in response to this toxin.

HYPOTHESIS

In this thesis project, we hypothesized that Toll-like receptor expression is increased in COPD patients compared to control subjects; and this contributes to the pathogenesis of the disease via activation of $CD8^+$ T cells and epithelial cells in response to cigarette smoke exposure.

OBJECTIVES

The first objective of this study was to evaluate the expression of TLR4 and TLR9 in COPD patients compared to control subjects. Next, we wanted to localize the TLR expression to $CD8^+$ T cells as well as epithelial cells. Finally, we wanted to determine the effect of cigarette smoke on the aforementioned cell types by using both human samples and primary cells.

CHAPTER 2: Cigarette smoke increases TLR4 and TLR9 expression and induces cytokine production from $CD8^+$ T cells in chronic obstructive pulmonary disease

Jessica Nadigel[1], David Préfontaine[1], Carolyn J. Baglole[1], Jean Bourbeau[2], François Maltais[3], David H. Eidelman[1], and Qutayba Hamid[1]

[1]Meakins-Christie Laboratories, Faculty of Medicine, McGill University, Montreal, Qc, [2]Respiratory Division, Research Institute of McGill University Health Centre, Montreal, Qc, and [3]Respiratory Division, Laval University, Quebec, Qc

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ABSTRACT

Cigarette smoke is a major risk factor for chronic obstructive pulmonary disease (COPD), an inflammatory lung disorder. COPD is characterized by an increase in $CD8^+$ T cells within the central and peripheral airways. We hypothesized that the $CD8^+$ T cells in COPD patients have increased Toll-like receptor (TLR) expression compared to control subjects due to the exposure of cigarette smoke in the airways.

Endobronchial biopsies and peripheral blood were obtained from COPD patients and control subjects. TLR4 and TLR9 expression was assessed by immunostaining of the lung tissue and flow cytometry of the peripheral blood. $CD8^+$ T cells isolated from peripheral blood were treated with or without cigarette smoke condensate (CSC) as well as TLR4 and TLR9 inhibitors. PCR and western blotting were used to determine TLR4 and TLR9 expression, while cytokine secretion from these cells was detected using electrochemiluminescence technology.

No difference was observed in the overall expression of TLR4 and TLR9 in the lung tissue and peripheral blood of COPD patients compared to control subjects. However, COPD patients had increased TLR4 and TLR9 expression on lung $CD8^+$ T cells. Exposure of $CD8^+$ T cells to CSC resulted in an increase of TLR4 and TLR9 protein expression. CSC exposure also caused the activation of $CD8^+$ T cells, resulting in the production of IL-1β, IL-6, IL-10, IL-12p70, TNF-α and IFN-γ. Furthermore, inhibition of TLR4 or TLR9 significantly attenuated the production of TNF-α and IL-10.

Our results demonstrate increased expression of TLR4 and TLR9 on lung $CD8^+$ T cells in COPD. $CD8^+$ T cells exposed to CSC increased TLR4 and TLR9 levels and increased cytokine production. These results provide a new perspective on the role of $CD8^+$ T cells in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide [3], with more than 80% of COPD cases caused by cigarette smoking [315]. The chronic inflammation observed in COPD is characterized by proinflammatory cytokine production and recruitment of several cell types to the lungs, including cells of the innate immune response, such as neutrophils and macrophages [114], as well as those of the adaptive immune response, namely T and B lymphocytes [70, 111]. $CD8^+$ T cells are regarded as a hallmark cell of COPD, and are increased in both the central [107] and peripheral [66] airways of COPD patients. $CD8^+$ T cells found within the airways are generally located within the submucosa and invading the epithelium [139, 316]. Unfortunately, the role of $CD8^+$ T cells in COPD and the mechanisms by which they are recruited to the lung are still generally unknown. While it can be speculated that these cytotoxic T cells promote injury to the already damaged lung, they could also contribute towards protecting the lung by sensing invading microbes and using their cytotoxic abilities to eliminate infected cells.

Toll-like receptors (TLR), a key component of the innate immune system, sense foreign microbes via pathogen-associated molecular patterns. Although largely found on innate immune and structural cells [245, 313], TLRs are also present on T cells, thereby contributing to the adaptive immune response [194, 195, 314, 317]. TLR4, which recognizes Gram-negative bacteria, and TLR9, which binds unmethylated CpG motifs, are two well-studied TLRs. Activation of TLR4 or TLR9, results in signal transduction cascades involving downstream

pathways, including nuclear factor of kappa B (NF-κB) and c-Jun N-terminal kinase (JNK) [243]. This ultimately results in the production of inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α and IL-10 which can then go on to modulate inflammatory responses [210, 318, 319].

There is growing interest in the potential role of TLRs in COPD pathogenesis [320, 321], including the relationship between cigarette smoke exposure and the expression of TLRs on epithelial cells [24, 274]. In our study, we investigated the expression of TLR4 and TLR9 on $CD8^+$ T cells, an important cell type in COPD pathogenesis. We report for the first time increased expression of TLR4 and TLR9 on $CD8^+$ T cells in lung tissue of COPD patients compared to control subjects. Moreover, our data further demonstrates that cigarette smoke exposure induces TLR4 and TLR9 expression on $CD8^+$ T cells, which results in increased production of cytokines, including IL-1β, IL-6, IL-10, IL-12p70, TNF-α and IFN-γ. Cigarette smoke activation of TLRs on $CD8^+$ T cells and the resulting increased cytokine production represents a mechanism by which $CD8^+$ T cells can contribute to the pathogenesis of COPD.

METHODS

Study subjects

Endobronchial biopsies from eight COPD patients and five aged-matched control subjects were received from the Tissue Bank of the Respiratory Health Network of the FRSQ, MUHC site. Peripheral blood was obtained from nine COPD patients and eight control subjects recruited at the Montreal Chest Institute. Each participant gave a total of 20 ml of peripheral blood and underwent spirometry. Control subjects represented healthy volunteers, either non-smokers or ex-smokers, with normal lung function. Participant details can be found in Table 1. This study was reviewed and approved by The Biomedical C Research Ethics Board of the Montreal Chest Institute, and written informed consent was obtained from all subjects.

Immunostaining

Endobronchial biopsies were taken from the segmental and subsegmental carinas of the right upper lobe and immediately fixed in paraformaldehyde. Biopsies were then embedded in paraffin and cut into 5 μm sections. The sections were de-paraffinized in xylene, rehydrated in ethanol and washed with phosphate-buffered saline (PBS). Antigen retrieval was performed using citrate buffer (pH 6); the sections were permeabilized with 0.2% Triton X-100 (in PBS) and then incubated with 5% hydrogen peroxide (in PBS). After washing, the sections were blocked with Universal Blocking Solution (Dako, Burlington, ON) for 30 minutes. Slides were incubated overnight at 4ºC with a rabbit polyclonal IgG antibody

against TLR4 or TLR9 (1.25 μg/ml; Abcam, Cambridge, MA). Slides were then incubated with a biotinylated secondary antibody raised in goat (1:100), followed by incubation with SA-HRP (Vector Laboratories, Burlington, ON). The immunostainings were developed using the Liquid DAB+ Substrate Chromogen System (Dako) according to the manufacturer's instructions and counterstained with hematoxylin and lithium carbonate. Within the submucosa, the number of immune cells positive for TLR4 or TLR9 were quantified and expressed as the number of positive immune cells per square millimeter of subepithelium.

To co-localize TLR4 or TLR9 to $CD8^+$ cells, double immunofluorescence was performed on endobronchial biopsies embedded in OCT. Blocks were cut into 5 μm sections and fixed with 70% ethanol for 30 seconds. After washing with PBS, sections were permeabilized and blocked as above. Sections were incubated overnight at 4ºC with a monoclonal IgG1κ CD8 antibody (clone C8/144B, Dako) at a concentration of 2 mg/ml. After washing, the sections were incubated with a rabbit anti-mouse AlexaFluor 555 (Invitrogen, Carlsbad, CA) secondary antibody for 1 hour in the dark at a concentration of 5 μg/ml. Next, the sections were incubated with either the TLR4 or TLR9 antibody as described above for 2 hours at room temperature. A goat anti-rabbit AlexaFluor 488 (Invitrogen) secondary antibody was used at a concentration of 5 μg/ml for 1 hour in the dark. Sections were then washed and mounted using PermaFluor™ Aqueous Mounting Medium (Thermo Scientific, Waltham, MA). The absolute numbers of the single- and double-stained cells were counted and results were expressed as the percentage of $CD8^+$ T cells expressing TLR4 or TLR9. All slides were imaged using the Image-Pro Plus 6.2 system (Media Cybernetics, Bethesda, MD).

Peripheral blood mononuclear cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were separated from the peripheral blood using Ficoll-Paque Plus (GE Healthcare, Baie D'Urfé, QC), which separates cells using density gradient centrifugation. Ten milliliters of Ficoll-Pacque Plus were aliquoted into 50 ml tubes. Approximately 10 ml of peripheral blood was mixed with 20 ml of PBS and was then carefully layered on top of the Ficoll in the 50 ml tubes. Samples were then centrifuged at 1,500 rpm for 30 minutes, without using the brakes of the centrifuge. The PBMC layer was then collected using a Pasteur pipette. The PBMCs were then washed twice in PBS and centrifuged for 10 minutes at 1,500 rpm. Cells were then maintained in RPMI-1640 medium (Thermo Scientific) supplemented with 10% v/v fetal bovine serum (FBS; HyClone, Logan, Utah), 4 mM L-glutamine (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), and 1.5% 1M HEPES (Invitrogen) and incubated at 37ºC with 5% CO_2 overnight.

Flow cytometry cell staining of PBMC

PBMCs were first incubated with intravenous immunoglobulin to prevent any non-specific binding. Cells were then stained with an AlexaFluor 488-conjugated mouse IgG2a antibody against TLR4, clone HTA125 (AbDSerotec, Kidlington, Oxford), or with a FITC-conjugated mouse IgG2a antibody against TLR9, clone 5G5 (Abcam). All cells were stained with a CD8 antibody conjugated to APC, mouse IgG1κ, clone RPA-T8 (BD Pharmingen™, Mississauga, ON,) and a CD3 antibody conjugated to PE, mouse IgG1κ, clone

UCHT1 (BD Pharmingen™). All corresponding isotype control antibodies were used. Cells stained for TLR9 were permeabilized with Cytofix/Cytoperm (BD Pharmingen™). A total of 25,000 events were acquired by flow cytometry (FACSCalibur, BD Bioscience) and analyzed using CellQuest™ Pro Software. Data are presented as the percentage of positively stained cells for each subject.

$CD8^+$ T cell isolation and cigarette smoke condensate treatment

$CD8^+$ T cells were isolated from PBMCs using the $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, DE) according to the manufacturer's protocol. Briefly, PBMCs were suspended in buffer (PBS containing 0.5% w/v BSA and 2 mM EDTA, pH 7.2) and incubated with a biotin-antibody cocktail containing antibodies against CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCR γ/δ and CD235a for 10 minutes at 4ºC. Next, a microbead-conjugated antibody against CD14 and biotin was added for 15 minutes at 4ºC. Cells were then washed in 2 ml of buffer and centrifuged at 1,500 rpm for 5 minutes and then resuspended in 0.5 ml of buffer. The cell suspension was separated using LS columns with the QuadroMACS™ (Miltenyi Biotec) cell separator. The enriched $CD8^+$ T cells passed through the column and were collected. These cells were then washed with PBS by centrifuging at 1,500 rpm for 10 minutes prior to continuing with the experiment. The $CD8^+$ T cell purity was $\geq$ 95% as confirmed by flow cytometry staining using the anti-CD8 antibody as described previously.

Once purified, the $CD8^+$ T cells were incubated with cigarette smoke condensate (CSC), which was received as a gift from Imperial Tobacco Canada, generated as previously reported [322]. In brief, CSC was generated by smoking 1R4F research cigarettes on a Cerulean ASM 500 smoking machine. Condensates were collected on filter pads and extracted using DMSO to yield 15 mg TPM/ml. The CSC was then stored at -80ºC and only defrosted prior to use. The $CD8^+$ T cells were treated with CSC at either 10 or 50 μg/ml for 24 hours. $CD8^+$ T cells were also pretreated with or without a TLR4 neutralizing antibody (10 μg/ml; clone HTA125, eBioscience, San Diego, CA) or chloroquine (20 μg/ml; Invivogen, San Diego, CA) for 1 hour followed by CSC at 50 μg/ml for 24 hours. Cells receiving DMSO were used as the control as DMSO was the solvent for the CSC.

Protein quantification and immunoblotting

$CD8^+$ T cells were lysed in 100 μL of lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton x-100, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.27 M sucrose, 5 mM sodium pyrophosphate decahydrate and Complete Mini protease inhibitor cocktail). Protein concentrations were quantified using the BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Fifty micrograms of protein were boiled and separated on a 10% Pro-Pure Next Gel with Pro-Pure Running Buffer (Amresco, Solon, OH). After transferring proteins to nitrocellulose, membranes were blocked for 1 hour at room temperature in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE). Blots were then

incubated with an anti-TLR4 antibody (H-80; Santa Cruz, Santa Cruz, CA) at a concentration of 0.2 μg/ml or anti-TLR9 antibody (Abcam) at a concentration of 1 μg/ml overnight at 4ºC. Goat anti-rabbit IgG (DyLight^{TM}800, Thermo Scientific) or goat anti-mouse IgG (DyLight^{TM}680, Thermo Scientific) antibody was applied for 1 hour in the dark at room temperature (1:15,000). The signal was detected and quantified using a Li-Cor Odyssey imaging system. All samples were normalized to GAPDH (Millipore, Billerica, MA) and expressed as a ratio relative to the DMSO sample.

Cytokine protein quantification

Supernatants were collected 24 hours after $CD8^+$ T cells were treated with CSC and stored at -80ºC until used. The Human ProInflammatory 7-Plex Tissue Culture Kit was purchased from Meso Scale Discovery (MSD; Gaithersburg, MD) and used according to the manufacturer's protocol. Briefly, a 150 μl of a 1% (w/v) blocking solution (prepared by company) was added to each well of the MSD plate which was then left to incubate at room temperature for 1 hour while shaking. After washing with PBS and 0.05% Tween-20, 25 μl of samples or calibrators were added to each well and incubated for 2 hours at room temperature while shaking. The detection antibody (25 μl per well) was then added and left to incubate again for 2 hours while shaking. Next, the plate was washed with PBS and 0.05% Tween-20 and then 150 μl of 2X Read Buffer T (provided by company) was added to each well. The MSD plate was then read using the SECTOR® Imager 2400. The kit allowed for the quantification of IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and IFN-γ. The lower limits of detection for this plate

were as follows: IL-1β, 0.0199 pg/ml; IL-6, 0.0158 pg/ml; IL-8, 0.0125 pg/ml; IL-10, 0.0794 pg/ml; IL-12p70, 0.0199 pg/ml; TNF-α, 0.3162 pg/ml, and IFN-γ, 0.0 pg/ml. The upper limit of detection was 10,000 pg/ml for all seven cytokines.

Statistical analysis

For statistical analysis between two groups, a t-test was used. Comparison between more than two groups was performed by ANOVA, followed by a Bonferonni multiple comparison test. A p-value of < 0.05 was considered statistically significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.0001$). Data are expressed as mean ± standard error of mean.

RESULTS

TLR4 and TLR9 are expressed in COPD patients and control subjects

Activation of TLRs can lead to the production of proinflammatory mediators, many of which are increased in COPD. However, there is little information on the expression of TLR4 or TLR9 in the lungs of COPD patients. Therefore, we first examined the expression of TLR4 and TLR9 by immunohistochemistry in endobronchial biopsies from COPD patients and control subjects. TLR4 is expressed in the airway epithelium and inflammatory cells in both healthy controls (Figure 1A) as well as COPD patients (Figure 1B). In addition, immunoreactivity of TLR9 was also found in the epithelium and the immune cells within the subepithelial layer in both control subjects (Figure 1C) and patients with COPD (Figure 1D). The epithelium was scored according to the extent of staining, thus the percentage of epithelial cells showing positive immune reactivity. There was no significant difference between controls and COPD patients in the percentage of the epithelial cells expressing TLR4 (Figure 2A) or TLR9 (Figure 2B). In order to further quantify the staining in the subepithelium, the number of positive immune cells per square millimeter were counted from both COPD and control biopsies for each of the TLRs of interest. There was also no significant difference in the number of immune cells expressing TLR4 (Figure 2C) or TLR9 (Figure 2D) in COPD biopsies compared to control biopsies.

Co-localization of TLR4 and TLR9 on lung $CD8^+$ T cells in COPD

Although the total lung expression of TLR4 and TLR9 did not differ significantly between COPD patients and control subjects, we wanted to examine the expression of TLR4 and TLR9 more closely in lung $CD8^+$ T cells. Therefore, we performed immunofluorescence staining on endobronchial biopsies obtained from both COPD and control subjects. Slides were stained for the presence of $CD8^+$ T cells, (Figures 3A, 3D), and stained with either TLR4 (Figure 3B) or TLR9 (Figure 3E) antibodies. An overlay of the images shows co-localization of CD8 immunoreactivity and TLR4 (Figure 3C) and TLR9 (Figure 3F) positive cells.

Quantification of the staining was performed by counting the absolute numbers of single-stained $CD8^+$ T cells compared to the double-stained $CD8^+$ T cells, resulting in a percentage of $CD8^+$ T cells which express TLR4 and TLR9. In the lung tissue of COPD patients, there was a significant increase in the percentage of $CD8^+$ T cells expressing TLR4 (Figure 4A) ($p < 0.0001$) and TLR9 (Figure 4B) ($p < 0.0001$) when compared to control subjects. COPD patients had approximately 90% of their $CD8^+$ T cells co-expressing TLR4 and TLR9. The number of cells co-expressing CD8 and TLR4 or TLR9 was substantially lower in control subjects compared to COPD patients, with less than 20% of $CD8^+$ T cells expressing TLR4 or TLR9.

Expression of TLR4 and TLR9 on peripheral blood mononuclear cells

Next, we wanted to investigate TLR4 and TLR9 expression in peripheral blood to observe if the expression was similar to that of the lung. Peripheral blood

mononuclear cells (PBMC) were isolated and stained with TLR4 and TLR9 antibodies. Flow cytometry was used to determine that COPD patients had a significantly higher percentage of total PBMCs which expressed TLR4 (Figure 5A) ($p < 0.05$) and TLR9 (Figure 5B) ($p < 0.05$) compared to aged-matched control subjects. To determine if the PBMCs expressing TLR4 or TLR9 included $CD8^+$ T cells, we stained the PBMCs with CD8, TLR4 or TLR9 and CD3. A CD3 antibody was used to ensure that the population of analyzed cells consisted of true T cells expressing CD8. It was found that the percentage of $CD8^+$ T cells which expressed TLR4 (Figure 5C) and TLR9 (Figure 5D) were not statistically different between COPD patients and control subjects. In addition, the overall expression of TLR4 and TLR9 was quite minimal on peripheral blood $CD8^+$ T cells. This indicates that, in contrast to what was observed in the lungs of COPD patients, $CD8^+$ T cells do not greatly contribute to TLR expression in the blood. While approximately 90% of the COPD lung $CD8^+$ T cells expressed these receptors, this was only observed in about 2% of peripheral blood $CD8^+$ T cells. This raises the possibility that there may be a factor in the lungs of COPD patients not found within in the blood, which can induce TLR4 and TLR9 expression on $CD8^+$ T cells. Furthermore, this factor would also have to be absent from the lungs and blood of control subjects as these subjects had low TLR expression in general.

Cigarette smoke condensate upregulates TLR4 and TLR9 protein expression

Our findings led us to speculate that cigarette smoke may be the contributing factor that can induce TLR4 and TLR9 expression on $CD8^+$ T cells.

Cigarette smoke is the primary risk factor for COPD, and considering the location of $CD8^+$ T cells within the lung tissue, one would expect that these cytotoxic cells are exposed to smoke. Cigarette smoke exposure would also explain the lack of co-localization found in the peripheral blood. To test this hypothesis, $CD8^+$ T cells isolated from the peripheral blood of COPD patients were treated with cigarette smoke condensate (CSC) for 24 hours, mimicking the effects of cigarette smoke exposure to the lungs. Cytotoxicity was determined using trypan blue staining to demonstrate that greater than 80% of cells remained viable at concentrations of CSC up to 50 μg/ml (Addendum Figure 2); this concentration approximates human exposures [323-325].

TLR4 and TLR9 gene expression was assessed by qRT-PCR, and there was no significant change in TLR4 or TLR9 expression after 24 hours of CSC exposure (Addendum Figure 3). A similar finding was previously observed when epithelial cells were treated with cigarette smoke [24]. In contrast, the expression of both TLR4 (Figure 6A, 6B) and TLR9 (Figure 6A, 6C) protein was significantly increased ($p < 0.05$) from baseline after a CSC exposure of 50 μg/ml, suggesting these receptors are post-transcriptionally regulated during CSC treatment.

CSC induces cytokine production from $CD8^+$ T cells

Next, we wanted to investigate the activation of $CD8^+$ T cells by CSC to induce cytokine production. COPD- and CD8-associated cytokines were measured in the supernatants of peripheral blood $CD8^+$ T cells treated with CSC, DMSO, or medium for 24 hours. There was no change in the cytokine production

between the cells given media alone or those treated with DMSO. However, there was a significant increase in IL-1β, IL-10, IL-12p70, TNF-α and IFN-γ (Figures 7A,D-F) ($p < 0.05$) at the highest concentration (50 μg/ml) of CSC when compared to the DMSO-treated cells, while IL-6 was only significantly different between the two smoke concentrations (Figure 7B). There was no change in IL-8 production (Figure 7C). It should be noted that the concentrations of all cytokines released was relatively low, suggesting that $CD8^+$ T cells may lack the ability to secrete significant levels of cytokines in COPD. However, these cytokine values correspond to what is observed in the bronchoalveolar lavage fluid of patients with bronchiectasis, another type of chronic lung disease [326]. The significant increase in cytokine production at 50 μg/ml cannot be ignored and may be critical to the inflammatory setting. The increased expression of TLR4 and TLR9 on $CD8^+$ T cells, concomitant with CSC-induced release of inflammatory cytokines, suggests that the CSC is activating the TLR pathways to induce the aforementioned cytokines.

Blocking the TLR4 and TLR9 pathways inhibits cytokine production

To determine if the CSC is inducing cytokine production through the activation of TLR4 and TLR9, we utilized well-described inhibitors of TLR4 [327] and TLR9 [328]. One hour prior to CSC treatment (50 μg/ml), $CD8^+$ T cells were treated with a TLR4 neutralizing antibody (10 μg/ml) or chloroquine (20 μg/ml). Chloroquine prevents endosomal acidification and is a commonly-used TLR9 signaling inhibitor [329]. Chloroquine significantly reduced CSC-induced TNF-α production (Figure 8A) ($p < 0.0001$). IL-10 production was significantly

reduced by blocking either the TLR4 or TLR9 pathways (Figure 8C) ($p < 0.05$). As was observed in Figure 7, there was no change in IL-8 production (Figure 8B). Also, inhibition of TLR4 or TLR9 did not significantly reduce IL-1β, IL-6, IL-12p70 and IFN-γ. Collectively, our data suggests that CSC induces cytokine production from $CD8^+$ T cells through the activation of TLR4 and TLR9. This finding highlights the potential importance of TLRs on $CD8^+$ T cells in promoting inflammation in the lungs of COPD patients.

Table 1: Characterization of smoking status and demographics of COPD patients and control subjects

		Control	COPD
Biopsy		(n = 5)	(n = 8)
	Age (years)	54.8 ± 7.16	65.0 ± 17.33
	Sex (M:F)	4:1	6:2
	FEV_1 (L)	3.69 ± 0.89	2.31± 0.84
	FEV_1/FVC (ratio)	0.83 ± 0.01	0.56 ± 0.08
	GOLD (I/II/III/IV)	0/0/0/0	3/3/2/0
	Smoking History		
	Pack Years	3.4 ± 4.77	38.25 ± 14.76
	Current smokers	0	5
	Ex-smokers	2	3
	Non-smokers	3	0
Blood		(n = 8)	(n = 9)
	Age (years)	59.86 ± 7.15	64.63 ± 4.44
	Sex (M:F)	5:3	6:3
	FEV_1 (L)	3.14 ± 0.63	1.21 ± 0.570
	FEV_1/FVC	0.79 ± 0.03	0.59 ± 0.25
	GOLD (I/II/III/IV)	0/0/0/0	0/4/4/0
	Smoking History		
	Pack Years	13.59 ± 24.33	62.06 ± 29.67
	Current smokers	0	1
	Ex-smokers	4	8
	Non-smokers	4	0

FEV_1: Forced expiratory volume in 1 second, FVC: Forced vital capacity

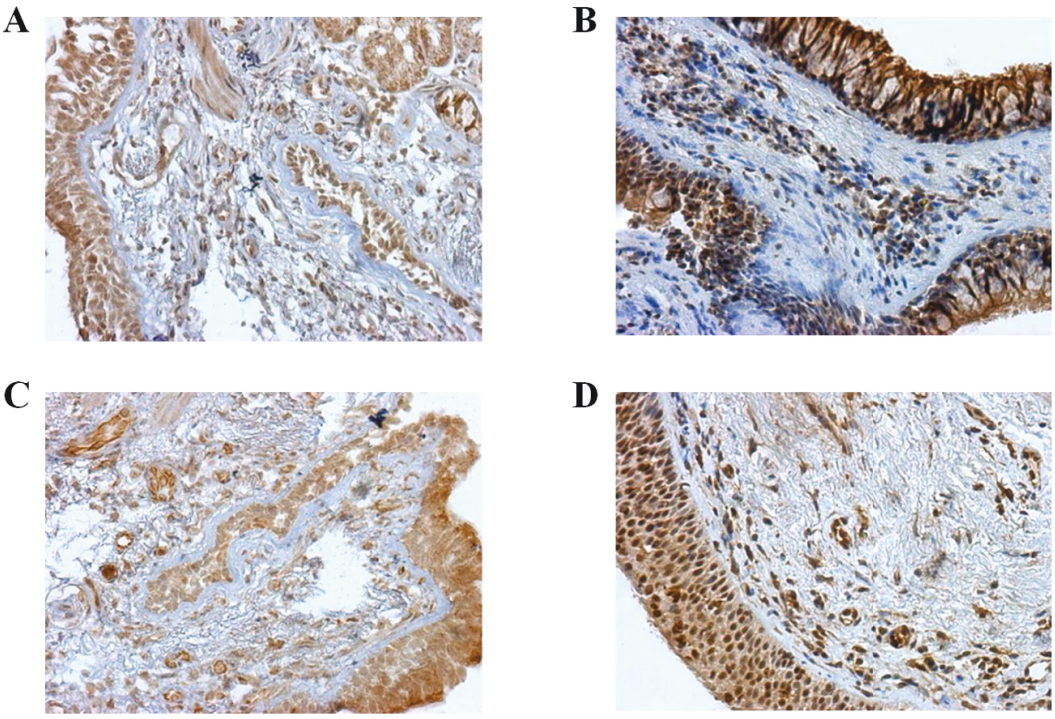

Figure 1.
TLR4 and TLR9 are expressed in lung tissue. Endobronchial biopsy tissue sections from COPD patients and control subjects were processed by immunohistochemistry as described in Methods. Representative examples show similar immunoreactivity for TLR4 in the epithelium and inflammatory cells of control subjects (A) and COPD patients (B). TLR9 was also expressed at comparable levels in the epithelium and inflammatory cells in healthy controls (C) and COPD patients (D). All magnifications are 200x.

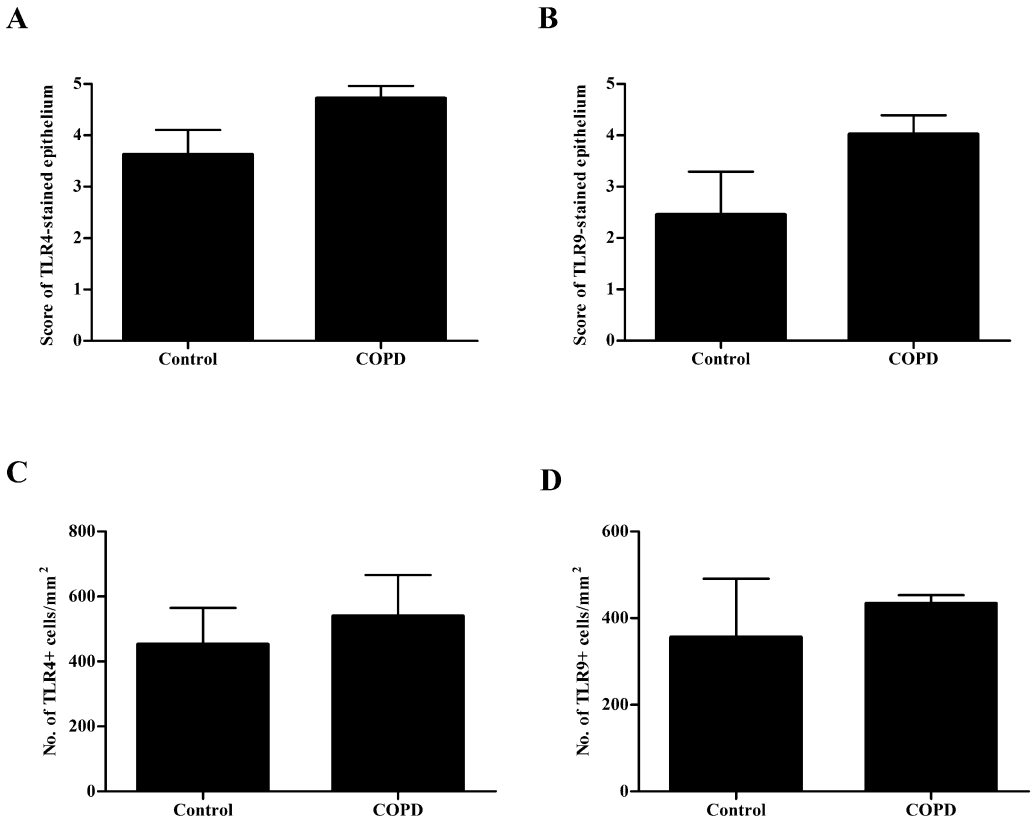

Figure 2.
Quantification of TLR4 and TLR9 in COPD and control subjects. The degree of epithelial staining of TLR4 (A) and TLR9 (B) were scored according to a scale (1 = no staining; 5 = 100% staining) following immunostaining. The numbers of the positively stained immune cells were also counted, and the area of the tissue was measured. There was no significant difference in the percentage of TLR4- (A) or TLR9- (B) stained epithelium between the two groups. There was also no significant difference in the total number of inflammatory cells expressing TLR4 (C) and TLR9 (D) between the control group and COPD patients, when expressed as cells/mm^2 tissue.

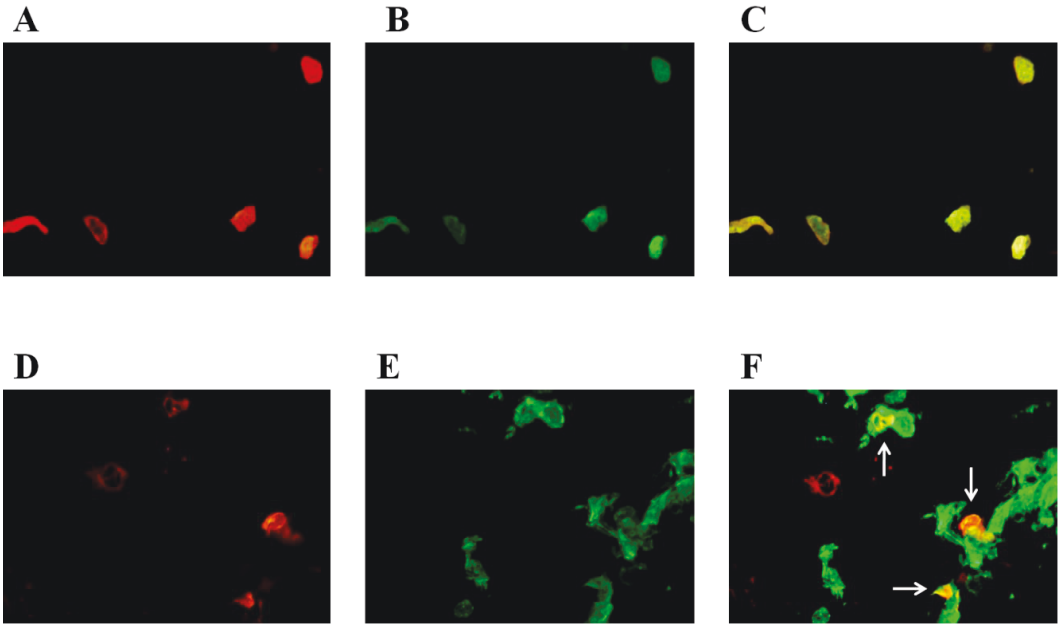

Figure 3.
Lung $CD8^+$ T cells express TLR4 and TLR9 in COPD patients. Representative immunofluorescent images from frozen endobronchial biopsies of COPD patients demonstrate the co-localization of CD8 (red fluorescence, panels A and D) and TLR4 (green fluorescence, panel B) or TLR9 (green fluorescence, panel E). The merged images show $CD8^+$ T cells expressing TLR4 or TLR9 (arrows, panels C and F, respectively). All magnifications are 400x.

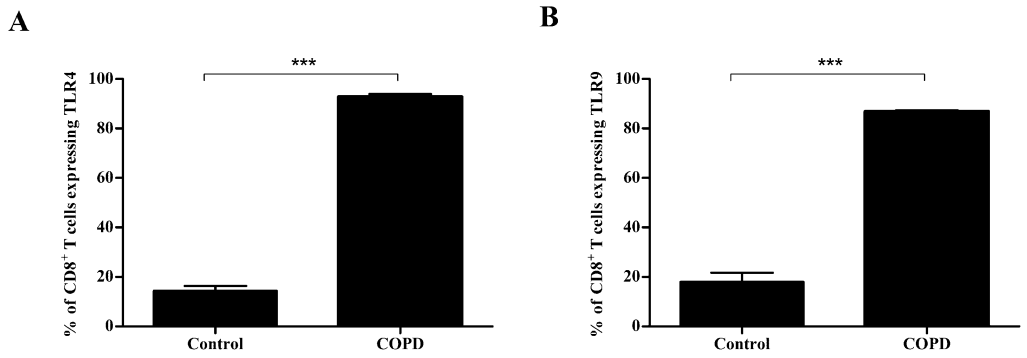

Figure 4.
Quantitative analysis of the number of $CD8^+$ T cells expressing TLR4 or TLR9 in endobronchial biopsies. The absolute numbers of the singled-stained $CD8^+$ T cells were counted and compared to the absolute numbers of $CD8^+$ T cells expressing TLR4 or TLR9 from COPD patients (n = 6) and healthy controls (n = 5). The percentage of $CD8^+$ T cells expressing TLR4 (A) was significantly higher in COPD patients (92.76 ± 1.18) compared to control subjects (14.38 ± 2.00). The percentage of $CD8^+$ T cells expressing TLR9 (B) was also significantly increased in COPD patients (86.88 ± 0.51) compared to healthy controls (17.97 ± 3.74). Data are represented as mean ± SEM. *** indicate $p < 0.0001$.

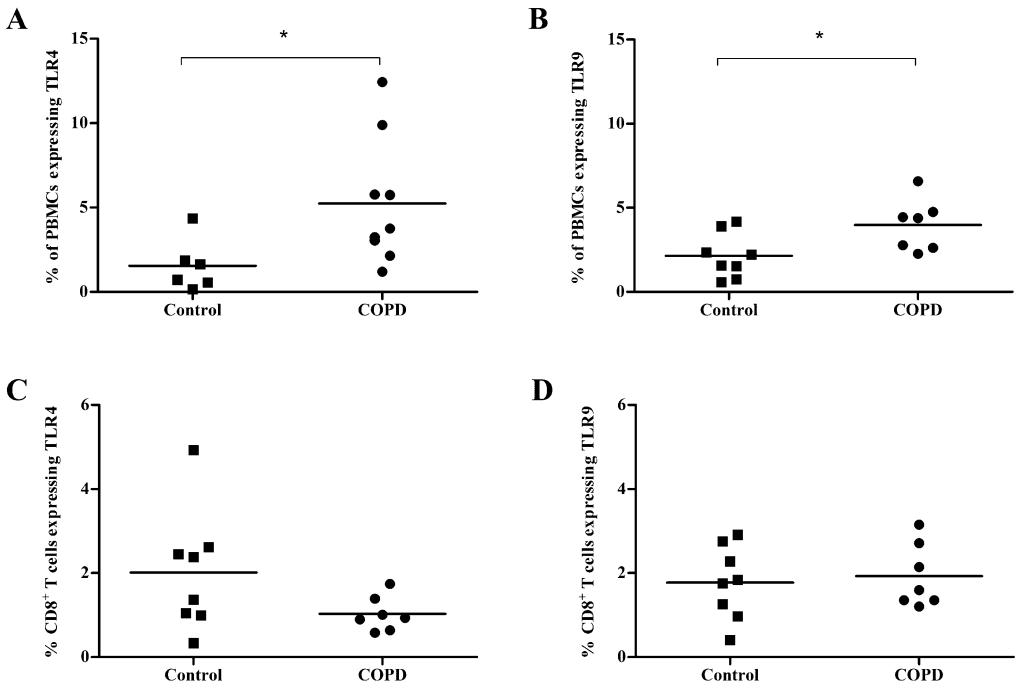

Figure 5.
The expression of TLR4 and TLR9 on $CD8^+$ T cells from peripheral blood. Peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood of COPD patients (n = 7-9) and control subjects (n = 6-8). The percentage of cells positive for CD8, TLR4 and TLR9 were determined by flow cytometry. TLR4 (A) and TLR9 (B) expression in PBMCs was significantly increased in COPD patients compared to healthy controls. PBMCs were double-stained with CD8 and either TLR4 and TLR9 and expressed as the number of $CD8^+$ T cells expressing the receptor. There was no significant difference in the percent of $CD8^+$ T cells expressing TLR4 (C) or TLR9 (D) in COPD patients vs. healthy controls. Each data point represents an individual patient. * indicate $p < 0.05$.

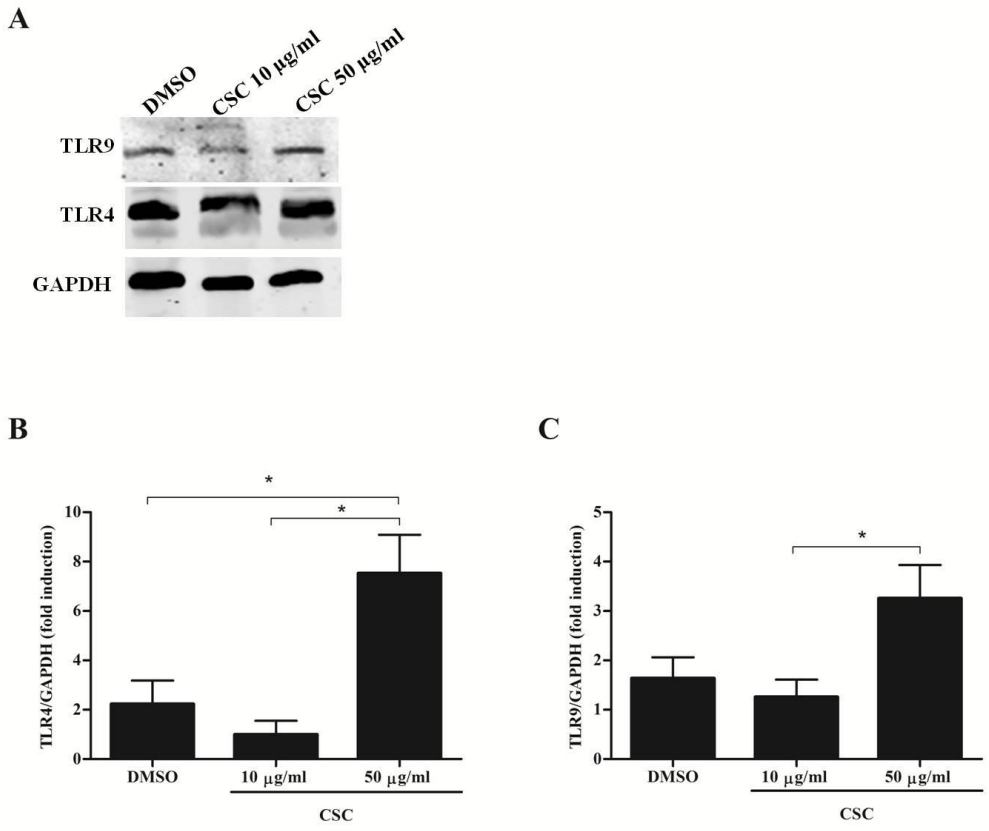

Figure 6.
TLR4 and TLR9 protein expression on $CD8^+$ T cells is upregulated by CSC. $CD8^+$ T cells isolated from the peripheral blood of COPD patients (n = 4-5) and treated with CSC (10 µg/ml or 50 µg/ml) or DMSO for 24 hours. A representative western blot nitrocellulose membrane expressing TLR4, TLR9 and GAPDH (A). Western blot analysis revealed an increase in TLR4 (B) and TLR9 (C) protein expression with a CSC treatment of 50 µg/ml. Individual samples were normalized to GAPDH and then expressed as a relative ratio of treated compared to DMSO sample. Data are represented as mean ± SEM. * indicates $p < 0.05$.

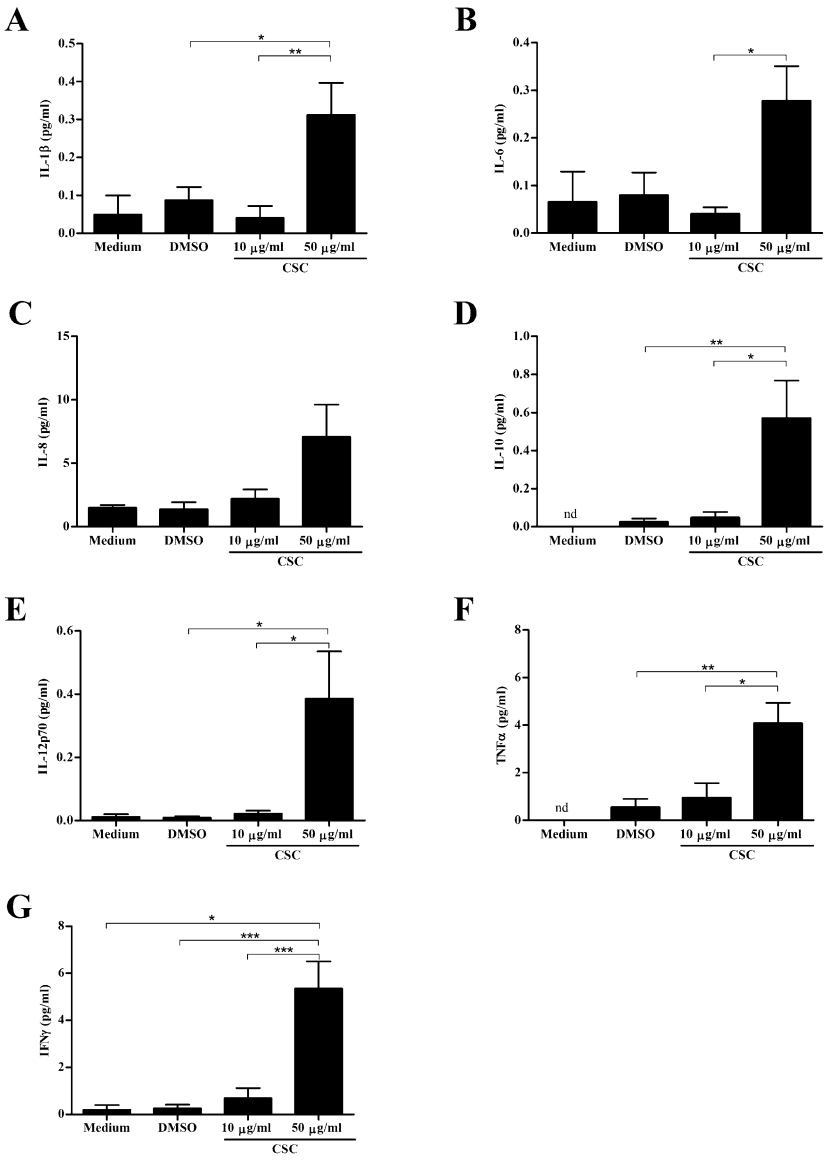

Figure 7.
CSC induces cytokine production from $CD8^+$ T cells. $CD8^+$ T cells from COPD patients (n = 10) were treated with CSC (10 or 50 μg/ml), DMSO, or medium for 24 hours. The supernatants were collected and cytokine expression was examined using the Human ProInflammatory 7-Plex Tissue Culture Plate from Meso Scale Discovery. There was no significant change in cytokine production between the untreated cells or those which were treated with DMSO. At 50 μg/ml of CSC treatment, there was a significant increase in cytokine expression for IL-1β (A), IL-10 (D), IL-12p70 (E), TNF-α (F), and IFN-γ (G) compared to DMSO. There was no change in IL-8 (C) expression. There was also a significant increase in cytokine production at 50 μg/ml compared to cells treated with 10 μg/ml of CSC for IL-1β (A), IL-6 (B) IL-10 (D), IL-12p70 (E), TNF-α (F), and IFN-γ (G). Data are represented as mean ± SEM. * indicates $p < 0.05$; ** indicates $p < 0.01$ and *** indicates $p < 0.0001$. nd = not determined.

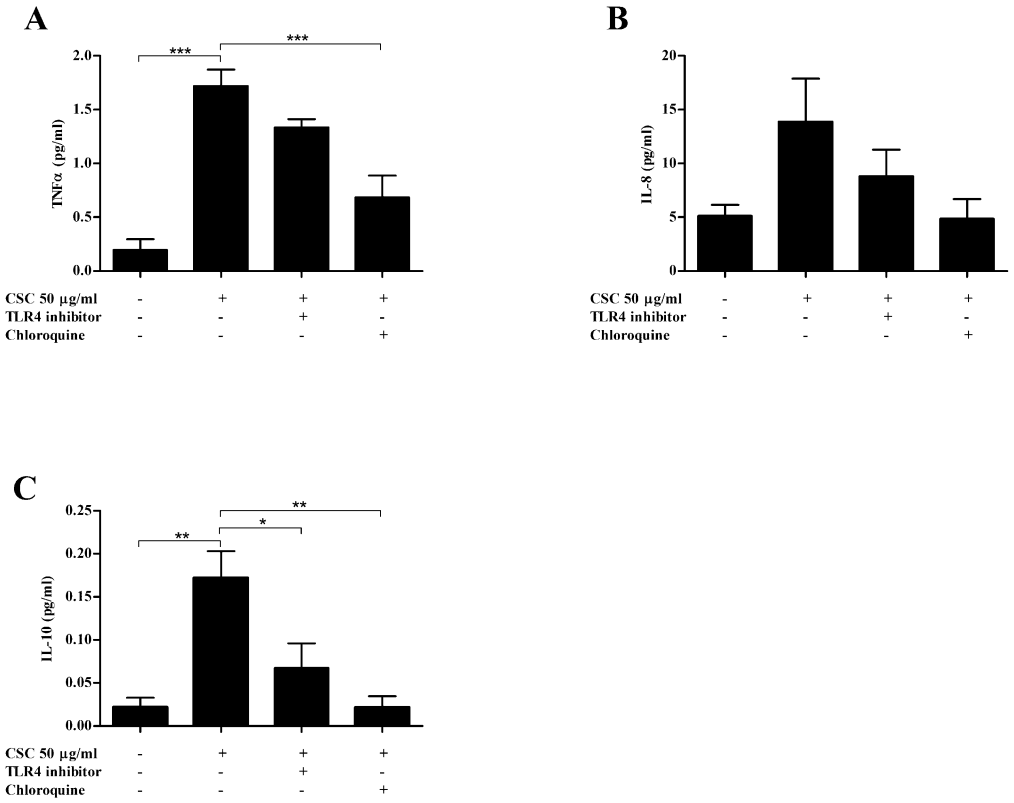

Figure 8.
Inhibitors of the TLR4 and TLR9 pathways reduce cytokine secretion from $CD8^+$ T cells. $CD8^+$ T cells were incubated for 1 hour with or without a TLR4 neutralizing antibody (10 µg/ml) or chloroquine (20 µg/ml). Cells were then treated with either DMSO or CSC (50 µg/ml) for 24 hours. The supernatants were analyzed for cytokine expression using the Human ProInflammatory 7-Plex Tissue Culture Plate. (A) Chloroquine significantly reduced CSC-induced TNF-α expression. (B) IL-8 expression was not significantly increased by CSC, however chloroquine was able to reduce IL-8 levels back to baseline values. (C) Blocking the TLR4 and TLR9 pathways significantly attenuated the CSC-induced production of IL-10. Data are represented as mean ± SEM. * indicates $p < 0.05$; ** indicates $p < 0.01$, and *** indicates $p < 0.0001$.

DISCUSSION

During the past few decades, the mortality rate of COPD has been steadily climbing and it is estimated that COPD will become the third leading cause of mortality by 2030 [330]. Therefore, current research efforts are focusing on the cellular mechanisms of COPD in an attempt to identify potential therapeutic targets. Current pharmacological treatments include bronchodilators and inhaled corticosteroids, which according to the TORCH [331] and UPLIFT [332] clinical trials, do not prevent the decline in lung function in these patients. It has been speculated that inhibiting TLRs, key cellular targets involved in initiating and maintaining an inflammatory response, could hinder the FEV_1 decline and thus function as a therapy for COPD patients [333]. Inhibiting TLR activation would reduce the production of cytokines, thus dampening inflammation, and thereby potentially improving lung function or at least stopping its decline. Consequently, studies have investigated the expression of TLRs on both immune and epithelial cells in COPD [274, 312, 320, 321]. There is some discrepancy between whether TLRs are up or downregulated, however this seems to vary according to the cell type being examined.

$CD8^+$ T cells are the hallmark cells of COPD and are increased in the airways of these patients. Although it is known that the cytotoxic potential of these cells is correlated to the FEV_1 [152] and largely mediated by perforin [136], the role of $CD8^+$ T cells in the pathogenesis of COPD still remains unclear. Our study is the first to investigate the role of TLR4 and TLR9 on $CD8^+$ T cells and we demonstrate the differential expression of TLR4 and TLR9 on $CD8^+$ T cells

from COPD patients. Here, we found there were significantly more TLR4- and TLR9-expressing $CD8^+$ T cells isolated from the lungs of COPD patients compared to control subjects. Interestingly, there was a clear difference between lung and peripheral blood $CD8^+$ T cells in TLR expression.

Surprisingly, our initial results revealed that there was no significant difference in TLR4 and TLR9 immunoreactivity in inflammatory cells between COPD and control lung tissue. Although there were fewer inflammatory cells in our age-matched controls, almost all the immune cells expressed TLR4/TLR9. Consequently, with more cells in the COPD biopsies, fewer of these cells expressed the receptors. The fact that we observed no significant change may have been due to the variability of our samples as well as our small sample population. It is rather difficult to recruit healthy control subjects which match the age of our COPD patients and are willing to undergo bronchoscopy.

Interestingly, we found that 90% of the lung $CD8^+$ T cells expressed TLR4 and TLR9 in COPD patients, compared to only approximately 20% in control subjects. This indicates that there is a certain selectivity over which cells express these receptors in the diseased state. Under COPD conditions, the lung seems to function similarly to the intestine; the cells expressing the receptors are more specific, allowing TLR activation to become more precise, perhaps to better control persistent lung inflammation.

PBMCs from COPD patients showed increased TLR4 and TLR9 expression compared to control subjects. Monocytes are known to both express TLRs [195] and increase in number during chronic inflammation, and may therefore account for the elevated percentage of TLRs in PBMCs in the COPD

patients. Further studies focusing on specific cell types in PBMCs, would help to clarify which cells are responsible for the increase. Contrary to the lung, the percentage of peripheral blood $CD8^+$ T cells expressing TLR4 or TLR9 was not significantly different between COPD patients and control subjects. It may be that peripheral blood $CD8^+$ T cells from COPD patients may change receptor expression once they have migrated to the lung. With approximately 2% of peripheral blood $CD8^+$ T cells expressing TLR4 or TLR9, we speculate that a trigger induces an increase (approximately 90%) in the number of $CD8^+$ T cells that express TLR4 and TLR9 in the lung. Due to the proximity of $CD8^+$ T cells to the epithelium, it is probable that cigarette smoke can cause TLR upregulation on $CD8^+$ T cells. Cigarette smoke induces inflammation in a wide variety of cell types, which may be mediated via activation of TLRs. In support of this, recent studies have shown that cigarette smoke-induced inflammation is both TLR4-dependent [26] and can induce TLR9 expression on neutrophils [28]. Here, we have demonstrated that TLR4 and TLR9 are increased by cigarette smoke exposure in $CD8^+$ T cells from COPD patients, further demonstrating that components of cigarette smoke are able to activate T cells.

It was interesting to note that $CD8^+$ T cells exposed to cigarette smoke condensate (CSC) for 24 hours did not change their TLR4 or TLR9 mRNA expression, while the protein levels of both receptors were significantly increased. These results are consistent with those from epithelial cells, which when treated for 18 hours with a 10% cigarette smoke medium did not significantly change TLR mRNA expression despite an upregulation in TLR4 protein expression [24]. When neutrophils were exposed to smoke, TLR9 mRNA expression was

increased at 5 hours, but showed decreased expression at 24 hours [28]. These results, combined with our data, suggest that TLR expression may be post-transcriptionally regulated in $CD8^+$ T cells.

As for the protein data, previous results have demonstrated that LPS can induce TLR4 protein expression on $CD8^+$ T cells [334], and ODN can induce TLR9 activation on $CD8^+$ T cells [335] as well as protein expression in other cells, such as neutrophils [28]. We hypothesize that the cigarette smoke is acting similarly by activating TLR4 and TLR9.

It is widely known that TLR activation results in a signal transduction cascade that acts through several pathways including NF-κB and JNK, which subsequently bind to target DNA sequences to induce cytokine expression. We hypothesized that activation of TLR4 and TLR9 by cigarette smoke would induce cytokine release by $CD8^+$ T cells. Using an established model of *in vitro* cigarette smoke exposure, $CD8^+$ T cells exposed to CSC significantly increased their levels of IL-1β, IL-6, IL-10, IL-12p70, TNF-α and IFN-γ, but not IL-8. Although IL-8 expression is known to be increased in COPD, our data suggest that $CD8^+$ T cells are not the major source of pulmonary IL-8 production. Other lymphocytes exposed to cigarette smoke also have negligible IL-8 production compared to other peripheral blood cells [22], suggesting that the high IL-8 levels found in the lungs of COPD patients are likely derived from airway epithelial cells [336]. It should be noted that $CD8^+$ T cells are known producers of IL-12. Monocytes and B lymphocytes are known to secrete IL-12, especially after stimulation with bacteria, and it has been shown that certain T lymphocytes can potentially produce IL-12 under these conditions as well [337]. In addition, it has previously

been reported that $CD8^+$ T cells exposed to LPS express TNF-α and IFN-γ in a dose-dependent manner [334], similar to our data. This implies that components in our CSC are acting as TLR ligands, activating the receptors and causing the release of cytokines. To further investigate this, we used a TLR4 neutralizing antibody and chloroquine, a TLR9 signaling inhibitor, to block both of these pathways. Chloroquine significantly reduced both TNF-α and IL-10 production, whereas the TLR4 neutralizing antibody significantly inhibited IL-10. IL-10, a potent anti-inflammatory cytokine, is known to inhibit the synthesis of inflammatory cytokines, including TNF-α [338]. It would not be beneficial to block a pathway that inhibits IL-10 release; however it should be noted that regulatory T cells [339] and macrophages [340] are the main producers of IL-10, not $CD8^+$ T cells. Therefore, one would have to look at the effect of these TLR inhibitors on these cells to fully understand how the use of these inhibitors would affect anti-inflammatory processes. Inhibiting TLR4 and TLR9 modestly reduced the levels of IL-1β, IL-6, IL-8, IL-12p70 and IFN-γ, suggesting that other receptors play a role in the global induction of inflammatory cytokines due to smoke exposure. It is well-described that inflammatory cytokines can be produced through several pathways including, NF-κB [341] and p38 MAP kinase [342]. It is therefore not surprising that inhibiting only two of the receptors would not suppress all cytokine release. However, our findings do highlight the importance of these receptors once cigarette smoke has entered the lungs of patients.

$CD8^+$ T cells are one of the most important cell types in COPD. Considering the lack of knowledge concerning the role these cells are truly playing in this disease, it is important to fully understand the phenotype of the

$CD8^+$ T cells which have migrated to the lungs of these patients. We have clearly shown the distinction between the $CD8^+$ T cells expressed in the peripheral blood and those found in the lung in terms of TLR4 and TLR9 expression. Furthermore, we demonstrate for the first time that CSC is responsible for the upregulation of these receptors on lung $CD8^+$ T cells as well as the resulting cytokine expression. This increased cytokine expression can cause the recruitment of other inflammatory cells to the lung, further perpetuating the damage and inflammation observed in COPD patients. Blocking these receptors may prove to be useful in the future as a potential therapy.

CHAPTER 2 ADDENDUM

This addendum contains unpublished data related to the article "Cigarette smoke increases TLR4 and TLR9 expression and induces cytokine production from $CD8^+$ T cells in chronic obstructive pulmonary disease". The article focuses on the expression of both TLR4 and TLR9 on $CD8^+$ T cells. It was observed that lung $CD8^+$ T cells from COPD patients have increased TLR4 and TLR9 expression compared to control subjects. Once the $CD8^+$ T cells were exposed to cigarette smoke, there was an increase in the protein expression of TLR4 and TLR9, along with significant cytokine expression. In addition, inhibiting both TLR4 and TLR9 was seen to partially reduce the amount of cytokines released from these cells.

Due to the limitations of the journal, certain figures were excluded from the article. These include the purity of our $CD8^+$ T cell samples and the cytotoxicity of the cigarette smoke condensate on $CD8^+$ T cells. I also investigated the mRNA expression of TLR4, TLR9, certain TLR4 co-factors, and proinflammatory cytokines from $CD8^+$ T cells subsequent to cigarette smoke exposure.

ADDITIONAL METHODS

RNA extraction and reverse transcription

Twenty-four hours after the $CD8^+$ T cells were exposed to CSC, total RNA was isolated. The cells were lysed with 250 µl of RLT buffer (RNeasy Mini Kit, Qiagen, Mississauga, ON, CA) supplemented with 1 % (v/v) β-mercaptoethanol. RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, 250 µl of 70% ethanol was added to the lysates, and together these were pipetted onto the RNeasy Mini column. After several washing steps, the RNA is eluted within 35 µl of RNase-free water. The RNA was quantified using the NanoDrop (Thermo Scientific). A total of 500 ng of RNA was reverse transcribed into complementary DNA (cDNA) using Oligo $(dT)_{12-18}$ primers according to the manufacturer's instructions. This was achieved by combining the RNA with the Oligo $(dT)_{12-18}$ primers and heating the sample for 5 minutes at 65ºC. Next, RNase-free water, 0.1 M dithiothreitol (DTT), dNTP mix (dATP, dCTP, dGTP, dTTP), Superscript II reverse transcriptase (Invitrogen), and RNaseOUT (Invitrogen) was added. Together, these are heated at 42ºC for 1 hour and then at 70ºC for 15 minutes. The cDNA was stored at -20ºC until use.

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Power SYBR® Green (Applied Biosystems). All transcript sequences were designed using the Primer3

software (http://frodo.wi.mit.edu/) and purchased from Invitrogen. The PCR reactions each contained 1 µl of cDNA and 9 µl of master mix (0.5 µl of 5 µM sense and anti-sense primers, 3 µl of RNase-free water and 5 µl of Power SYBR® Green). The run method began with a holding stage of 10 minutes at 95ºC and then cycled 50 times (95ºC for 20 seconds, 58ºC for 40 seconds, and 72ºC for 30 seconds) and terminated with a melting curve. Results were analyzed using the 7500 Software v2.0 (Applied Biosystems). All samples were normalized to GAPDH and expressed as a ratio.

Statistical analysis

An ANOVA followed by a Bonferonni multiple comparison test was performed to compare differences between multiple groups. A p-value of < 0 .05 was considered statistically significant (***$p < 0.0001$). Data are expressed as mean ± standard error of mean.

Table 1: Primer sequence and amplicon length for qRT-PCR

Gene	Amplicon length (bp)	Sense	Antisense
CD14	202	GCCGCTGTGTAGGAAAGAAG	GCTGAGGTTCGGAGAAGTTG
GAPDH	138	AGCAAATGCCTCCTGCACCACC	CCGGAGGGGCCATCCACAGTC
IFN-γ	118	AGCTCTGCATCGTTTTGGGTT	GTTCCATTATCCGCTACATCTGAA
IL-1β	139	CCACATTCAGCACAGGACTCT	TACATCAGCACCTCTCAAGCA
IL-6	154	ACCTTCCAAAGATGGCTGAAA	GCTCTGGCTTGTTCCTCACTAC
IL-8	196	GTGCAGTTTTGCCAAGGAGT	CTCTGCACCCAGTTTTCCTT
MD-2	169	GAATCTTCCAAAGCGCAAAG	GCTCCCAGAAATAGCTTCAAC
TLR4	151	CAACCAAGAACCTGGACCTG	GAGAGGTGGCTTAGGCTCTG
TLR9	488	GTGACAGATCCAAGGTGAAGT	CTTCCTCTACAAATGCATCACT
TNF-α	200	TCAAGCCTCTTCTCCTTCCTG	TCAGCTTGAGGGTTTGCTAC

RESULTS

The purity and cytotoxicity of $CD8^+$ T cells

Once the $CD8^+$ T cells were isolated from the PBMCs, flow cytometry was used to determine the purity of the cells. The cells were stained with a CD8 antibody (or isotype control) in order to determine the percentage of cells which were positively stained for CD8. A representative dot plot (Figure 1) shows that 94.28% of the cells are $CD8^+$ T cells. Normally, the CD8 purity ranged between 90-95%.

In order to ensure that the amount of CSC used to treat the cells was not toxic, a cytotoxicity test was performed. $CD8^+$ T cells were treated with CSC or DMSO for 24 hours and then the Countess® Automated Cell Counter was used to determine the cell viability. This cell counter can calculate the absolute number of cells as well as determine the number of dead cells using the trypan blue method. DMSO and CSC concentrations 1-50 μg/ml resulted in over 90% viability of the cells, while a concentration CSC 100 μg/ml resulted in less than 80% viability (Figure 2). Therefore, all CSC experiments using $CD8^+$ T cells used CSC concentrations between 1-50 μg/ml.

The effect of cigarette smoke condensate on gene expression

$CD8^+$ T cells treated with CSC for 24 hours were lysed in order to obtain mRNA. Gene expression was assessed by qRT-PCR. There was no significant change in TLR4 (Figure 3A) or TLR9 (Figure 3B) expression after 24 hours of CSC exposure. The TLR-associated molecules MD-2 (Figure 3C) and CD14

(Figure 3D), show a slight increase, but do not reach significant levels either. The gene expression for several inflammatory cytokines was also analyzed. After 24 hours of cigarette smoke exposure, IL-1β (Figure 4A), IL-6 (Figure 4B), IL-8 (Figure 4C), and IFN-γ (Figure 4E) were not significantly increased, however CSC 50 μg/ml greatly induced TNF-α (Figure 4D) mRNA expression ($p < 0.0001$).

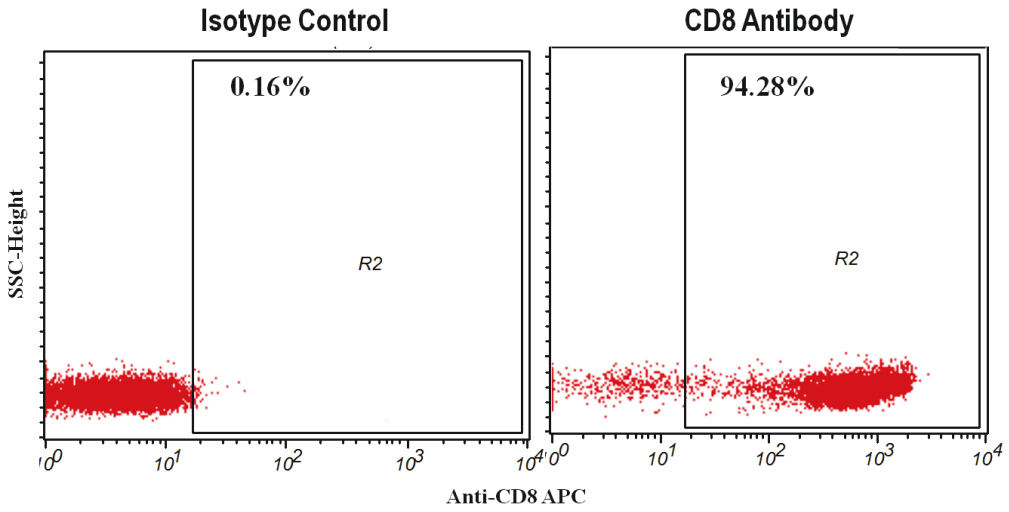

Figure 1.
Determining $CD8^+$ T cell purity. Once the $CD8^+$ T cells were isolated from the PBMCs using the $CD8^+$ T cell Isolation Kit, they were incubated with an anti-CD8 antibody, or isotype control, and analyzed by flow cytometry. Representative dot plots show a $CD8^+$ T cell purity of 94.28%.

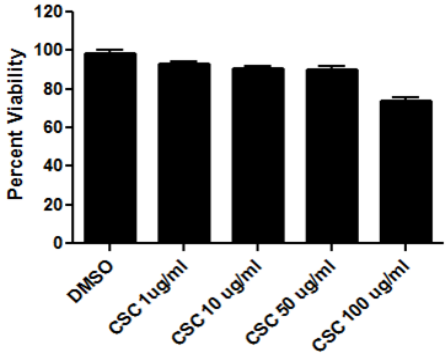

Figure 2.
Viability of $CD8^+$ T cells following 24 hours of cigarette smoke condensate exposure. $CD8^+$ T cells (n = 3) were exposed to CSC (1-100 µg/ml) or DMSO for 24 hours. Viability was determined using the Countess® Automated Cell Counter, which uses the trypan blue exclusion method to determine cell viability. The percent viability was greater than 90% for CSC 1-50 µg/ml, while CSC 100 µg/ml resulted in more than 20% cell death.

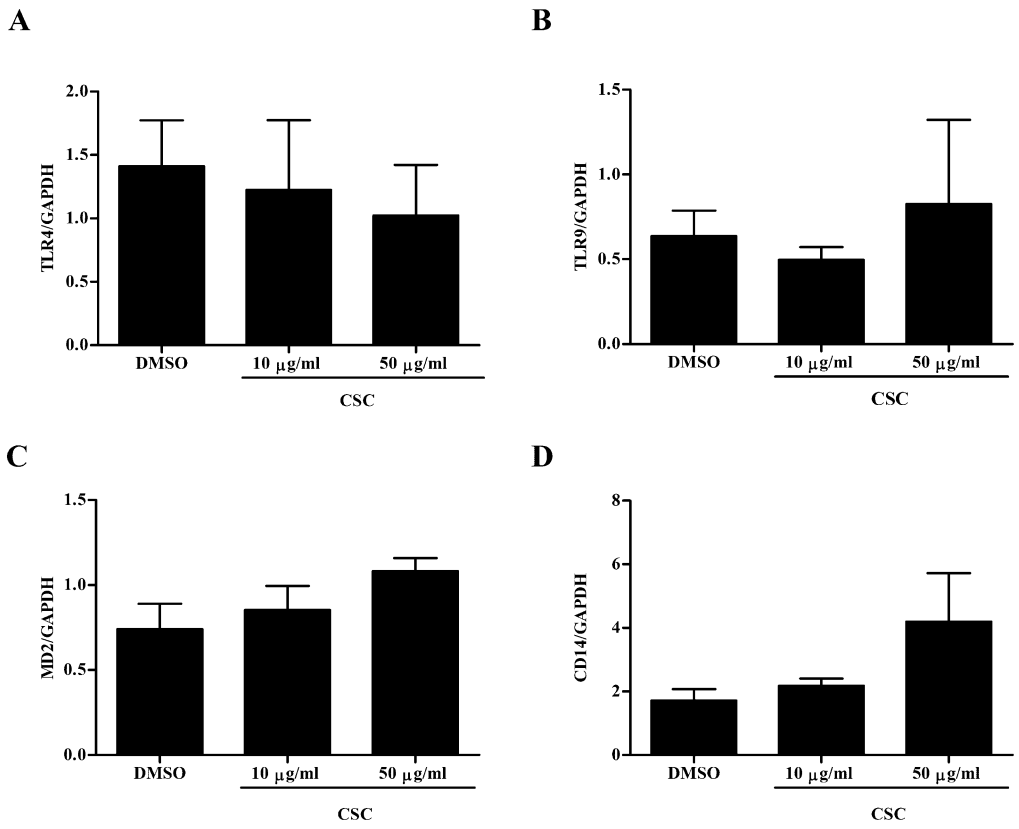

Figure 3.
$CD8^+$ T cells do not induce mRNA expression of TLR4, TLR9, and TLR-associated molecules in response to cigarette smoke. $CD8^+$ T cells were isolated from the peripheral blood of COPD patients (n = 6). Cells were treated with CSC (10 or 50 µg/ml) or DMSO for 24 hours and analyzed by qRT-PCR. CSC did not increase the mRNA expression of TLR4 (A), TLR9 (B), MD-2 (C) or CD14 (D) when compared to DMSO-treated cells. All samples are analyzed relative to GAPDH. Data are represented as mean ± SEM.

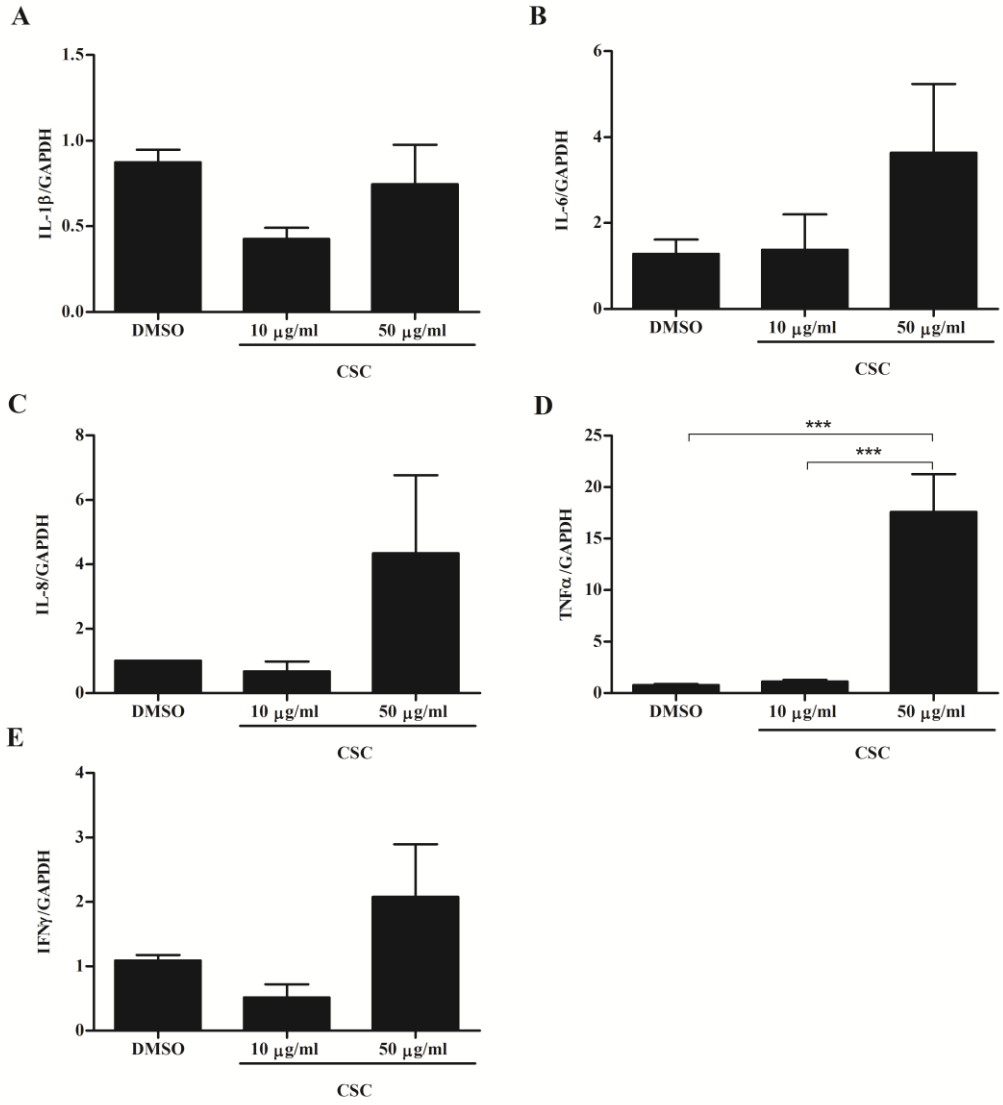

Figure 4.
Cigarette smoke condensate can induce TNF-α mRNA expression. $CD8^+$ T cells were isolated from the peripheral blood of COPD patients (n = 6). Cells were treated with CSC (10 or 50 µg/ml) or DMSO for 24 hours and analyzed by qRT-PCR. CSC did not increase the mRNA expression of IL-1β (A), IL-6 (B), IL-8 (C) and IFN-γ (E), while TNF-α (D) expression was significantly increased at CSC 50 µg/ml. All samples are analyzed relative to GAPDH. Data are represented as mean ± SEM. *** indicates $p < 0.0001$.

DISCUSSION

$CD8^+$ T cells comprise approximately 28% of lymphocytes from peripheral blood [343]. In order to ensure the most pure $CD8^+$ T cell population, flow cytometry was performed following MACS separation of our cells. In each of the experiments, I was able to obtain purity between 90-95%, which is consistent with the manufacturer's protocol. Prior to beginning the experiments, I wanted to determine the cytotoxicity of the CSC. Isolated $CD8^+$ T cells were cultured for 24 hours with increasing concentrations of CSC (1-100 μg/ml). CSC concentrations of 1-50 μg/ml did not induce a great deal of cell death, while 100 μg/ml caused the viability of the cells to decrease more than 20%. For this reason, a CSC concentration of 1-50 μg/ml was used for all subsequent experiments.

It was interesting to note that $CD8^+$ T cells exposed to CSC for 24 hours did not change their TLR4 or TLR9 mRNA expression, while I previously showed that the protein levels of both receptors were significantly increased. These results are consistent with those from epithelial cells, which when treated for 18 hours with a 10% cigarette smoke medium, did not significantly change their TLR mRNA expression, while there was an upregulation of TLR4 protein expression. Furthermore, LPS-treated epithelial cells also showed no change in mRNA expression, despite an increase in TLR4 protein expression [24]. Alternatively, monocytes and polymorphonuclear leukocytes had significantly increased TLR4 mRNA after LPS exposure [245]. When neutrophils were exposed to smoke, TLR9 mRNA expression was increased at 5 hours, but

decreased after 24 hours [28]. Other data suggests that CpG oligodeoxynucleotides (ODN) decrease TLR9 mRNA expression in both B cells and plasmacytoid dendritic cells [194]. As would be expected, TLR4 and TLR9 regulation is not comparable between all cell types. It seems that their regulation is most likely dependent on cell type as well as experimental conditions, such as the length of time cells are treated with cigarette smoke.

In addition, I also investigated two TLR4-associated molecules, MD-2 and CD14. The MD-2 molecule is associated with the extracellular domain of TLR4 and was shown to be essential in conferring LPS responsiveness [344, 345]. CD14 is normally found as a glycosylphosphatidylinositol-anchored membrane protein, although a soluble form of CD14 has been identified as well [346]. CD14 can bind LPS and help to mediate LPS-induced activation [347, 348]. It was thought that perhaps these molecules, which are greatly involved in LPS-induced signaling of TLR4, would also be involved in smoke-induced signaling. However, I did not observe any significant increase in expression of these co-factors after 24 hours of cigarette smoke exposure.

Cigarette smoke was able to induce significant mRNA levels of TNF-α after 24 hours of exposure, while IL-1β, IL-6, IL-8, and IFN-γ all remained unchanged. This is not surprising, as 24 hours is likely too long to see any changes in mRNA expression, which normally peaks around 4 hours post treatment. For example, macrophages treated with cigarette smoke for 4 hours had increased IL-8 mRNA levels [23]. When taken together, our results suggest that at the 24 hour time point, mRNA transcription is no longer involved in cigarette smoke-induced inflammation from $CD8^+$ T cells. In our model it seems that at this

point the protein levels of TLR4, TLR9, and inflammatory-associated cytokines have all been increased and the transcription of RNA is no longer essential to the inflammatory process.

CHAPTER TRANSITION

In the first part of this thesis I investigated the expression of TLR4 and TLR9 in patients with COPD compared to healthy control subjects. I found that $CD8^+$ T cells from the lungs of COPD patients have increased TLR4 and TLR9 expression. I also demonstrated that this increased expression was attributed to cigarette smoke exposure. Furthermore, cigarette smoke was also able to induce $CD8^+$ T cells to produce inflammatory cytokines; and TLR4 and TLR9 inhibitors were able to reduce cytokine production, thereby suggesting a new avenue to target inflammation in COPD patients.

In examining the expression of TLR4 and TLR9 on endobronchial biopsies, I observed that there was no difference in TLR4 or TLR9 expression in the epithelium of COPD patients compared to healthy controls. This finding was quite surprising as it was assumed that TLR expression on epithelial cells would be modulated in cigarette smoke-induced inflammation. I therefore wanted to investigate how cigarette smoke affects these cells in terms of TLR4 expression and regulation *in vitro*. In addition, we wanted to determine if there is a difference in the way normal human bronchial epithelial cells and COPD-derived bronchial epithelial cells respond to cigarette smoke.

CHAPTER 3: Cigarette smoke induces IL-8 production from epithelial cells without increasing TLR4 protein

Jessica Nadigel[1], Séverine Audusseau[1], Carolyn J. Baglole[1], David H. Eidelman[1], and Qutayba Hamid[1]

[1]Meakins-Christie Laboratories, Faculty of Medicine, McGill University, Montreal, Qc,

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ABSTRACT

Cigarette smoke is the principal cause of chronic obstructive pulmonary disease (COPD), a disorder characterised by airway inflammation and remodelling. As epithelial cells are the first line of defense against foreign material, the response of normal epithelial cells to smoke has been extensively studied. However, little is known about how epithelial cells derived from COPD patients respond to ongoing smoke exposure. This study was aimed at comparing the response of normal human bronchial epithelial cells (NHBE) and COPD-diseased human bronchial epithelial cells (DHBE) to cigarette smoke.

NHBE and DHBE cells were treated with cigarette smoke condensate (CSC) for 24 hours and IL-8 and TLR4 were both used as measures of epithelial cell activation. To elucidate the mechanism of CSC activation, cells were pretreated with either CLI-095, a TLR4 inhibitor, or the signaling pathway inhibitors PD184352, Helenalin, or PI-103, which inhibit the ERK1/2, NF-κB and PI3K pathways, respectively. Western blotting and ELISA were both used to determine which signaling pathways were activated.

NHBE cells increased IL-8 production in a dose-dependent manner in response to CSC, while DHBE cells did not show any significant change in IL-8 production. Comparatively, the DHBE cells had a much lower production of IL-8 than the NHBE cells. CSC exposure had no effect on TLR4 expression in either epithelial cell type. However, CLI-095 and PD184352 were able to attenuate IL-8 secretion from NHBE cells, suggesting that CSC-induced inflammation is both TLR4- and ERK1/2- dependent.

These results demonstrate that NHBE and DHBE cells differentially respond to cigarette smoke. DHBE cells exhibit reduced IL-8 release, indicating that COPD is associated with a diminished capacity of airway epithelial cells to respond to toxic material.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is associated with airway inflammation, which is at best, only partially reversible. The principal cause of COPD is chronic, long term inhalation of cigarette smoke [315]. Bacteria and viruses are also causative factors, and are known to induce exacerbations as well [349, 350]. COPD is currently the fourth leading cause of mortality in the United States and its prevalence continues to escalate [3].

Airway epithelial cells serve as the first line of defense against foreign materials and infectious agents, forming an efficient physical barrier and employing a variety of physiological mechanisms to efficiently deal with exogenous threats including secretion of mucus, secretion of antimicrobials and immunological factors and mucociliary clearance [156]. A hallmark of the pathological changes in the airways of COPD patients, is altered appearance of the epithelium, including squamous cell metaplasia and goblet cell hyperplasia [351]. The physiological significance of the these changes, which are believed to be the result of the inflammatory response in the face of ongoing, long-term exposure to cigarette smoke, remain relatively unexplored.

Studies in smokers using a variety of techniques have found the inflammatory response in COPD to be characterized by the presence of increased production of numerous cytokines and chemokines, with IL-8 being particularly prominent [174, 352]. Airway epithelial cells are a major source of IL-8, which is a potent chemoattractant for neutrophils. Under infectious conditions, the release of IL-8 causes the recruitment of inflammatory cells, primarily neutrophils, to the

site of injury [353, 354]. IL-8 secretion is extremely important in initiating the inflammatory response, which first begins with recruiting and activating leukocytes to eliminate any foreign invaders. Therefore, it is not surprising that increased IL-8 production has been associated with various inflammatory diseases, including COPD [355, 356]. Furthermore, IL-8, along with IL-1β and TNF-α, are known to be released by normal epithelial cells following cigarette smoke exposure [20, 21, 24, 173, 179]. Although the precise components of cigarette smoke that are responsible for the increased IL-8 production are unknown, there is reason to be believe that the mechanism involves increased oxidative stress, as cigarette smoke-induced IL-8 production was attenuated when inhibitors of reactive oxygen species were used [25]. Cigarette smoke contains more than 4000 compounds that incite inflammation and oxidative stress and are capable of activating numerous cellular signaling pathways that lead to the release of inflammatory mediators [20, 357].

Cigarette smoke is also known to activate Toll-like receptors (TLR), a key component of the innate immune system. Of particular interest is TLR4, which is expressed on airway epithelial cells [252] and is triggered by exposure to the lipopolysaccharide (LPS) component of the outer cell membrane of Gram-negative bacteria. Activation of TLR4 leads to release of proinflammatory cytokines [24-26] through activation of signaling pathways involving nuclear factor-κB (NF-κB) [24, 358] and extracellular signal-regulated kinase (ERK) 1/2 [359, 360]. TLR4 mediated activation of these pathways is a key component of innate immunity against bacterial infection.

Despite the long established evidence that COPD is associated with major changes in epithelial morphology, the physiological impact of these changes on the response to exposure to foreign materials has not been widely studied. In regard to cigarette smoke exposure, previous studies have used normal human epithelial cells, with the hope of inducing a COPD-like phenotype. We are unaware of any previous study that has sought to investigate the response to cigarette smoke in epithelial cells from patients with COPD. The goal of the present study was to compare the response to cigarette smoke of between normal and COPD human airway epithelial cells. Our data suggests that in response to cigarette smoke, normal epithelial cells produce more IL-8 than epithelial cells derived from patients with COPD, while there is no change in TLR4 expression from either epithelial cell type. This marked decrease in production of IL-8 from COPD-derived epithelial cells suggests that COPD is associated with a blunting of the capacity of epithelial cells to respond to foreign insults.

METHODS

Reagents

BEBM (Bronchial Epithelial Cell Basal Medium) and BEGM (Bronchial Epithelial Growth Medium) Bulletkit were purchased from Lonza/Clonetics (Walkersville, MD). CLI-095, a TLR4 signaling inhibitor, was purchased from InvivoGen (San Diego, CA). Helenalin, a NF-κB inhibitor was purchased from Enzo Life Sciences (Farmington, NY); PD184352, an inhibitor of extracellular signal-regulated kinase (ERK) 1/2, was purchased from US Biological (Swampscott, MA); PI-103, a phosphoinositide 3-kinase (PI3K) inhibitor was purchased from Cayman Chemical (Ann Arbor, MI). The p44/42 mitogen-activated protein kinase (MAPK) ERK1/2 antibody (clone 137F5) and the Phosphorylated-p44/42 MAPK ERK1/2 (Thr202/Tyr204) (clone E10) were purchased from Cell Signaling (Danvers, MA). The TLR4 antibody (clone H-80) was purchased from Santa Cruz (Santa Cruz, CA), and the GAPDH antibody (clone 6C5) from Millipore (Billerica, MA).

Human bronchial epithelial cell culture

Normal human bronchial epithelial cells (NHBE) and COPD-diseased human bronchial epithelial cells (DHBE), were purchased from Lonza/Clonetics. Cells were grown on collagen-coated plates using 1% collagen type I (from rat tail; Nalgene Culture 3D Matrix, Rochester, NY). Cells were cultured in BEBM medium supplemented with the BEGM Bulletkit. Once confluent, cells were washed with PBS and starved for 24 hours in starving medium (99% BEBM

medium with 1% BEBM medium supplemented with BEGM Bulletkit) prior to treatments.

Treatment of cells

Cigarette smoke condensate (CSC) was received as a gift from Imperial Tobacco Canada and was generated as previously reported [322]. Initially, both types of cells were treated with CSC at various concentrations (1 – 50 μg/ml) for 24 hours. For all subsequent experiments, 10 μg/ml of CSC was used. CLI-095, a pharmacological inhibitor of TLR4 signaling was also used. CLI-095 inhibits TLR4 activation by blocking the intracellular domain of TLR4, thus preventing any further downstream signaling. Here, cells were pretreated for 1 hour at 37ºC with CLI-095 (1 – 10 μM), followed by CSC (10 μg/ml) or control (DMSO) incubated for 24 hours. In separate experiments, cells were treated with or without the signaling inhibitors, PD184352 (2 μM), Helenalin (1 μM), or PI-103 (5 μM) for 1 hour at 37ºC, followed by CSC at 10 μg/ml or DMSO. To determine ERK1/2 activation, cells were treated with CSC (10 μg/ml) for 0 – 60 minutes and protein lysates were collected for immunoblotting.

Protein quantification and immunoblotting

Epithelial cells were lysed in 100 μL of lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton x-100, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.27 M sucrose, 5 mM sodium pyrophosphate decahydrate and protease inhibitors). The concentrations of the protein were quantified using the BCA Protein Assay Kit

(Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Twenty-five micrograms of protein were then boiled and separated on a 10% Pro-Pure Next Gel using Pro-Pure Running Buffer (Amresco, Solon, OH). After transferring proteins onto nitrocellulose, membranes were blocked for 1 hour at room temperature in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE). Blots were then incubated with an anti-TLR4 antibody (0.2 μg/ml) and GAPDH (1:2000) or total p44/42 MAPK ERK1/2 (1:1000) and phosphorylated-p44/42 MAPK ERK1/2 (1:2000) overnight at 4ºC. Goat anti-rabbit IgG (DyLight^{TM}800, Thermo Scientific) or goat anti-mouse IgG (DyLight^{TM}680, Thermo Scientific) antibody was applied for 1 hour in the dark at room temperature (1:15,000). The signal was detected and quantified using a Li-Cor Odyssey imaging system. Samples were normalized to GAPDH or total p44/42 MAPK ERK1/2 and expressed as a ratio relative to the control sample.

Quantification of IL-8

After the 24 hour treatment with either CSC, CLI-095, or pathway inhibitors, supernatants were collected and stored at -80ºC. IL-8 concentrations were quantified using the Human IL-8 ELISA MAX Standard kit (Biolegend, San Diego, CA) according to the manufacturer's protocol. Briefly, the capture antibody was diluted in coating buffer (1:200) and applied to a 96-well plate overnight. After washing, the plate was blocked with assay diluent to reduce non-specific binding. Next, standards and samples were added to each well and incubated for 2 hours at room temperature after which the detection antibody was added for 1 hour at room temperature while shaking. After washing, avidin-HRP

was added to each well and left to incubate for 30 minutes at room temperature. The TMB substrate solution was added to each well for 15 minutes in the dark. A stop solution was then added to inhibit the reaction and the absorbance was immediately read on a plate reader at 450 nm.

Statistical analysis

All comparisons were between more than two groups and therefore an ANOVA, followed by a Bonferonni multiple comparison test was used. A p-value of < 0.05 was considered statistically significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.0001$). Data are expressed as mean ± standard error of mean.

RESULTS

Normal human bronchial epithelial cells are morphologically distinct from COPD epithelial cells

COPD is associated with remodeling of the airway epithelium, as epithelial cells undergo squamous metaplasia and goblet cell hyperplasia. Therefore, we therefore first examined the morphological difference between normal human bronchial epithelial cells (NHBE) compared to COPD-diseased human bronchial epithelial cells (DHBE), prior to any type of stimulation. NHBE cells (Figure 1A) show a normal cobblestone-like appearance typical of epithelial cells, while DHBE cells (Figure 1B) appear to undergo squamous metaplasia as their cells tend to be larger in size, with distinct nuclei.

Cigarette smoke condensate induces IL-8 production without increasing TLR4 protein expression

Airway epithelial cells are the first type of cells to come into contact with cigarette smoke. Although several studies have investigated IL-8 release by epithelial cells after cigarette smoke exposure [20, 24], none have examined for differences between normal and COPD-derived epithelial cells. We therefore wanted to determine if normal epithelial cells respond differently to cigarette smoke condensate (CSC) than epithelial cells isolated from a patient with COPD.

In order to determine CSC cytotoxicity, a MTT assay was performed. Both types of cells were treated with CSC (or DMSO as a control) at various concentrations (1-100 μg/ml) for 24 hours. The viability of the cells was greater

than 90% for the CSC concentrations 1 – 50 μg/ml. At the highest concentration of CSC (100 μg/ml), there was approximately 30% cell death (Addendum Figure 1). Therefore only concentrations of CSC 1-50 μg/ml were used for all subsequent experiments.

Both NHBE and COPD epithelial cells were stimulated with CSC (1-50 μg/ml) for 24 hours and supernatants were examined for IL-8 secretion. The production of IL-8 by NHBE cells increased in a dose-dependent manner (Figure 2A). The level of IL-8 was significantly greater following exposure to CSC 10 and 50 μg/ml ($p < 0.01$) when compared to the control. In contrast, IL-8 failed to significantly increase in DHBE cells after exposure to CSC (Figure 2B). These data suggest that not only is there a dramatic difference in their morphology, but that NHBE and DHBE cells exhibit a significant difference in IL-8 release. Interestingly, when the cells were treated with LPS, similar results were observed with NHBE cells secreting more IL-8 than the DHBE cells (Addendum Figure 4).

We have previously shown that CSC was able to induce TLR4 expression on $CD8^+$ T cells as well as activate these receptors to produce inflammatory cytokines [361]. Therefore, we wanted to determine if TLR4 expression was increased on epithelial cells in a similar manner. After 24 hours of CSC exposure, NHBE cells (Figure 2C) and DHBE cells (Figure 2D) had no significant change in TLR4 expression. Interestingly, we found that, although CSC was able to increase the amount of IL-8 released, there was no upregulation of TLR4 in NHBE cells.

CLI-095, a TLR4 inhibitor, can attenuate IL-8 production from normal human bronchial epithelial cells

Although no differences in TLR4 expression were detected after CSC exposure, our group and others have shown that cigarette-induced inflammation is at least partially dependent on TLR4 [26, 27, 361]. This raises the possibility that differences in response to cigarette smoke in NHBE and DHBE cells might be a function of differences in signalling downstream from the TLR4 receptor. To explore this possibility, NHBE and DHBE cells were pretreated for 1 hour with or without CLI-095 (1-10 μM), a pharmacological inhibitor of TLR4 [362], followed by treatment with CSC 10 μg/ml for 24 hours. Once again, the CSC significantly increased IL-8 production from NHBE cells, and CLI-095 was able to inhibit this production (Figure 3A). CLI-095 attenuated IL-8 production with concentrations between 3 μM ($p < 0.05$) and 10 μM ($p < 0.0001$), demonstrating that IL-8 release from normal epithelial cells is TLR4-dependent despite the fact that TLR4 is not upregulated. DHBE cells treated with CSC did not induce significant levels of IL-8, and CLI-095 had no significant effect on IL-8 production in DHBE cells (Figure 3B).

Cigarette smoke-induced IL-8 is attenuated by PD184352, an inhibitor of the ERK1/2 signaling pathway

Although TLR4 normally signals through the NF-κB pathway, it has been observed that other pathways are activated by TLR4, including MAPK pathways [363]. To determine which signaling pathways may be involved in the CSC-induced IL-8 release, we used three pharmacological inhibitors targeting different

cellular pathways: PD184352, an inhibitor of the ERK1/2 pathway; Helenalin, which inhibits the NF-κB pathway; and PI-103, an inhibitor of the PI3K pathway. NHBE and DHBE cells were treated with or without these inhibitors for 1 hour prior to exposure to CSC 10 μg/ml for 24 hours. PD184352 was the only inhibitor which significantly decreased IL-8 produced from NHBE cells (Figure 4A) ($p < 0.0001$). Neither Helenalin nor PI-103 reduced the levels of IL-8 upon CSC exposure. Although it did not reach statistical significance, DHBE cells (Figure 4B) responded similarly to NHBE cells, with PD184352 reducing the levels of IL-8 below baseline. Helenalin and PI-103 had no significant effect on IL-8 production. These results suggest that in NHBE cells ERK1/2 is a dominant pathway involved in cigarette smoke-induced IL-8 production.

CSC activates the ERK1/2 signaling pathway

After observing that PD184352 inhibits IL-8 production, we verified that CSC induced activation of the ERK1/2 pathway. Once activated, ERK becomes phosphorylated. Therefore, to determine if the ERK1/2 signaling pathway is activated, we compared the amount of phosphorylated ERK1/2 to the amount of total ERK protein, following stimulation of NHBE and DHBE cells with CSC 10 μg/ml (0 – 60 minutes). Figure 5A typifies immunoblot analysis which indicates that ERK activation peaks 15 minutes after exposure. Thereafter, ERK1/2 activation decreases until 60 minutes. CSC can induce NHBE cells (Figure 5B) and DHBE cells (Figure 5C) to activate the ERK1/2 signaling pathway, with the highest activation at 15 minutes post CSC exposure.

PD184352 inhibits CSC-induced ERK1/2 activation

Next, to ensure that the PD184352 specifically inhibits CSC-induced ERK1/2 activation, NHBE and DHBE cells were treated with DMSO, CSC, CSC in combination with PD184352 or PD184352 alone for 15 minutes**.** CSC induced slight ERK1/2 activation in both NHBE and DHBE cells, and all activation was abolished when PD184352 was added (Figure 6). When taken together, we conclude that CSC activates the TLR4 pathway, which uses the ERK1/2 signaling pathway to induce IL-8 production.

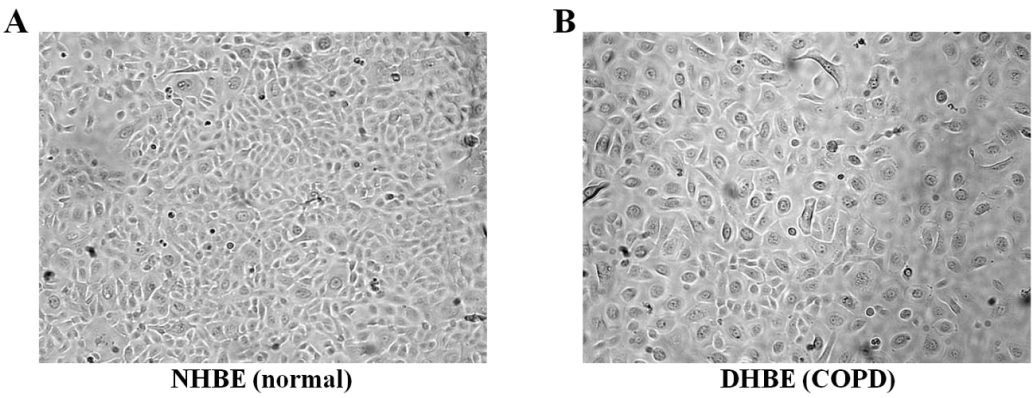


Figure 1.
The difference in morphology of NHBE cells compared to DHBE cells. NHBE cells (n = 3) and DHBE cells (n = 3) on their second passage were grown to 100% confluence. A representative image demonstrates the difference in appearance of the cells. The NHBE cells (A) have a distinctive cobblestone appearance, typical of airway epithelial cells, while the DHBE cells (B) appear larger in size with distinct, round nuclei. All magnifications are 200x.

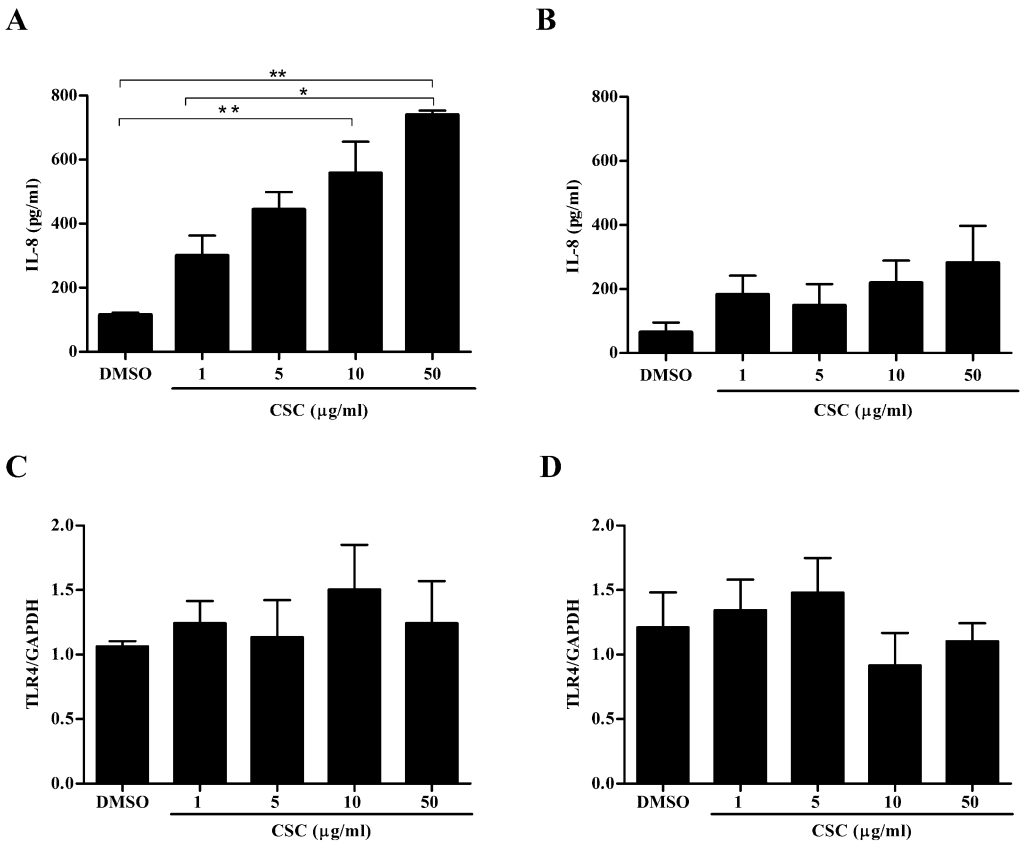


Figure 2.
Epithelial cells induce IL-8 production with no change in TLR4 expression in response to CSC. NHBE (n = 4-5) and DHBE (n = 6-7) cells were treated with various concentrations of CSC (1-50 µg/ml) or with DMSO for 24 hours. Protein lysates and supernatants were collected. IL-8 secretion was measured using the Human IL-8 ELISA MAX Standard kit and NHBE cells (A) show a dose-dependent increase in IL-8, which reached significance at CSC 10 µg/ml and CSC 50 µg/ml, when compared to the control DMSO sample. DHBE cells (B) do not significantly increase IL-8 secretion after CSC treatment. Furthermore, NHBE cells were able to induce more IL-8 production than the DHBE cells. Western blot analysis revealed that there is no increase in total TLR4 protein expression in NHBE cells (C) or DHBE cells (D). Individual samples were normalized to GAPDH and then expressed as a relative ratio of treated samples compared to DMSO sample. Data are represented as mean ± SEM. * indicates $p < 0.05$ and ** indicates $p < 0.01$.

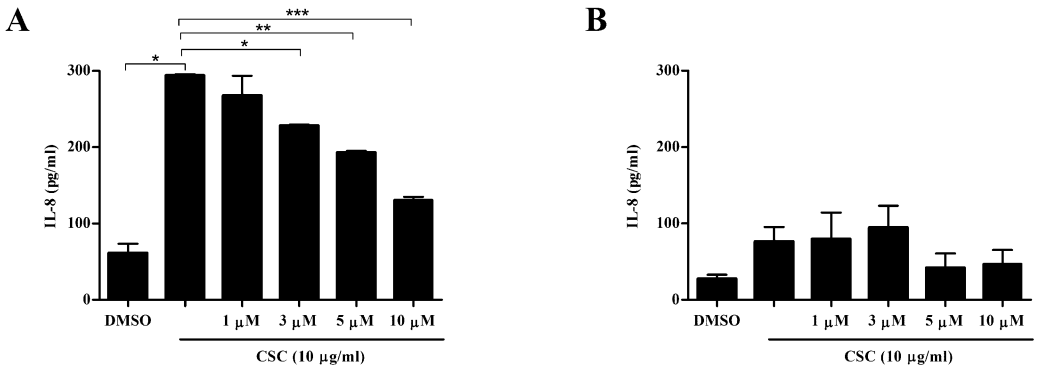


Figure 3.
IL-8 production from normal human bronchial epithelial cells is TLR4-dependent. NHBE (n = 4) and DHBE (n = 5) cells were treated with or without the TLR4 inhibitor, CLI-095 (1 – 10 μM) for 1 hour at 37ºC. Next, cells were treated with CSC 10 μg/ml (or DMSO as control) for 24 hours. Supernatants were collected and analyzed for IL-8 production through ELISA. CSC significantly increased IL-8 production from NHBE cells when compared to DMSO and CLI-095 was able to attenuate IL-8 production at 3, 5, and 10 μM concentrations (A). CSC was unable to significantly increase IL-8 production from DHBE cells and therefore CLI-095 did not have any significant change on the amount of IL-8 released (B), although the highest concentrations of CLI-095 seem to modestly reduce IL-8 release. Data are represented as mean ± SEM. * indicates $p < 0.05$; ** indicates $p < 0.01$ and *** indicates $p < 0.0001$.

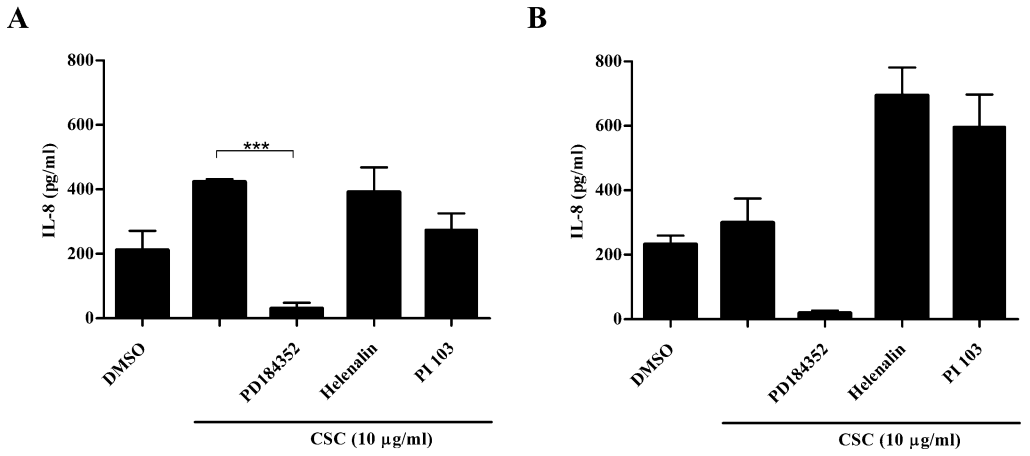


Figure 4.
IL-8 production in response to CSC is mediated through the ERK1/2 signaling pathway. NHBE (n = 4) and DHBE (n = 3) cells were pre-treated for 1 hour at 37ºC with one of the following pathway inhibitors: PD184352 (2 µM), which inhibits ERK1/2; Helenalin (1 µM), which inhibits NF-κB; and PI-103 (5 µM), which inhibits PI3K. Next, cells were incubated with CSC (10 µg/ml) or DMSO for 24 hours, the supernatants were collected and an IL-8 ELISA was performed. IL-8 production from NHBE cells (A) was significantly inhibited with PD184352. The production of IL-8 from DHBE cells (B) was reduced to below baseline levels with PD184352, however significance was not reached. Helenalin and PI-103 did not have any significant effect on the cells. Data are represented as mean ± SEM. *** indicates $p < 0.0001$.

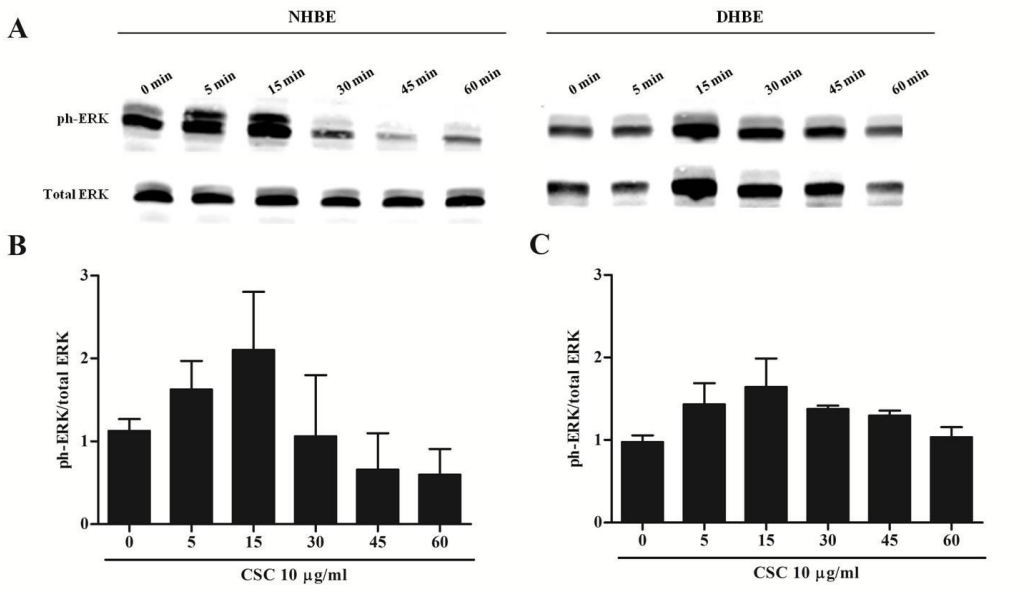


Figure 5.
CSC activates the ERK1/2 signaling pathway. NHBE (n = 3) and DHBE (n = 4) cells were treated with CSC (10 µg/ml) for 0 – 60 minutes and protein lysates were collected for immunoblotting. A representative western blot nitrocellulose membrane expressing phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK1/2 protein (A). Western blot analysis revealed that CSC induced ERK1/2 activation, which reached its peak at 15 minutes for NHBE cells (B). DHBE cells (C) show ERK1/2 activation, with a slight peak at 15 minutes. Individual samples were normalized to total ERK1/2 and then expressed as a relative ratio compared to the 0 minute sample. Data are represented as mean ± SEM.

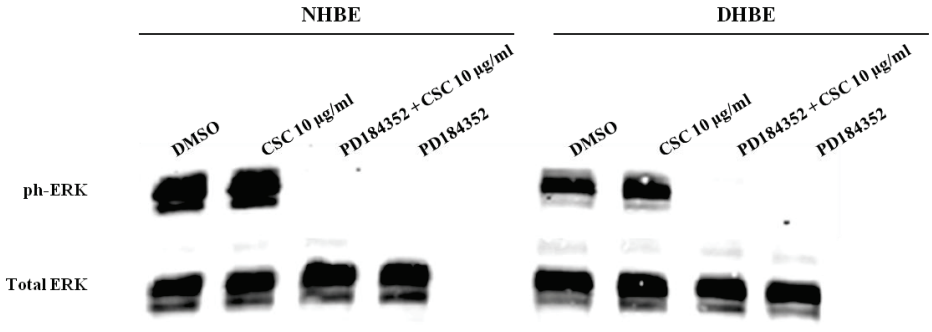


Figure 6.
CSC-induced ERK1/2 activation is inhibited by PD184352. Cells were treated with CSC (10 µg/ml), CSC + PD184352, PD184352 alone, or DMSO as a control, for 15 minutes at 37ºC, whereupon protein lysates were collected. A representative western blot nitrocellulose membrane showing phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK1/2 protein indicates that CSC can activate ERK1/2, and that PD184352 specifically inhibits the CSC-induced activation of the ERK1/2 signaling pathway.

DISCUSSION

COPD is currently a leading cause of morbidity and mortality worldwide and its importance is expected to grow because of the widespread use of cigarettes in the developing world [364]. Cigarette smoke-induced inflammation has been widely studied in an effort to understand the pathogenesis of COPD. Airway epithelial cells are the first cells exposed to cigarette smoke and therefore initiate the inflammatory process. In an individual afflicted with COPD, epithelial cells induce several cytokines including, IL-8, IL-1β, TNF-α and TGF-β, as well as matrix metalloproteases and antioxidants, including glutathione [20, 92]. This inflammatory and oxidative stress response is perpetuated by the continued release of mediators by other cell types, thereby amplifying the inflammatory response. Several studies have focused their efforts on understanding how cigarette smoke affects epithelial cells, especially in terms of TLR activation. It was observed that epithelial cells exposed to cigarette smoke produce IL-8 via the activation of TLRs and the NF-κB and MAPK signaling pathways [24, 359, 360, 365]. However, these studies used either transformed cell lines or primary epithelial cells from subjects without COPD. Rusznak et al. have attempted to look at the difference between normal and COPD-derived epithelial cells, and did so in terms of cell permeability and glutathione release [178]. We therefore wanted to evaluate if epithelial cells from a COPD patient would increase inflammatory mediator production utilizing the same signal transduction pathways in response to cigarette smoke.

We first observed a striking difference in morphology between the primary NHBE and DHBE cells. Although both were grown to confluence in identical media, they exhibited a distinct appearance. NHBE cells had a typical, cobblestone appearance that is normally observed with epithelial cells grown in culture, while DHBE cells were larger and rounder, with larger nuclei. Remodeling of the airway epithelium has been extensively studied in COPD, with squamous metaplasia being one the main features [184, 366]. Once the epithelium is injured, this feature of remodelling occurs in order to protect and maintain the integrity of the epithelial cells [184, 367], however it has been shown that squamous metaplasia is also correlated with the severity of airway obstruction and can therefore add to the complications of COPD [368]. DHBE cells were received from three patients and all of them had the same morphology indicating remodeling had occurred.

It is noteworthy that only the NHBE cells had a significant increase in IL-8 production after CSC exposure. This data was consistent with previous studies which showed that normal epithelial cells release IL-8 following cigarette smoke exposure [20, 25]. In contrast, DHBE did not significantly increase IL-8 production. This was surprising, as COPD is a chronic inflammatory disease associated with increased IL-8 production [352]. Furthermore, using immunostaining, studies have revealed that at baseline levels, COPD patients have increased IL-8 expression compared to normal subjects [174]. This suggests that there is actually more IL-8 present within the cells of COPD patients, however there is likely a problem with the release mechanism of IL-8 from these cells, essentially allowing NHBE cells to release more IL-8 in response to CSC.

Our data suggests that the DHBE cells have changed both their morphology as well as how they respond to certain stimuli. This may be a type of protective mechanism by which DHBE cells have become less responsive to cigarette smoke in order to dampen the inflammation which is already uncontrollable in COPD patients. However, the failure of the COPD-derived epithelial cells to marshal a strong IL-8 response following CSC exposure indicates a potential malfunction in their immune response. IL-8 is needed under these types of conditions to recruit inflammatory cells to sites of injury to help eliminate pathogens and repair tissue.

Next, we decided to look at TLR4 protein expression on both types of cells. At basal levels, there was no difference in TLR4 expression between NHBE and DHBE cells. Furthermore, CSC was unable to induce any statistically significant change in the TLR4 expression on either type of epithelial cell. Similarly, Guillot et al. found that the expression of TLR4 was not regulated by LPS in epithelial cells. They demonstrated that treating normal epithelial cells with LPS did induce IL-8 production without an increase in TLR4 protein expression [253]. In addition, they also showed that TLR4 can be found within vesicles in the cell. Taking this into consideration, perhaps for the NHBE cells more TLR4 is trafficked to the cell surface in response to CSC without the total amount of protein changing. This would allow more receptors to be activated thus increasing the amount of IL-8 released. Or, perhaps the receptors already present on the cell membrane increase their responsiveness reducing the need for more receptors to translocate to the membrane. As for the DHBE cells, it is known that cells undergoing squamous metaplasia lose some of their secretory abilities [184]. Therefore, these cells may not be able to produce as much IL-8 as normal

epithelial cells or perhaps the TLR4 receptors on the DHBE cells have become desensitized over time, limiting the amount of IL-8 they are able to release.

Interestingly, we demonstrated that CSC-induced IL-8 production is still TLR4-dependent. CLI-095, a TLR4 inhibitor that blocks the intracellular portion of TLR4 from signaling [362], was able to attenuate IL-8 production. CLI-095 reduced the amount of IL-8 released in a dose-dependent manner from NHBE cells treated with CSC. Although the DHBE cells responded slightly to the highest concentrations of CLI-095, this was not significant, as CSC alone did not boost IL-8 production. Nonetheless, we demonstrated that CSC-induced IL-8 production is mediated via TLR4.

Next, we demonstrated that the activation of TLR4 in NHBE cells signals through the MAPK pathway, specifically ERK1/2. Inhibition of ERK1/2 abolished IL-8 production from NHBE cells after cigarette smoke exposure. Although the ERK1/2 pathway has been implicated in cigarette smoke-induced inflammation [359, 360], numerous studies have identified the NF-κB pathway, as activation of this pathway is strongly linked to inflammation caused by cigarette smoke exposure [358, 365, 369]. It was therefore quite surprising that Helenalin, an NF-κB pathway inhibitor, did not have any significant effect on IL-8 release from either NHBE or DHBE cells. The reason for these differences may be partially due to the type of cell being studied. Although we used primary cells from healthy donors or COPD patients, many of the other studies used cell lines, which could lead to differences in signaling paradigms. In addition, experimental conditions, such as the method of generating cigarette smoke for *in vitro*

assessment, (condensate versus extract), potentially may have led to activation of different signalling pathways.

We went on to confirm that the CSC could induce ERK1/2 activation when the epithelial cells were treated with CSC for 0 - 60 minutes. We saw an increase in ERK1/2 activation for up to 15 minutes, and then it decreased. This was observed for both types of epithelial cells, although the ERK1/2 activation was less pronounced in the DHBE cells. Once it was established that the ERK1/2 signaling pathway was most activated at 15 minutes, cells were treated with CSC alone, CSC plus PD184352, PD184352 alone or DMSO for 15 minutes. This was done to ensure that PD184352 was specifically inhibiting the ERK1/2 signaling pathway. Using western blotting, we demonstrated the inhibitor was sufficiently specific so as to be able to completely block the phosphorylation of ERK1/2, even when cells were also treated with CSC.

Previous studies have investigated the effect of cigarette smoke on airway epithelial cells in terms of the signaling pathways involved. Here, we take it one step further, as we demonstrate the how epithelial cells from COPD patients react to cigarette smoke. For the first time we show that although there may be overall inflammation in COPD patients, NHBE cells can actually produce more IL-8 in response to cigarette smoke than DHBE cells. Our data suggests that NHBE cells are responsive to the cigarette smoke exposure and can induce a normal immune response. In contrast, DHBE cells have either become desensitized to the smoke or have changed their phenotype to the point where they fail to mount a proper immune response. This lack of a reaction to cigarette smoke is extremely detrimental to COPD patients as they cannot properly rid the airways of any

invading pathogens and may help to explain the ongoing inflammation observed within the lungs of these patients. More research in phenotyping epithelial cells derived from COPD patients is needed to properly understand how cigarette smoke-induced inflammation propagates in COPD.

CHAPTER 3 ADDENDUM

The additional data presented in this addendum is related to the results presented in the manuscript "Cigarette smoke induces IL-8 production from epithelial cells without increasing TLR4 protein", which is to be submitted. The manuscript highlights the difference in the production of IL-8 from normal human bronchial epithelial cells (NHBE) compared to COPD-diseased human bronchial epithelial cells (DHBE) in response to cigarette smoke condensate (CSC). It was observed that NHBE cells produce more IL-8 in response to CSC compared to DHBE cells, suggesting that epithelial cells from COPD patients fail to mount a proper immune response. In addition, we demonstrated that the increased IL-8 production from NHBE cells is at least partially mediated though TLR4 and ERK1/2 signaling pathways.

Certain figures were excluded from the manuscript, as they did not add any significant information. These include the cytotoxicity of the CSC on epithelial cells, the difference in diameter between NHBE and DHBE cells, and the effect of the signaling pathway inhibitors on the cells without CSC. We also investigated the response of both these types of epithelial cells to LPS. In addition, we examined if CSC could induce these cells to produce inflammatory cytokines as well as cause $CD8^+$ T cell migration

ADDITIONAL METHODS

Cell Viability

MTT assay (Enzo Life Sciences, Brockville, ON) was used to assess the viability of the cells following CSC exposure. Cells were cultured in duplicate in a 96-well plate. Once the cells reached confluence, they were treated with 140 µl/well of CSC (1-100 µg/ml) for 24 hours. Next, 10 µl of a 5mg/ml MTT (in PBS) was added to each well for 4 hours at 37ºC. The insoluble precipitate was dissolved using 200 µl of DMSO and the optical density was read at 510 nm.

Treatment of cells

NHBE and DHBE cells were treated with CSC (1 – 50 µg/ml) or LPS (0.05 – 5 µg/ml) from *Salmonella Minnesota* R595 (Sigma, Oakville, ON) for 24 hours. The cells were also treated with signaling inhibitors PD184352 (2 µM), Helenalin (1 µM), PI-103 (5 µM), or DMSO as a control, for 24 hours to determine if these inhibitors have any effect on IL-8 production from the cells. All supernatants and cell lysates were collected.

In addition, the Scepter Handheld Automated Cell Counter (Millipore, Billerica, MA) was used to calculate cell counts. This cell counter can also determine the diameter of the cells, which is displayed as a histogram, and was therefore used to determine the size of the NHBE and DHBE cells.

Cytokine protein quantification

Supernatants were collected 24 hours after NHBE and DHBE were treated with CSC (1 and 10 μg/ml) and stored at -80ºC. The Human ProInflammatory 7-Plex Tissue Culture Kit was purchased from Meso Scale Discovery (MSD) and used according to the manufacturer's protocol. Briefly, a 150 μl of a 1% (w/v) blocking solution, (prepared by company), was added to each well of the MSD plate which was then left to incubate at room temperature for 1 hour while shaking. After washing with PBS and 0.05% Tween-20, 25 μl of samples or calibrators were added to each well and incubated for 2 hours at room temperature while shaking. Twenty-five μl of detection antibody was then added and left to incubate for 2 hours while shaking. Next, the plate was washed with PBS and 0.05% Tween-20 and then 150 μl of 2X Read Buffer T was added to each well and the MSD plate was then read using the SECTOR® Imager 2400. The kit allowed for the quantification of IL-1β, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ. The lower limits of detection for this plate were as follows: IL-1β, 0.0199 pg/ml; IL-6, 0.0158 pg/ml; IL-10, 0.0794 pg/ml; IL-12p70, 0.0199 pg/ml; TNF-α, 0.3162 pg/ml, and IFN-γ, 0.0 pg/ml. The upper limit of detection was 10,000 pg/ml for all cytokines.

$CD8^+$ T cell migration

Peripheral blood was taken from COPD patients (n = 3) and PBMCs were isolated using Ficoll-Paque Plus (GE Healthcare). $CD8^+$ T cells were then isolated from the PBMCs using the $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol and as previously described [361]. Once isolated,

$CD8^+$ T cells were resuspended (1 x 10^6 cells/ml) in RPMI-1640 medium (Thermo Scientific) supplemented with 10% v/v fetal bovine serum, L-glutamine, penicillin, streptomycin, sodium pyruvate, and HEPES. Migration assays were performed using a 48-well micro Chemotaxis Chamber (Neuro Probe, Inc, Cabin John, Md) with a 5 μm-pore polycarbonate membrane (Millipore) which was treated with 0.01% Collagen type I (rat tail; Nalgene Culture 3D Matrix, Rochester, NY) solution in 0.01N HCl and was placed between the 2 chambers. $CD8^+$ T cells were added to the upper chamber, while supernatants from NHBE and DHBE cells, previously treated with CSC (1, 5, 10, 50 μg/ml), were added to the lower chamber. A preliminary experiment was performed in order to determine the optimal time for migration, and this was observed at 3 hours. After 3 hours of incubation at 37ºC, the membrane was removed and cells were removed from the upper side. Cells that have migrated to the lower side of the membrane are fixed using 4 % paraformaldehyde and stained using the Hemi 3kit (Fisher, Kalamazoo, MI). The membrane was stained for 30 seconds in each solution. Five random fields were used to count the number of migrated cells at a magnification of 400x.

Statistical analysis

All comparisons were between more than two groups and therefore an ANOVA, followed by a Bonferonni multiple comparison test was used. A p-value of < 0 .05 was considered statistically significant (*$p < 0.05$; **$p < 0.01$). Data are expressed as mean ± standard error of mean.

RESULTS

The cytotoxicity of cigarette smoke condensate on epithelial cells

The MTT assay was used to determine the cytotoxicity of the CSC. Cells were treated with increasing concentrations of CSC (1 – 100 μg/ml) or DMSO for 24 hours and viability of the cells was analyzed. CSC did not affect cell viability at CSC concentrations of 1 – 50 μg/ml, however there was approximately 25% cell death with CSC 100 μg/ml (Figure 1). Therefore, all experiments which involved cigarette smoke condensate and epithelial cells used CSC concentrations between 1 – 50 μg/ml.

COPD epithelial cells have a larger diameter than normal epithelial cells

I previously described the morphological distinction observed between the normal human bronchial epithelial cells (NHBE) and the COPD-diseased human bronchial epithelial cells (DHBE). Here, I describe the difference in the diameter that was observed between the two epithelial cell types. The Scepter Handheld Automated Cell Counter can determine the diameter of the cells that it counts. The NHBE cells have an approximate diameter of 8 μm, while the DHBE cells have two distinct populations, one similar to the NHBE cells, and another population which has an approximate diameter of 14 μm (Figure 2). Considering that the DHBE cells are from COPD patients, one can assume that the population of cells which have increased in size are those epithelial cells which have been altered due to the disease, and have undergone squamous metaplasia. This also

confirms the difference in appearance that is observed when looking at the DHBE cells compared to the NHBE cells.

Pathway inhibitors do not affect IL-8 production from epithelial cells

I previously demonstrated that epithelial cells treated with the pathway inhibitor PD184352 prior to CSC treatment could reduce the amount of IL-8 released. Here, I wanted to determine if the pathway inhibitors alone had any effect on IL-8 production. NHBE cells were treated with either CSC or one of the pathway inhibitors alone. Figure 3 shows that the pathway inhibitors do not induce IL-8 production. This indicates that PD184352 is truly inhibiting CSC-induced IL-8 production.

Epithelial cells are unresponsive to LPS

I have previously shown that epithelial cells produce IL-8 in response to CSC. Here, I wanted to determine if LPS could cause an induction of IL-8 as well. NHBE and DHBE cells were stimulated with LPS for 24 hours and supernatants were collected for ELISA. LPS does not significantly upregulate IL-8 production from NHBE cells (Figure 4A) or DHBE cells (Figure 4B). However, both types of cells seem to show a small, dose-dependent, increasing trend. Furthermore, it is evident that NHBE cells produce more IL-8, even at baseline, compared to the DHBE cells, suggesting that the COPD-derived epithelial cells have an altered phenotype. Similar to what was observed with CSC, DHBE cannot elicit a proper immune response to this stimulus.

CSC induces inflammatory cytokine production from normal epithelial cells

I previously observed that NHBE were able to significantly increase IL-8 production in response to cigarette smoke. Therefore, I investigated if CSC could differentially induce the production of other inflammatory cytokines. All cells were treated with CSC for 24 hours and the supernatants were collected. NHBE cells significantly increased the levels of IL-1β, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ (Figure 5 A, C, E, G, I, K) after CSC exposure, while there was no significant change in production from the DHBE cells (Figure 5 B, D, F, H, J, L). This suggests that DHBE cells have an altered phenotype and do not respond as readily to cigarette smoke as NHBE cells. Under normal conditions epithelial cells should mount an immune response when in contact with a pathogen and unfortunately, the DHBE cells are unable to do so. This may be a repair mechanism in play in order to keep the persistent inflammation in COPD under control.

$CD8^+$ T cell migration in response to CSC-exposed epithelial cells

The role of $CD8^+$ T cells in COPD still remains unclear. In addition, why these T lymphocytes in particular migrate to the lungs of these patients also remains a mystery. I thought that perhaps cigarette smoke was inducing airway epithelial cells to secrete certain chemokines which would induce $CD8^+$ T cell migration.

NHBE and DHBE cells were treated with CSC for 24 hours and the resulting supernatant was used for the $CD8^+$ T cell migration. $CD8^+$ T cells from COPD patients were isolated from peripheral blood and loaded onto the Boyden

chamber for 3 hours of migration. Regrettably, the $CD8^+$ T cells did not migrate in response to the lowest doses of CSC-exposed epithelial cell supernatants (Figure 6). However, there seems to be a slight increase in the percent of cells which migrated at the highest concentrations of CSC, indicating that high doses of CSC may induce epithelial cells to secrete certain factors which cause the migration of these cells to the lung.

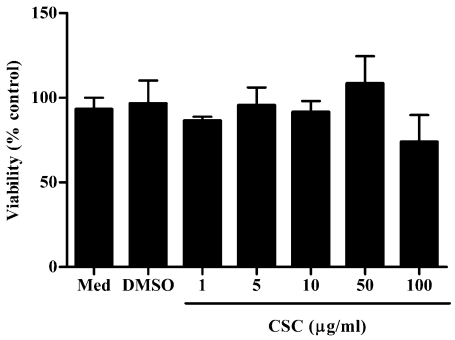


Figure 1.
Epithelial cell viability after cigarette smoke condensate exposure. Epithelial cells (n = 3) were exposed to CSC (1-100 µg/ml) or DMSO for 24 hours. Viability was determined using the colorimetric MTT assay. The percent viability was greater than 88% for CSC 1-50 µg/ml, while CSC 100 µg/ml caused more than 25% cell death. Data are represented as mean ± SEM.

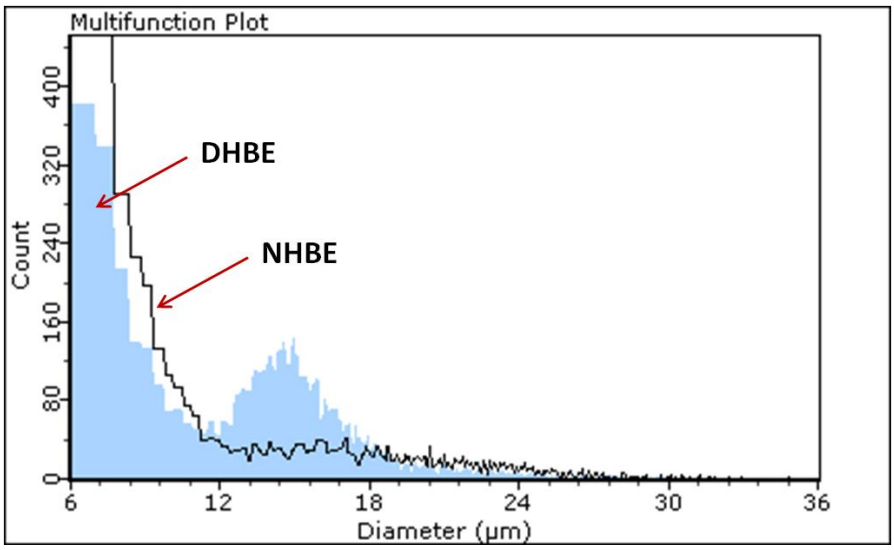


Figure 2.
COPD epithelial cells have a larger diameter than normal epithelial cells. A representative example taken from the Scepter Handheld Automated Cell Counter, which determines the diameter of counted cells. The NHBE cells (solid black line) have an approximate diameter of 8 µm, while DHBE cells (blue coloured region) have two distinct populations. One population has an average diameter similar to the NHBE cells, while the smaller population of cells has an approximate diameter of 14 µm. This suggests that DHBE cells begin as NHBE cells, however over time, due to the disease, they transform into larger cells.

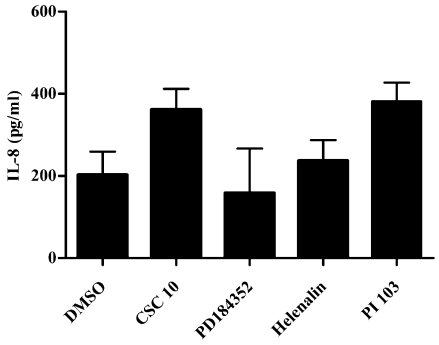


Figure 3.
Pathway inhibitors do not affect basal IL-8 production. NHBE cells (n = 4) were treated for 24 hours with either CSC (10 μg/ml); PD184352 (2 μM), which inhibits ERK1/2; Helenalin (1 μM), which inhibits NF-κB; PI-103 (5 μM), which inhibits PI3K; or DMSO as a control. Supernatants were collected and an IL-8 ELISA was performed. This figure shows that each of the pathway inhibitors does not affect IL-8 production when they are given alone to the cells. Data are represented as mean ± SEM.

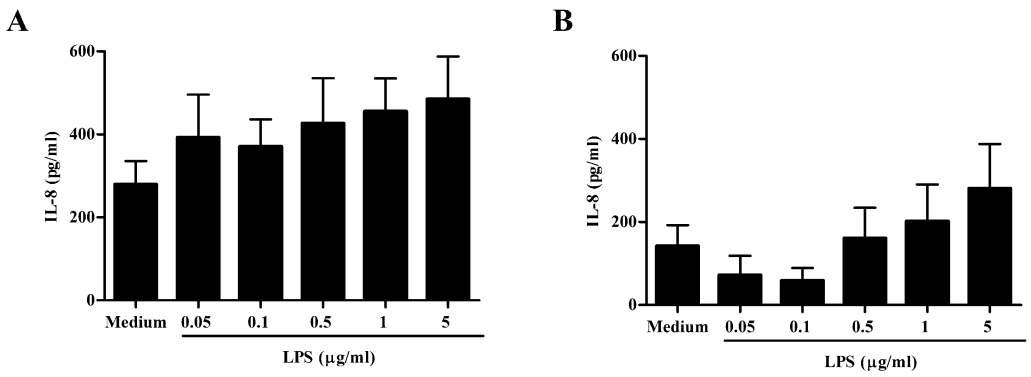
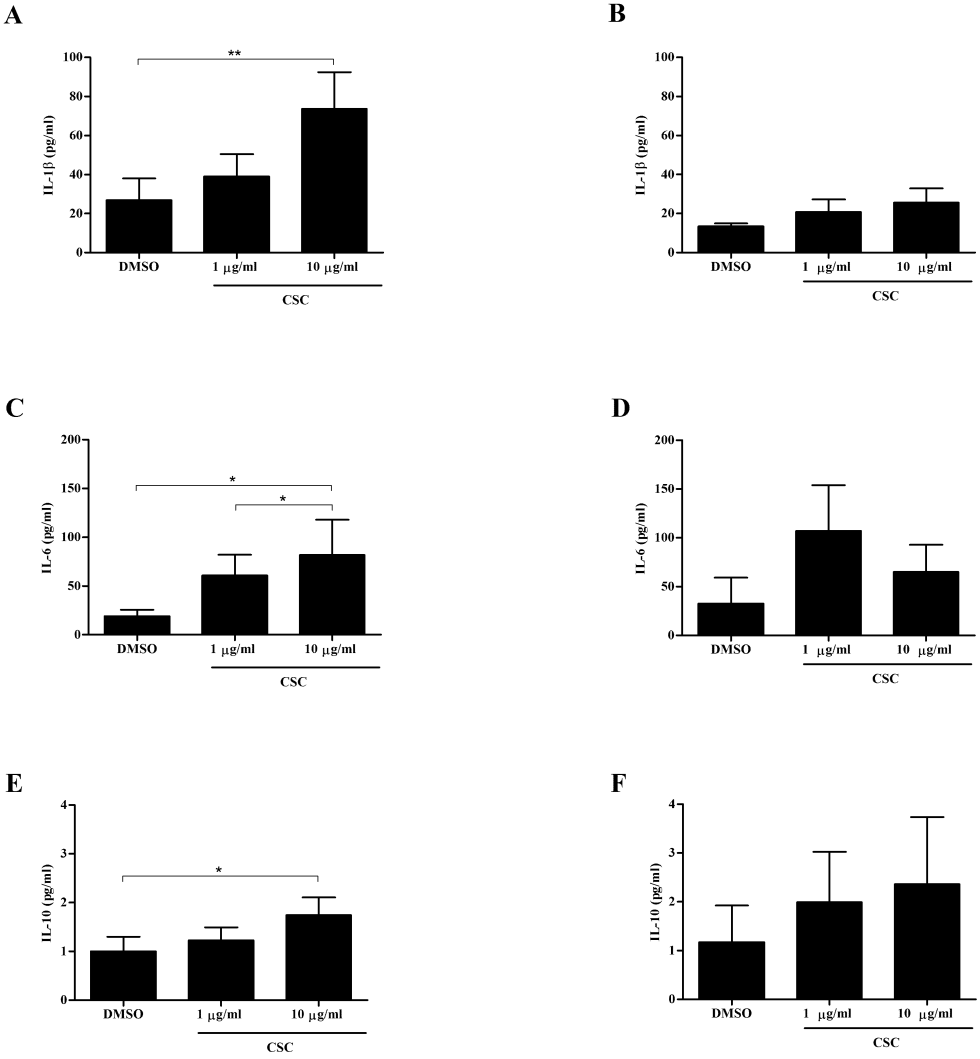


Figure 4.
LPS does not significantly increase IL-8 production from epithelial cells. NHBE cells (n = 6) and DHBE cells (n = 4) were treated with LPS (0.05 – 5 µg/ml) for 24 hours. Supernatants were collected and IL-8 secretion was measured using the Human IL-8 ELISA MAX Standard kit. IL-8 production from NHBE cells (A) and DHBE cells (B) was not significantly increased, although there seems to be a slight dose-dependent increasing trend. Data are represented as mean ± SEM.


A
B
C
D
E
F
IL-1β (pg/ml)
IL-6 (pg/ml)
IL-10 (pg/ml)
DMSO
1 μg/ml
10 μg/ml
CSC
*
**

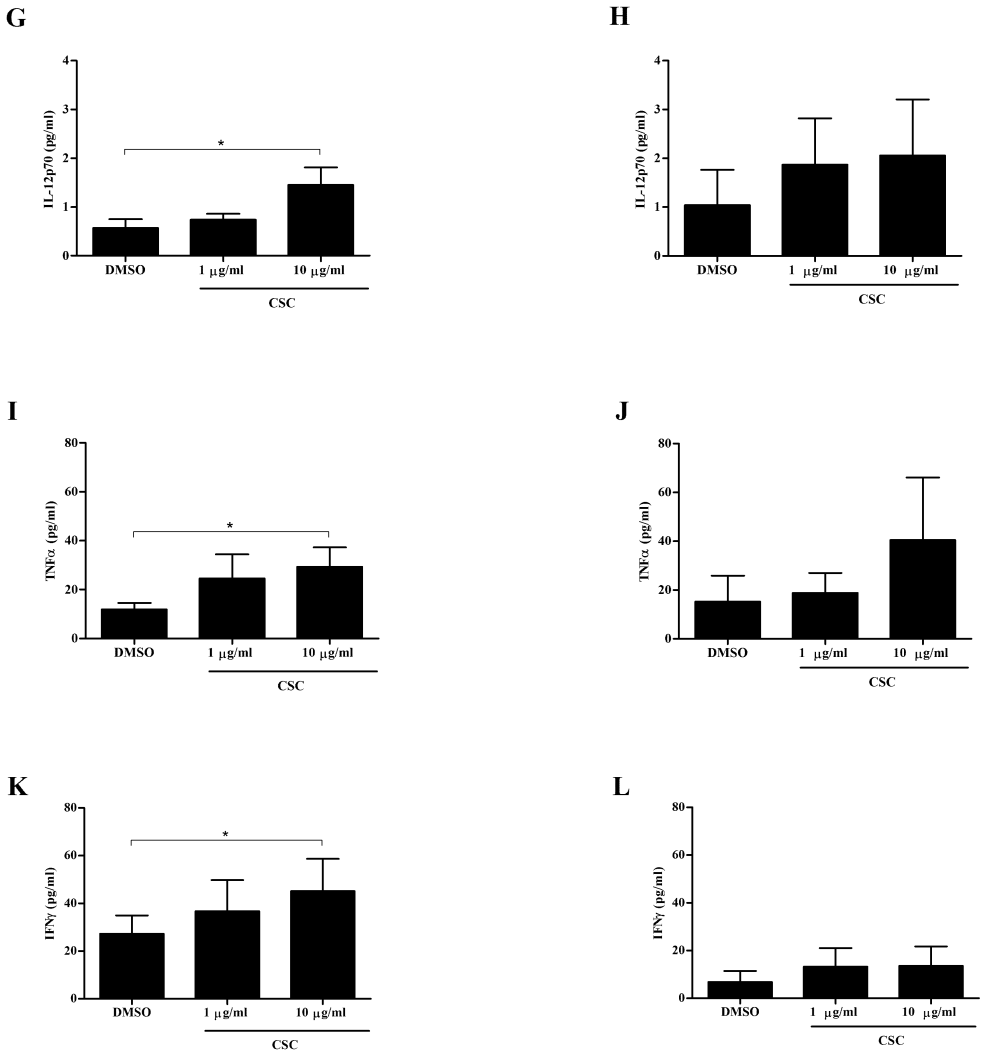


Figure 5.
CSC induces cytokine production from normal epithelial cells. NHBE cells (n = 5) and DHBE cells (n = 3) were treated with CSC (1 or 10 μg/ml) or DMSO for 24 hours. The supernatants were collected and cytokine expression was examined using the Human ProInflammatory 7-Plex Tissue Culture Plate from Meso Scale Discovery. At 10 μg/ml of CSC treatment, there was a significant increase in cytokine expression from NHBE cells for IL-1β (A), IL-6 (C), IL-10 (E), IL-12p70 (G), TNF-α (I), and IFN-γ (K) compared to DMSO. As for the DHBE cells, there was no change in expression for IL-1β (B), IL-6 (D), IL-10 (F), IL-12p70 (H), TNF-α (J), and IFN-γ (L). Data are represented as mean ± SEM. * indicates $p < 0.05$; ** indicates $p < 0.01$.

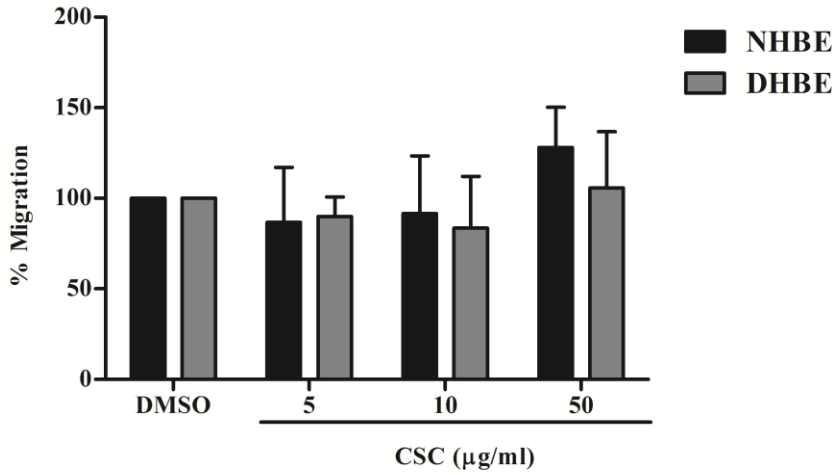


Figure 6.
$CD8^+$ T cell migration in response to CSC-exposed epithelial cells. NHBE cells (n = 3) and DHBE cells (n = 3) were previously treated with CSC (5 - 10 µg/ml) or DMSO for 24 hours. The supernatants were collected and then distributed on the lower section of the Boyden chamber. $CD8^+$ T cells were isolated and distributed on the upper section of the Boyden chamber. $CD8^+$ T cells were allowed to migrate towards the epithelial cell supernatants for 3 hours. $CD8^+$ T cells did not increase migration in response to epithelial cells exposed to CSC.

DISCUSSION

The MTT assay is a colorimetric assay in which the tetrazolium salt is reduced to form insoluble purple formazan crystals in metabolically active cells. I used this method to determine the cytotoxicity of the CSC on the airway epithelial cells. All concentrations of CSC had viability levels greater than 85%, except CSC 100 μg/ml, which caused almost 25% cell death, and was therefore not used for any subsequent experiments.

The Sceptor Handheld Automated Cell Counter is a type of cell counter that graphs histograms of the diameter of the cells that it counts. I previously described how the NHBE and DHBE cells have a different morphology, suspecting that these DHBE cells are undergoing squamous metaplasia. This device was able to confirm our suspicions, as one can see that the NHBE and DHBE histograms are different. The NHBE cells have an average cell diameter of 8 μM, while the DHBE cells have two distinct populations of cells; those that also have an average diameter of 8 μM, while a smaller population, which have likely undergone metaplasia, have a diameter of 14 μM.

I previously demonstrated that PD184352 can inhibit cigarette smoke-induced IL-8 production from epithelial cells. To ensure that the signaling pathway inhibitors did not have any effect on the cells themselves, I decided to treat the cells with the inhibitors alone. There was no significant difference between the control and the pathway inhibitors. Therefore, as it was suggested, PD184352 can specifically inhibit IL-8 production from epithelial cells exposed to cigarette smoke.

Our previous results showed the difference in CSC-induced IL-8 production from NHBE cells compared to DHBE. I wanted to see if other factors which induce TLR4 activation responded in a similar manner. NHBE and DHBE cells were treated with LPS for 24 hours. I determined that there was no significant increase in IL-8 production from either NHBE or DHBE epithelial cells, although there seems to be a dose-dependent increasing trend. In addition, I also observed that once again, the NHBE cells were able to induce more IL-8 in response to LPS than the DHBE cells. This suggests that there may be a difference in the response of TLR4 in both types of epithelial cells as both CSC and LPS are ligands for this receptor.

Finally, I decided to investigate if the epithelial cells could induce the expression of other inflammatory cytokines in response to cigarette smoke. The NHBE cells were able to significantly increase IL-1β, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ when exposed to CSC 10 μg/ml. The DHBE cells were not able to induce any significant change in cytokine expression. Once again, one sees that the DHBE cells secrete less cytokine levels than the NHBE cells. To the best of my knowledge, only one other study has examined the differences between normal epithelial cells and epithelial cells from a COPD patient in response to cigarette smoke. The study treated their cells for only 20 minutes with cigarette smoke or air, and looked at the production of IL-1β [178]. They too found that the normal epithelial cells released more IL-1β in response to cigarette smoke than the diseased cells, which confirms our findings. This suggests that the DHBE cells become desensitized to pathogens or toxicants in an attempt to reduce the extent of the inflammation.

For my last experiment I attempted to determine what causes $CD8^+$ T cells to migrate into the lungs of COPD patients. I hypothesized that the epithelial cells, which line the airways, will secrete certain chemokines, which may recruit these cells to migrate towards the lung. Certain factors, such as the chemokine receptor CXCR3, have been shown to play an important role in T cell migration in various diseases [370, 371]. I hypothesized that perhaps CSC could induce certain chemotactic factors. Regrettably, the supernatants from CSC-exposed epithelial cells were unable to significantly induce $CD8^+$ T cell migration. This data does suggest that higher concentrations of CSC may be able to induce migration, however further experiments will be needed to confirm this idea as well as identify which factors may play a role.

CHAPTER 4: DISCUSSION

4.1 Discussion of results

COPD is a complex and multifaceted inflammatory disease that is not completely understood. It was only 50 years ago that the British versus Dutch hypotheses [4, 5] were argued and more recently researchers were trying to classify COPD as an autoimmune disease [113]. Regardless of all the debates, throughout this last half century, great strides have been made in determining the risk factors for this disease. It was previously thought that repeated airway infection was the primary cause for the development of COPD. The groundbreaking study by Fletcher et al. revealed that this was not correct and that tobacco smoke was the main causative factor for COPD. After this study, it was thought that airway infections were an epiphenomenon, with little pathogenic significance. More recently, studies have revealed that infections are a likely contributor to the pathogenesis of COPD as well [34]. This suggests that there are still major aspects surrounding the pathogenesis of this disease that remain to be elucidated and more research is needed to fully understand the pathology of COPD.

The main objective of this thesis was to investigate the role of TLRs in COPD and more specifically, to see how they respond to cigarette smoke exposure. Given that these innate immune receptors normally function during bacterial and viral infections and are known to induce inflammatory cytokines, I thought that these receptors may be involved in regulating certain features of inflammation in COPD.

This project commenced by examining the expression of TLR4 and TLR9 in endobronchial biopsies from COPD patients and aged-matched healthy control subjects. Interestingly, I found that there was no difference in the total number of inflammatory cells expressing TLR4 or TLR9 in COPD patients compared to control subjects. There was also no difference in the extent of epithelium positively stained for TLR4 or TLR9 in COPD patients compared to control subjects. Moreover, normal human bronchial epithelial (NHBE) and COPD-diseased human bronchial epithelial (DHBE) cells, which were grown in culture, also expressed similar amounts of total TLR4 protein. Even when both types of epithelial cells were exposed to cigarette smoke, no change in the TLR4 protein expression was found, further confirming our results which were observed within lung biopsies.

After no difference was found in the number of cells expressing TLRs in the lung, I decided to examine which inflammatory cell types expressed these receptors. It was determined that 90% of $CD8^+$ T cells from the lungs of COPD patients expressed TLR4 and TLR9 compared to less than 20% in healthy control subjects. Next, I analyzed the expression of TLR4 and TLR9 in peripheral blood $CD8^+$ T cells from COPD patients and control subjects. It was revealed that less than 2% of these cells in both groups expressed either receptor. Therefore, there was a certain factor inducing TLR expression on lung $CD8^+$ T cells from COPD patients that was not present in control lungs nor peripheral blood from either group. I believed that cigarette smoke may be the factor inducing this TLR expression.

We found that cigarette smoke exposure was able to induce $CD8^+$ T cells to upregulate the TLR4 and TLR9 protein as well as induce inflammatory cytokine production. TLR4 and TLR9 inhibitors were able to attenuate the cytokine production, suggesting that cigarette smoke-induced inflammation is at least partially TLR-dependent. Similar results were observed with the epithelial cells, as cigarette smoke exposure was able to induce IL-8 production from both the NHBE and DHBE cells. The use of pathway inhibitors revealed that this mechanism is both TLR4- and ERK1/2- dependent, regardless of the fact that cigarette smoke exposure did not upregulate the TLR4 protein expression in the NHBE or DHBE cells. Furthermore, I demonstrated that cigarette smoke can induce more IL-8 production from NHBE cells compared to DHBE cells. This suggests that COPD-derived epithelial cells no longer respond to cigarette smoke or perhaps have an altered phenotype that fails to produce a proper immune response when exposed to a pathogen.

It was previously thought that the lung was a sterile environment. However, more recently it has been demonstrated that this is not correct and even normal, healthy lungs have a core microbiome. The paper by Erb-Downward et al. describes the differences in the lung microbiome of healthy controls compared to smokers, as well as COPD patients. It was observed that all individuals have a common core set of bacterial flora with some small differences between groups [372]. Even more interesting and somewhat unexpected, was that moderate and severe COPD patients tend to have less diversity in their lung microbiome. This suggests that although they may in fact have more resident bacteria within their lungs, they tend to be of similar bacterial communities. When considering the

amount of TLRs present within the lung it is remarkable to think that under normal bacterial conditions these receptors do not respond. These conserved innate immune receptors have adapted to the core microbiome and only become activated when foreign pathogens are inhaled into the lung proving how elegantly this system functions. This also suggests that these receptors have fine-tuned their role and if activated, are likely play a significant role in inflammation.

TLRs have previously been shown to be involved in COPD. A small number of studies have investigated the expression of TLRs in COPD and have suggested that there may be some benefit to using anti-TLR therapy to alleviate the severe inflammation observed within the lungs of these patients [22]. As would be expected, TLRs were found to have variable expression in COPD patients when compared to healthy controls. Monocytes from COPD patients and control subjects had similar expression of TLR4, while TLR2 was upregulated in patients with COPD [320]. Alveolar macrophages had decreased TLR2 expression in COPD patients [321], and neutrophils and macrophages taken from the BALF of COPD patients were found to express more TLR4 than cells taken from non-smokers and healthy smokers [312]. Our study was the first to investigate TLR expression on $CD8^+$ T cells in COPD; however other studies have examined the expression of TLRs on these cells in other conditions. Hepatitis C-infected patients undergoing therapy had significantly higher TLR9 expression on $CD8^+$ T cells than healthy control subjects, though there was no change in TLR4 expression between the two groups [373]. It was demonstrated that TLR4 was needed to trap and promote $CD8^+$ T cell adhesion in the liver in the presence of endotoxin [374] and the activation of TLR2 on $CD8^+$ T cells

increases cytotoxicity and cytokine production [375]. It was also observed that $CD8^+$ T cells increase their TLR4 expression after burn injuries in order to regulate the immune response [376]. T lymphocytes are not normally considered cells which express TLRs [194, 195], which is why our findings that cigarette smoke can significantly increase TLR4 and TLR9 expression on $CD8^+$ T cells, as well as induce these cells to produce a considerable amount of cytokines is extremely important. It implies that there is a specific role for these receptors in regulating the function of immune cells infiltrating into the lungs.

I decided to focus the first half of this project on $CD8^+$ T cells, as these are a predominant cell type in COPD. Interestingly, there is a considerable amount of information lacking regarding the role of these cells in the disease. The presence of $CD8^+$ T cells in the central and peripheral airways, parenchyma, and sputum has all been described [66, 107, 108, 137]. Data from humans has also revealed that the cytotoxic potential of these cells increases with disease severity [152]. A mouse study by Maeno et al. further demonstrated that $CD8^+$ T cells are necessary for emphysema-like tissue destruction [377]. Mice deficient in these cytotoxic cells have decreased inflammation in response to long-term cigarette smoke exposure. In addition, this study found that $CD8^+$ T cells had an indirect effect on tissue destruction, as a reduction in the number of $CD8^+$ led to decreased MMP activity, resulting in less tissue damage. This suggests that $CD8^+$ T cells are a central regulator of inflammation in COPD, which coincides with the results I obtained about inflammatory cytokine production. I found that at baseline, these cells produce negligible amounts of cytokines, however, once exposed to cigarette smoke, they were able to significantly increase their inflammatory cytokine

production. Normally, the potential of cytokine production from $CD8^+$ T cells is neglected, as $CD4^+$ T cells are the T cells which more commonly produce various types of cytokines, from both the Th1 and Th2 class [378, 379]. However, under the proper priming conditions, $CD8^+$ T cells can produce a wide array of inflammatory cytokines [380]. I found that cigarette smoke was able to activate these cells to produce IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and IFN-γ. If one can determine which other types of cytokines are being produced, one may be able to reduce the inflammation through anti-cytokine therapy. Further experiments also revealed that the use of TLR4 and TLR9 inhibitors were able to attenuate some of the cytokine production, suggesting that the cigarette smoke is activating TLR4 and TLR9 to induce their signaling pathways to produce cytokines. These results confirm a previous study which found that cigarette smoke-induced IL-8 expression from epithelial cells is attenuated when cells are pre-treated with TLR4 and TLR9 inhibitors [25]. Therefore, our data supports the idea that anti-TLR therapy may have the potential to downregulate inflammation in COPD.

Another question concerning the role of $CD8^+$ T cells in COPD, is why these cells in particular migrate to the lung. I hypothesized that epithelial cells exposed to cigarette smoke may induce chemokines, such as CCL5, CXCL9, and CXCL10, which are known to cause $CD8^+$ T cell migration [381-383]. In an attempt to answer this, I took supernatants from epithelial cells exposed to cigarette smoke and allowed $CD8^+$ T cells to migrate towards the supernatant. Unfortunately, there was no significant increase in migration towards the cigarette smoke-exposed supernatants, which means that something else must be causing

the recruitment of these cells to the lung. These cells are central to COPD and figuring out their role in the pathogenesis would help to fully understand the mechanisms behind the disease.

The large difference in expression of TLR4 and TLR9 on lung $CD8^+$ T from COPD patients compared to control subjects, as well as the fact that peripheral blood $CD8^+$ T cells had minimal TLR expression, led us to believe that cigarette smoke exposure to the lungs was inducing the increased TLR expression. In addition, a previous study had shown that cigarette smoke-induced inflammation was TLR4-dependent [26], further affirming our hypothesis. Our results did in fact reveal that exposing $CD8^+$ T cells to cigarette smoke upregulatated TLR4 and TLR9 expression. When the same experiment was performed with NHBE and DHBE cells, I did not observe an increase in TLR4 expression. This was not surprising as epithelial cells express low levels of TLR4 [193]. This could be due to the fact that epithelial cells are the first cells to come into contact with a pathogen and expressing high levels of TLRs on these cells would result in excessive inflammation that could not be regulated. It should be noted that under certain inflammatory conditions, such as in the combined presence of TNF-α and IFN-γ, TLR4 expression can be modestly increased on epithelial cells [384].

Although cigarette smoke was unable to increase the expression of TLR4 on the epithelial cells, it did induce IL-8 production. Similar results were observed when the cells were treated with LPS. It has been shown that both LPS and cigarette smoke condensate can induce IL-8 release from epithelial cells [20, 310, 385], however our results are the first to compare normal epithelial cells to

COPD-derived epithelial cells. Interestingly, I found that under both treatments, the normal epithelial cells produced more IL-8 than the diseased cells. These results are opposite to what was observed in a previous study done on asthmatic patients. Unstimulated airway epithelial cells from asthmatic subjects had higher IL-8 expression, as well as IL-6, and GM-CSF than control subjects [386]. The difference for this may be explained by the fact that COPD has a more sustained and different type of inflammation that cannot handle the increased cytokine release, while asthma is normally episodic in nature and during an exacerbation, more cytokine production may be released to control the inflammation.

The cigarette smoke-induced inflammatory response of epithelial cells was found to be TLR4-dependent, as a pharmacological inhibitor was able to reduce the amount of IL-8 secreted subsequent to cigarette smoke exposure. A study by Mortaz et al. found that a TLR4 blocking antibody had the same effect and was also able to inhibit IL-8 production in epithelial cells following smoke exposure [25]. Moreover, after testing several pathway inhibitors, I found that these epithelial cells activate TLR4 which then signals through the ERK1/2 pathway. The transcription factor NF-κB has been well-studied and has been shown to play a key role in regulating cytokine production in airway epithelial cells [358, 365, 387, 388]. It was therefore surprising that our data showed that the NF-κB pathway was not involved in this signaling process. However, a number of previous studies have corroborated our results in demonstrating the activation of ERK1/2 signaling pathway after cigarette smoke exposure, suggesting that this pathway is also critical for cytokine release in epithelial cells [359, 360]. The reason for the discrepancy with the lack of involvement of NF-κB may be due to

differences in primary cells compared to cell lines, the type of cigarette smoke used to treat the cells, as well as the length of time the cells are exposed to the cigarette smoke.

Cigarette smoke is made of over 4000 components and is therefore a very complex substance. Several carcinogens and oxidants can be found within the smoke and it is therefore next to impossible to determine which factors are causing the activation of the TLRs. It has been reported that LPS can found within cigarette smoke [45] and consequently, I thought that perhaps our cigarette smoke contained LPS, which could be the component activating TLR4. However, after testing our cigarette smoke for the presence of LPS, I found that there was no trace of it. This indicates that there is another component within the cigarette smoke condensate which is responsible for activating TLR4, as well as TLR9. There are several ligands which are known to bind and activate TLR4, while CpG DNA is the only known ligand for TLR9 [243]. Therefore, there must be more ligands which can activate these receptors that still remain to be elucidated. It is interesting to consider that these innate immune receptors, which have been so closely conserved from *Drosophila* to humans, are still not fully characterized. Determining which aspects of cigarette smoke can cause the activation of these receptors would be quite a daunting task, although extremely valuable. If it is possible to inhibit the binding of this factor to the TLRs, it may be possible to reduce the amount of inflammation that results from cigarette smoke.

4.2 Summary

In our first study, I wanted to investigate the expression of TLR4 and TLR9 in COPD patients compared to healthy control subjects. It was clearly demonstrated that although the total expression of these receptors does not change within the lung, the cells which express these receptors does change between healthy lungs and COPD lungs. I showed for the first time that 90% of lung $CD8^+$ T cells from COPD patients express TLR4 and TLR9 compared to less than 20% in control subjects. Further investigation demonstrated that cigarette smoke exposure was able to increase the TLR4 and TLR9 protein expression on $CD8^+$ T cells. Moreover, cigarette smoke was able to induce inflammatory cytokine production from $CD8^+$ T cells in a TLR-dependent manner, as TLR inhibitors were able to attenuate the cytokine production.

In our second set of experiments I investigated how normal and COPD-derived epithelial respond to cigarette smoke exposure. I demonstrated that normal epithelial cells significantly increase IL-8 production following cigarette smoke exposure, while the epithelial cells from COPD do not. Although the cigarette smoke was unable to upregulate the TLR4 protein expression on these cells, I did find that cigarette smoke activated the receptor, as a TLR4 pharmacological inhibitor was able to attenuate IL-8 production. In addition, using signaling pathway inhibitors, I demonstrated that cigarette smoke activates the ERK1/2 pathway to increase inflammatory cytokine production.

4.3 Future directions

As with all research, there is always more one can do to fully understand the entire story. There are a few experiments which could be completed to help fill in some of this story. At the end of this thesis, I was interested in the interactions between the $CD8^+$ T cells and epithelial cells. I hypothesized that cigarette smoke-exposed epithelial cells may secrete certain chemokines which induced $CD8^+$ T cell migration. Unfortunately, I did not get any significant results, but there is definitely more one can do in this area. Perhaps the cells need to migrate for a longer period of time, or the epithelial cells need a shorter stimulation time. Furthermore, one can analyze the epithelial cell supernatant to determine if any chemokines which induce T cell migration are present. In addition, most of our experiments were performed using peripheral blood and primary cells that were purchased. It would be interesting to separate lung tissue $CD8^+$ T cells and epithelial cells from the same individual and perform the same experiments. This may help to recreate a more "real" physiological setting.

Another interesting set of experiments could be done on the epithelial cells as well. I have demonstrated that COPD-derived epithelial cells have an altered phenotype compared to normal cells. It would be worthwhile to further investigate this as I have only studied how these cells respond to cigarette smoke and LPS, both of which activate TLR4. It would be interesting to look into how these cells respond to other bacteria and viruses and determine if other TLRs play a role.

Lastly, it would be extremely helpful if one were able to determine which factor(s) in cigarette smoke was inducing the inflammation. If this was discovered and targeted, one could potentially reduce the amount of local inflammation in the

lungs of COPD patients. Any further research which would help to expose more details about the pathogenesis of COPD would be extremely useful as there is still much unknown about this debilitating disease.

4.4 Implications of results

The current pharmacological therapies used to treat COPD patients are bronchodilators as well as inhaled corticosteroids. Unfortunately, studies have demonstrated that neither of these treatments prevents the decline in lung function observed within these patients [331, 332]. Therefore, there is presently a need for novel therapies which can hinder the decline in FEV_1. There has been speculation that inhibiting TLR activation may prove to be a favorable therapy. TLRs are crucial cellular targets that are at the forefront of initiating and maintaining inflammation. Blocking these receptors from activating and initiating a signaling cascade would result in attenuating certain inflammatory cytokines from being released. Thus, targeting these receptors in an attempt to hinder inflammation may also impede lung function decline.

The problem remains that these are innate immune receptors which can be found on all types of cells. One would not want to block all TLR receptors on all cell types as that could lead to other problems for the patient. It would be more favourable if it were possible to make an anti-TLR therapy that was selective for certain cells. Therefore, one could target the precise TLRs which are causing the inflammation on specific immune cells.

4.5 Conclusion

From our results one can conclude that TLRs have altered expression depending on cell type and location. I observed that under normal conditions, epithelial cells can respond to cigarette smoke exposure by inducing IL-8 to promote inflammation, as would be beneficial in a healthy lung. Conversely, COPD epithelial cells seem to induce less IL-8 production, indicating a change in the phenotype of these epithelial cells, most likely in an attempt to curb the amount of inflammation in an already diseased lung. In addition, cigarette smoke was also able to upregulate TLR4 and TLR9 expression on lung COPD $CD8^+$ T cells as well as induce these cells to secrete cytokines. One can therefore speculate that TLRs play a cell-specific role that is two-fold. First, these receptors have a role in the initiation of inflammation by smoking, as evidenced by our results on epithelial cells. It seems that TLR4 can function in the normal lung to try to rid the airways of pathogens by promoting inflammation. Secondly, once the development of COPD has begun and there is sustained inflammation, it might be that these receptors are upregulated, as well as activated on $CD8^+$ T cells, to induce more cytokine production. What is also interesting is that this dual, cell-specific role of TLRs begins with the epithelium, which is the first line of defense against any invading pathogen and once it has been breached, these receptors become activated on immune cells. Furthermore, our data demonstrates that epithelial cells in general, responded to lower amounts of cigarette smoke than the $CD8^+$ T cells. Normal epithelial cells were able to secrete significant amounts of IL-8 with a CSC concentration of 10 μg/ml, while $CD8^+$ T cells only produced significant amounts of cytokines at a CSC concentration of 50 μg/ml. Under

normal conditions, TLRs are activated on epithelial cells with lower concentrations as they are the first cell to be in direct contact with the smoke and this allows the inflammatory response to occur quickly and to clear the lung of the pathogen. Once a patient has COPD, it would take more cigarette smoke to induce the same response in epithelial cells, as was observed with the COPD epithelial cells. Moreover, being that the lung is already quite inflamed, the $CD8^+$ T cells also only respond to higher concentrations of cigarette smoke in order to try and reduce the amount of inflammation within the lungs of these patients. One can conclude that TLRs play a role in both the initiation and maintenance of inflammation in COPD. Therefore, the idea of anti-TLR therapy may be beneficial for alleviating the inflammation in patients with COPD.

STATEMENT OF ORIGINALITY

- To the best of our knowledge, this was the first study to investigate the expression of TLR4 and TLR9 on $CD8^+$ T cells. I found that the expression of TLR4 and TLR9 was increased on $CD8^+$ T cells from the lungs of COPD patients compared to healthy control subjects. Furthermore, I investigated the effect of cigarette smoke condensate on $CD8^+$ T cells and demonstrated that it was able to induce TLR4 and TLR9 expression on $CD8^+$ T cells as well as cause these cells to release various inflammatory cytokines, which has never been shown before.
- To the best of our knowledge, this was the first study to investigate the difference in cigarette smoke-induced IL-8 production in normal human bronchial epithelial cells (NHBE) compared COPD-diseased human bronchial epithelial cells (DHBE), in which we found that NHBE cells secrete more IL-8 in response to cigarette smoke than DHBE cells. In addition, I demonstrate for the first time that cigarette smoke-induced inflammation can be attenuated in both NHBE cells and DHBE cells by inhibiting the MAPK ERK1/2 signaling pathway.

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