IMMUNE RESPONSES ASSOCIATED WITH COMBINED EXPOSURE TO ALLERGEN AND DIESEL EXHAUST IN HUMAN ATOPIC SUBMUCOSA

by

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Abstract

Asthma is a chronic condition described by inflammation of the airways. There are no specific treatments for asthma yet but understanding the potential triggers of an asthma attack can effectively control this disease and make it more manageable for many sufferers. Therefore, there is intense interest in studying the negative impacts of air pollutants on respiratory diseases. Diesel exhaust (DE) is a primary source of emissions from motor vehicles and is also a significant cause of increased airway responsiveness in asthma. The main particulate fraction of diesel exhaust consists of fine particles ($PM_{2.5}$), which are inhaled efficiently into the lung. Inhalation of these particles can exacerbate asthma and trigger other harmful processes in the lung. The effect of traffic-related air pollution, such as DE on asthma exacerbations is well-established but the biological mechanism underlying this association is still not well understood. DE is thought to interact with allergen exposures to mediate adverse effects, but most of the studies done in this area are based on animal models and there remains poor appreciation of the mechanisms of allergen-DE synergy in human models. In this research project, we aim to elucidate if DE increases bronchial allergen-induced inflammation and cellular immune response in mild atopic asthmatic human subjects.

Volunteer participants were exposed to DE (300 $\mu g.m^{-3}$ of $PM_{2.5}$) or filtered air for two hours in a blinded crossover study design with a four-week washout period. One hour following either filtered air or DE exposure, subjects were exposed to allergen or saline via bronchoscopic segmental challenge. Forty-eight hours post-exposure, endobronchial biopsies were collected. Tissue sections were immunostained for tryptase, eosinophil cationic protein (ECP), neutrophil elastase (NE), CD138, CD4 and interleukin (IL)-4. The percent positivity of positive cells were quantified in the bronchial submucosa by Aperio ImageScope Software.

We have shown that *in vivo* allergen and DE co-exposure results in elevated CD4, IL-4, CD138 and NE in the respiratory submucosa of atopic subjects, while eosinophils and mast cells are not changed. Here we demonstrated, for the first time, the effect of DE exposure in promoting allergen-induced inflammatory responses directly within the lungs of atopic human.

Preface

This study is based on human controlled exposures, which were performed at the Air Pollution Exposure Laboratory (APEL) at the Vancouver General Hospital - Research Pavilion. Histological procedures and immunohistochemistry experiments were conducted at UBC Centre for Heart Lung Innovation (HLI) at St. Paul's Hospital and the Jack Bell Research Centre. The work presented in this thesis is covered under the Human Ethics Board of UBC certificate number H11-01831, project title "Effects of Co-Exposure to Air Pollution and Allergen". This human-controlled exposure study is registered at www.clinicaltrials.gov (Unique identifier: NCT01792232).

This thesis is an original intellectual product of the author, A. Hosseini, who was responsible for all major areas of concept development, data collection and analysis pertinent to this thesis, as well as thesis composition. Dr. K. McNagny and Dr. T. Hackett, were involved in the early stages of concept formation and contributed to thesis edits. Dr. C. Carlsten was the supervisory author on this project, designed and led the overall study that formed the basis for this work, and was involved throughout the project in concept formation and thesis edits.

A version of chapter 3 has been published. Hosseini A, Hirota JA, Hackett TL, McNagny KM, Wilson SJ, Carlsten C. Morphometric analysis of inflammation in bronchial biopsies following exposure to inhaled diesel exhaust and allergen challenge in atopic subjects. *Particle and Fibre Toxicology*. 2016 Jan 13;13(1):2. I assisted with monitoring of the subjects during voluntary exposures and was responsible for carrying out the immunohistochemistry experiments, performing image analysis and data collection, conducting the statistical analysis and data interpretation, and drafting the manuscript. Dr. C. Carlsten, Dr. J. Hirota, Dr. T. Hackett, Dr. K. McNagny and Dr. S. Wilson provided intellectual input throughout the study and contributed to revising the draft manuscript. Dr. C. Carlsten supervised the work, performed the bronchoscopy procedure, designed and coordinated the overall study that generated samples pertinent to this thesis.

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List of Abbreviations

AHR: Airway hyperresponsiveness
APC: Antigen presenting cell
BALF: Bronchoalveolar lavage fluid
CD: Cluster of differentiation
DE: Diesel exhaust
DEP: Diesel exhaust particle
EBx: Endobronchial biopsy
ECP: Eosinophil cationic protein
EDN: Eosinophil-derived neurotoxin
EPO: Eosinophil peroxidase
FA: Filtered air
FcεRI: High-affinity IgE receptor
FEV_1: Forced expiratory volume in 1 second
GATA3: GATA binding protein 3
GMA: Glycol methacrylate resin
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HDM: House dust mite
ICAM-1: Intra-cellular adhesion molecule-1
IFN-γ: Interferon gamma
IgE: Immunoglobulin E
IHC: Immunohistochemistry
IL: Interleukin
LTC4: Leukotriene C4
MBP: Major basic protein
PGD2: Prostaglandin D2
PM: Particulate matter
PMSF: Phenylmethylsulfonyl fluoride
ROS: Reactive oxygen species
SAC: Segmental allergen challenge
STAT6: Signal transducer and activator of transcription-6
T-bet: T-box transcription factor TBX21
TBS: Tris-buffered saline
Th2: T helper cell type-2
TNF: Tumor necrosis factor
Treg: Regulatory T cells
VCAM-1: Vascular cell adhesion molecule-1

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Dedication

This thesis is dedicated to my family and friends; in particular to my little angel Elina.

Chapter 1: Introduction

1.1 Exposure to air pollution: a global health concern

According to the world health organization (WHO), 7 million people died in 2012 as a result of air pollution exposure [1]. That is almost 1 in 8 deaths worldwide, making air pollution the biggest environmental contributor to mortality. This estimate has doubled since 2008, suggesting that air pollution is increasingly taking a toll on human health [2], although some of this increase may be due to better observational methods.

In recent years, numerous epidemiological studies have established a significant association between exposure to ambient air particulate matter (PM) and mortality and morbidity due to cardiovascular and respiratory diseases [3-5]. In particular, asthma is a major public health problem; it is estimated that 300 million people suffer from asthma around the globe, with 250,000 annual deaths and more than 2 million emergency room visits in U.S. [6, 7]. Asthma is commonly defined as a chronic inflammatory condition characterized by airway inflammation, reversible airway obstruction, and increased airway responsiveness, leading to symptoms such as wheezing, coughing, shortness of breath, and chest tightness [8]. Airway inflammation in asthmatic patients happens as a result of hypersensitivity of the respiratory tract to triggers like allergens and air pollutants resulting in accumulation of chronic inflammatory cells in the airway wall [9]. While asthma is typically a chronic disease, understanding the potential triggers of an asthma attack can effectively control it and make it more manageable for many sufferers [10]. Given such a large impact, and the consequent financial impact (Figure 1.1), there is intense interest in studying the negative impacts of air pollutants on respiratory diseases.

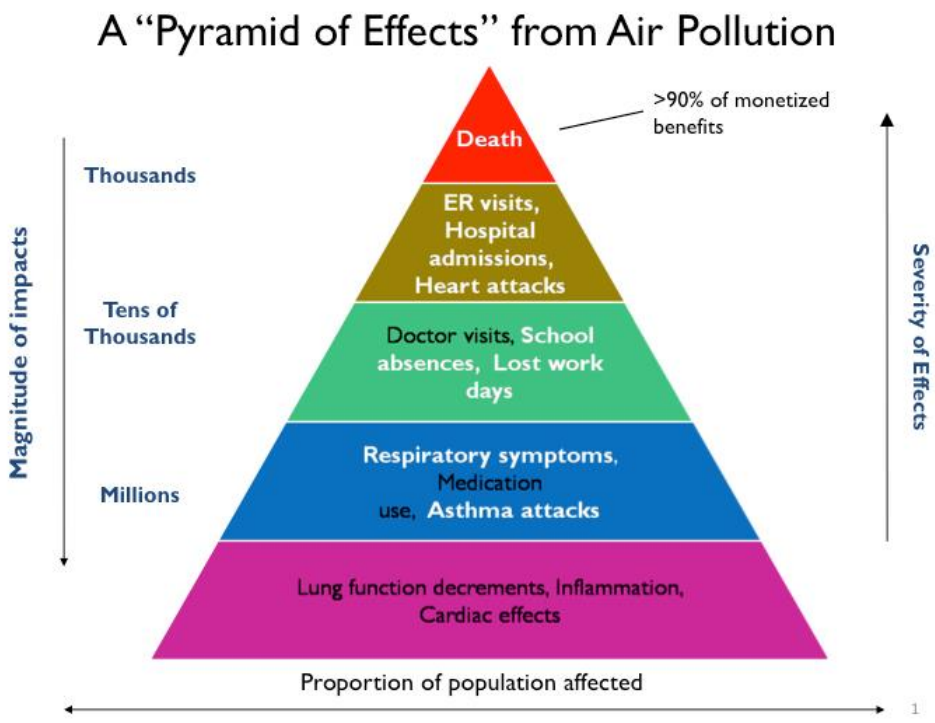

Figure 1.1 Health and economic effects of air pollution (Image accessed from http://www.epa.gov/benmap/how-benmap-ce-estimates-health-and-economic-effects-air-pollution)

Toxicological studies have shown that ambient airborne particulate matter (PM) can induce the production of certain cytokines and oxidants that initiate a cascade that can result in airway inflammation [11]. PM may have direct effects on the pulmonary system, including induction of inflammatory response, exacerbation of the existing airway disease or impairment of pulmonary defense mechanisms [12]. Epidemiologic reports have indicated that there is a higher prevalence of asthmatic and allergic symptoms between people who live closer to major roads [13-15]. Diesel exhaust (DE) is a principle contributor to ambient urban PM [16]. It has been suggested that exposure to diesel exhaust particles (DEPs) can trigger T-helper type 2 (Th2) immune response which is directly associated with developing and aggravating allergic

asthma and other respiratory diseases [17]. Consistent with observational studies, a great number of animal and human nasal models have demonstrated that DEPs can act as adjuvants to augment allergic immune responses [18-21]. The chronic airway inflammation in allergic asthma is characterized by activation of mast cells, T cells and infiltration of activated eosinophils and basophils [22]. Inhalation of aeroallergen, results in Th2 cell activation and secretion of inflammatory cytokines such as interleukin (IL)-4, IL-5, IL-9 and IL-13, which are considered to play an important role in the mucus hyper-secretion, thickening and contraction of airway smooth muscle in atopic asthmatic patients [22-25]. It has been hypothesized that DEPs impose a greater effect in the presence of environmental allergens but the exact mechanism behind this synergy is still not clear. More in-depth human studies with complex real-world exposure are needed to elucidate the biological mechanism of human lung immune response *in vivo* to DE and the potential contribution of DE to the pathogenesis of asthma. The knowledge gained in this study may help fill the gap in our understanding of the immunologic effects of DE on allergen-induced lung inflammation, which may lead to novel and more efficient treatments for allergic asthma; it can also provide further evidence justifying the importance of implementing strategies and imposing regulations to reduce the effects of air pollution on respiratory health in susceptible populations.

1.2 Diesel Exhaust characteristics and health effects of particulate matter (PM)

Diesel fuel is a mixture of hydrocarbons including polycyclic aromatic hydrocarbons (PAHs), which would produce only carbon dioxide (CO_2) and water vapor (H_2O) during an ideal combustion process [26]. Exhaust emissions from internal combustion engines consists of a complex mixture of wide range of organic and inorganic compounds in gaseous and particulate

phases. The main gases present in diesel exhaust in an incomplete combustion of diesel fuel are nitric oxides (NOx), sulfur oxides (SO_2) and carbon monoxide (CO) [27]. Diesel exhaust particles are fine particles composed of a center core of elemental carbon upon which particles such as carbon, PAHs, organic materials such as aliphatic hydrocarbons and bits of metallic compounds deposit [28]. (See Figure 1.2)

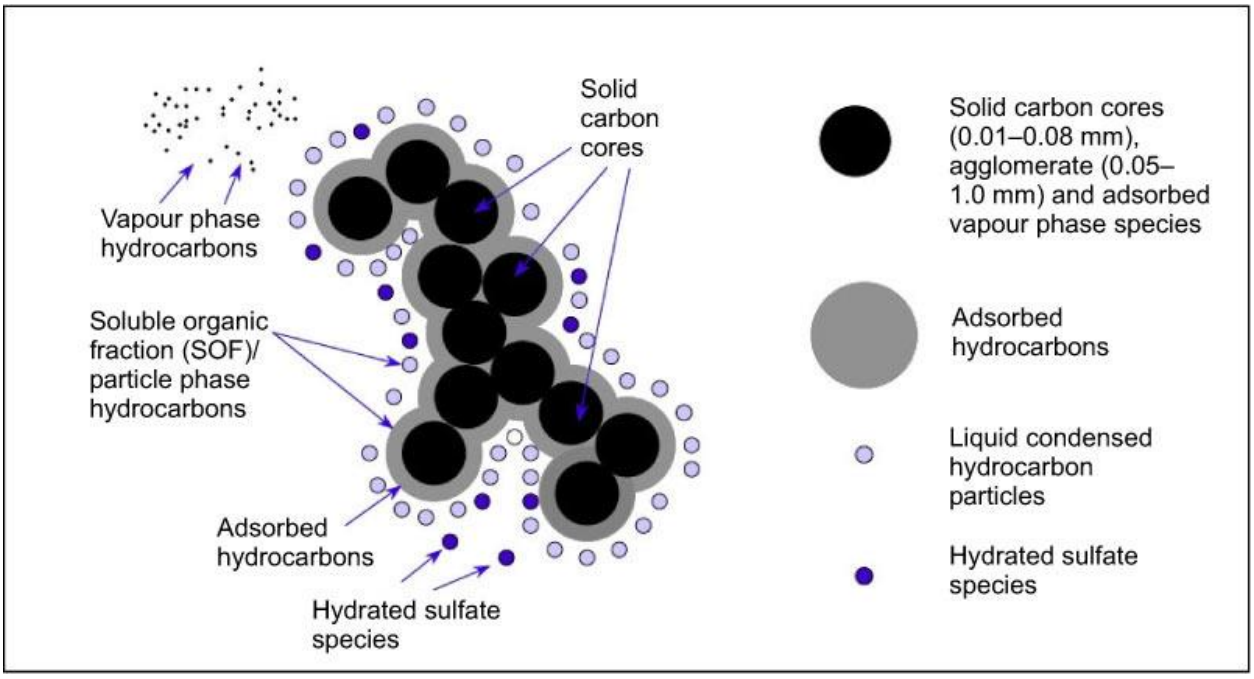

Figure 1.2 Structure and chemical composition of diesel particulate matter. "Schematic representation of diesel particulate matter (PM) formed during combustion of atomised fuel droplets. The resulting carbon cores agglomerate and adsorb species from the gas phase."[29]

Particulate matter (PM) is classified in three size groups according to their aerodynamic diameter: coarse (PM_{10}) with a diameter less than 10 µm, fine ($PM_{2.5}$) with a diameter less than 2.5 µm and ultrafine ($PM_{0.1}$) with a diameter less than 0.1 µm [30]. Arguably, smaller particles may be considered more threatening to human body systems because they can pass from many of the respiratory system defense barriers and penetrate deepest into pulmonary tissue. Recent

evidence suggests that they can enter into the blood circulation then circulate through the body, impairing a number of internal systems that may be unreachable to larger particles [31]. (See Figure 1.3)

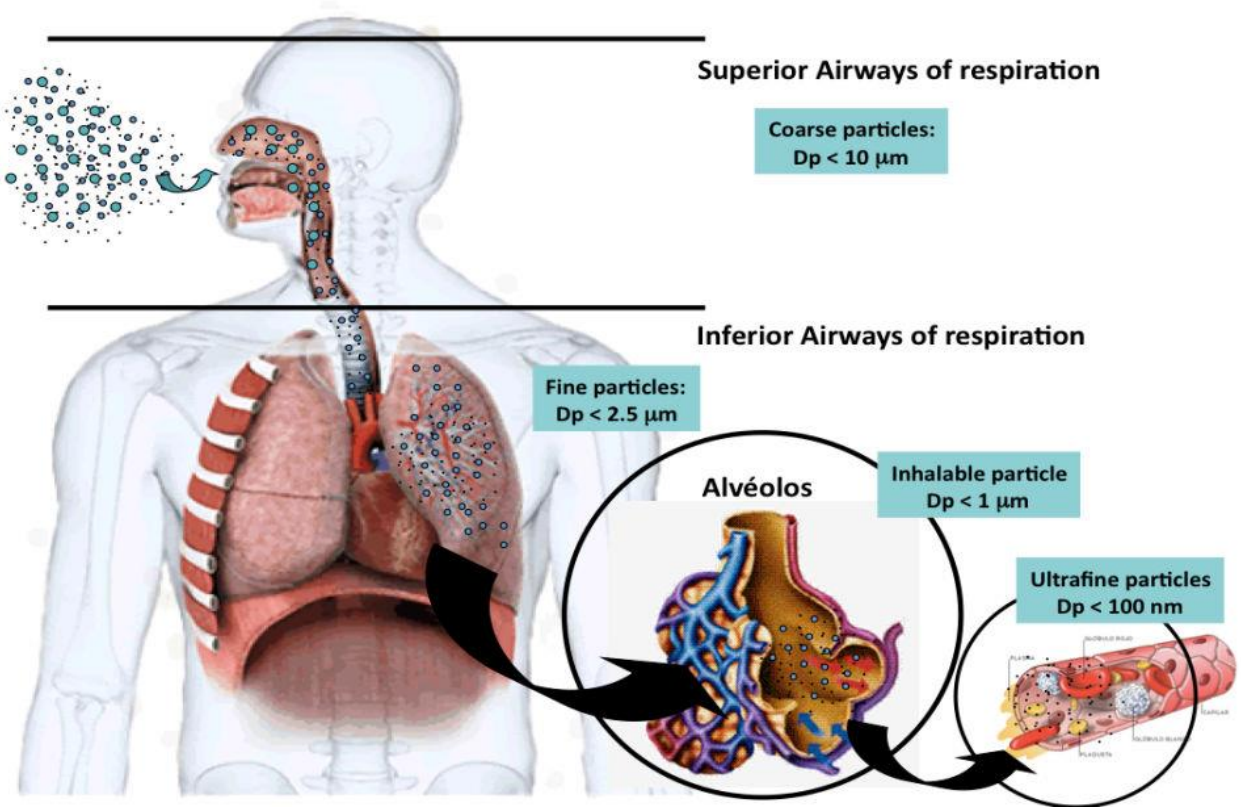

Figure 1.3 Deposition of inhaled airborne particulate matter (PM). "Represents the areas where particulate material from incomplete combustion processes is deposited in the body."[32]

Diesel exhaust is a prominent source of PM pollution; it consists of fine particles including a huge quantity of ultrafine particles. Diesel exhaust particles (DEP) deposit through diffusion and their deposition rate increases with exercise as opposed to breathing at rest and also depends directly to the duration of exposure [33]. It is also been shown that the rate of deposition is greater in the lungs of younger humans, the rate of deposition is nearly double in the lung of

two-year-old children compared to mature adults [34]. Increasing body of epidemiologic and experimental evidence suggest that inhaling the fine ambient particulate matter ($PM_{2.5}$) may partially account for the increased risk of developing cardiovascular and allergic airway diseases [35-37]. With the growing public health concerns, it is important to understand the pathological mechanisms that lead to the adverse effects of diesel exhaust particles on lung health. Experimental evidence suggests that complex organic molecules from DEPs impose their pro-inflammatory effect on the respiratory tract by acting as adjuvants to allergens. *In vitro* studies implicate reactive oxygen species (ROS) generated by exposure to DEPs, create an oxidative stress milieu within exposed cells [38].

1.3 Theories on rising prevalence of asthma and allergy

Over the past two decades, the rate of asthma and other allergic diseases has escalated in some parts of the world, coincident in part with communities' adoption of Westernization and urbanization (See Figure 1.4) [39]. Allergy and asthma result from a complex interaction between the environment and genetics. More than 100 genes have been linked with asthma, including IL-4, IL-5, STAT6, GM-CSF and IL-13, which are important in regulating inflammatory immune responses [40]. A change in the genetic susceptibility seems to be an implausible cause of the increasing incidence of asthma and atopic diseases since genetic alteration in a population needs a number of generations [41]. Thus, environmental factors such as life style and exposure to ambient air pollution may explain the increasing prevalence of respiratory hypersensitivity and asthma. According to "the hygiene hypothesis", the increased hygiene and overuse of chemical cleaners containing anti-microbial agents is one of the critical factors accounting for the current rise in allergic diseases [42]. Lack of early childhood exposure to

pathogenic microorganisms and bacteria interrupts the natural immune system development and results in severe reaction to allergens later in life [43].

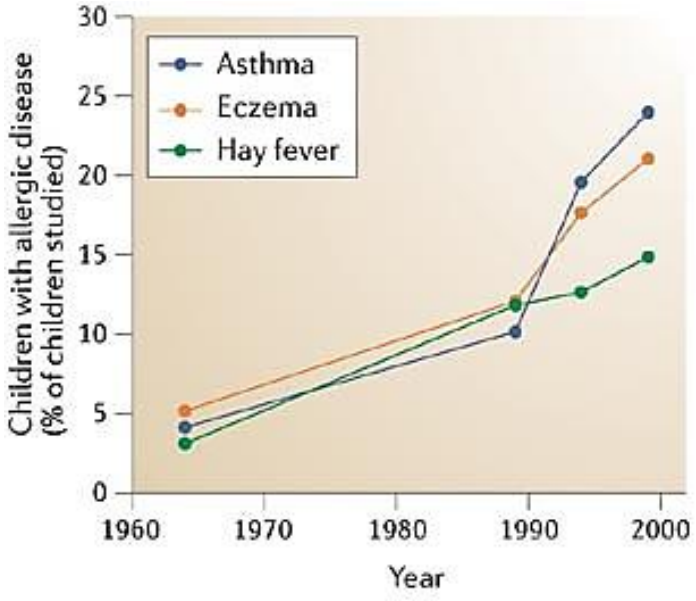

Figure 1.4 The increase in the prevalence of asthma and allergic disorders in recent decades. "The results of four cross-sectional surveys of school children aged 9–12 years in Aberdeen, United Kingdom, between 1964 and 1999 are shown."[44]

Epidemiology studies associate this increasing frequency of allergic respiratory diseases and bronchial asthma with the elevated level of air pollutants caused by industrial activities and vehicle exhausts and also with the increased exposure to allergens in our current life style [45-48]. Exposure to air pollution strengthens the airway reaction to inhaled allergens in vulnerable individuals. People who live in urban and industrialized areas tend to be further affected by allergic pulmonary diseases than those in rural areas [49-51]. Although a mixture of factors

mentioned, can potentially account for the increased asthma and allergy prevalence, this thesis focuses on the effects of exposure to DE on the augmentation of allergic airway inflammation.

1.4 Allergic asthma and ambient PM

Asthma is a chronic inflammatory disease of the airways, described by reversible airway narrowing and aggregation of lymphocytes and eosinophils in the lung [52, 53]. It can be categorized into two groups, allergic (atopic) and non-atopic asthma; these two groups are formally distinguished by the presence or absence of evidence of an allergy-driven immune response [54]. Atopic patients are sensitized to common aeroallergens derived from pollen grains, fungal spores, mite feces, pet dander and the condition is initiated by immunologic mechanisms mediated by specific IgE antibodies produced against these allergens [55].

It has been reported that 60% of nearly 20 million asthma cases in U.S. are allergy-driven [56]. Atopy is a crucial risk factor for asthma, however, asthma is a heterogeneous illness and various irritants beyond allergens including air pollution and exercise can trigger asthma attack. Allergic asthma is a Th2-cell mediated inflammatory disease with elevated levels of allergen-specific IgE in serum, which upon allergen challenge, stimulates airway eosinophilia, mucus over-production, and bronchoconstriction [57, 58]. Epidemiology studies have revealed that increases in ambient particle matter (PM) are associated with increased hospitalizations due to respiratory illness including exacerbation of pre-existing asthma [59-63]. Also, there is growing support linking air pollution with an increased risk of developing asthma [64-67]. The exact pathophysiological mechanisms underlying the adverse health outcomes from PM exposure are unclear. Investigations imply that PM-induced oxidative stress and inflammation may be the major mechanisms responsible for health effects [68-71].

1.5 Cellular and molecular mechanisms of allergic reactions

An allergen is an antigen that is perceived as a threat by the susceptible individual's immune system and activates an atypical immune response. When an allergen comes in contact with immune system, at the very first stage no allergic reaction occurs, as it only gets identified as an invader that needs to be attacked by the immune system in any future encounter. This stage in the mechanism of allergic reaction is known as sensitization in which macrophages surround the invading substance, digest and then present the allergen fragments to T-cells [72]. A signaling chemical called IL-4 is then released from T-cells; this cytokine is responsible for inducing the differentiation of naive T cells (Th0 cells) to Th2-cells which themselves produce additional IL-4 plus IL-5 and IL-13. IL-4 also triggers B-lymphocytes to produce IgE [73]. (See Figure 1.5)

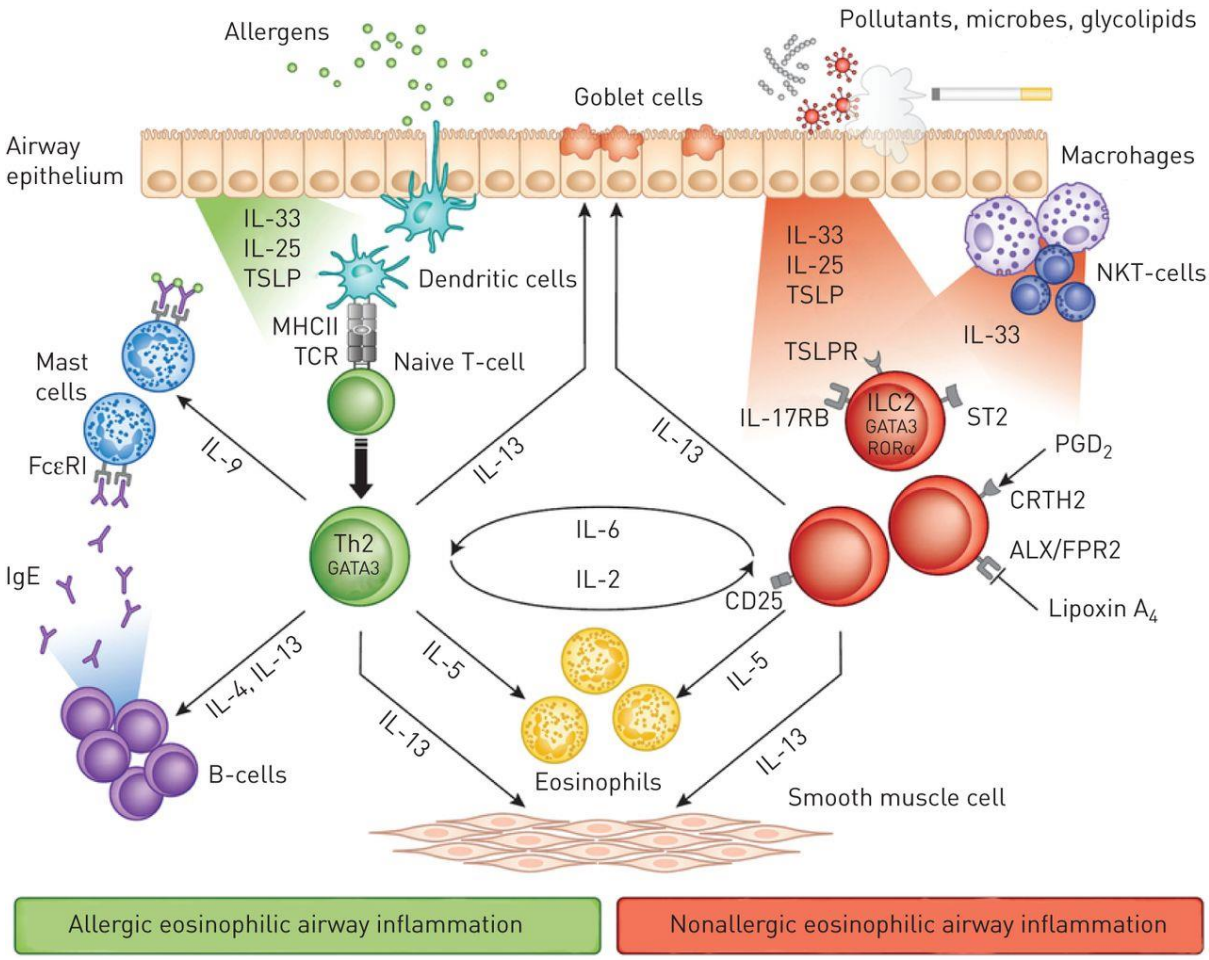

Figure 1.5 Pathways lead to airway inflammation in asthma. TH2 immune processes in the airways of people with asthma. The pathway begins with the development of TH2 cells and their production of the cytokines IL-4, IL-5 and IL-13. These cytokines stimulate allergic and eosinophilic inflammation as well as epithelial and smooth-muscle changes that contribute to asthma pathobiology.[74]

In the next phase of the allergic reaction, when the immune system confronts the allergen for the second time, IgE antibodies on mast cells (produced during the sensitization phase) identify the allergen and bind to the invader. The surface of mast cells contain unique receptors (FcεRI; high-affinity receptor for the Fc region of IgE) for binding IgE, when two or more molecules of IgE bound to the surface of a cell, the allergen attaches itself to both and cross-links the two IgEs [75]. In an atopic individual, exposure to the allergen cross-links enough IgE molecules to

trigger mast cell activation when the granules in the mast cells release its pre-formed inflammatory substances including histamine. Histamine stimulates mucus production and causes soreness, swelling, and inflammation [75]. In the next state of lgE-mediated allergic reaction, which happens a few hours after the exposure, tissue-resident mast cells and the adjacent cells secrete chemical messengers that call circulating basophils, eosinophils, and other cells to migrate into the affected tissue and help fight the invader substance. These recruited immune cells produce lipid mediators and cytokines that induce inflammation and tissue remodelling, engaging even more inflammatory cells [75, 76].

The three common allergens that we used in the present study are house dust mites (HDM), Birch pollens and Timothy grass pollens. Dust mites are small creatures from arthropods family measuring about 0.25–0.3 mm in length. Their life cycle is 10-19 days for male and up to 70 days for female mites; they feed on the flakes of shed human skin and can live and reproduce easily in warm, humid places. Dust mites are the main indoor allergens associated with asthma and other allergic conditions. The proteins within the bodies and feces of the mites trigger strong IgE antibody and T-cell responses in asthmatic patients with allergy to dust mites [77]. Birch allergy is in fact an allergy to the pollen of the birch tree. Birch is small to medium sized, slender and fast-growing tree that is usually found in the cool northern temperate areas and is known to trigger seasonal respiratory allergies. The allergy caused by birch pollen is seasonal; it strikes in mid spring and causes sneezing, nasal congestion coughing and shortness of breath [78]. Timothy grass is a hay crop native to Europe, North Africa and Northern Asia and it has been adapted for use in North America. Timothy flowers from early June to early September

and it grows 60 cm to 125 cm in height. Timothy is one of the world's most common grasses and the main source of the allergy is the protein content of its small pollens [79].

1.6 Previous controlled human exposure studies

A number of human exposure experiments has been performed using standard exposure chamber with controlled challenges by diluted diesel exhaust to investigate potential cardiopulmonary health effects of diesel exhaust exposure [80]. Exposure to elevated levels of DE was correlated with raises in symptoms and increased airway responsiveness [81]. Short-term exposure to DE (300μg.m^{-3} of PM_{10}) was shown to stimulate influx of inflammatory cells, including neutrophils, mast cells, CD4+ and CD8+ T lymphocytes in the bronchial mucosa at 6 hours post exposure [82]. The inflammatory response was accompanied by significant increases in neutrophils and B lymphocyte migration into the airways and activation of mast cells with histamine discharge. Moreover, up-regulated expression of adhesion molecules, intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) was discovered on the endothelial cells in sub-mucosal blood vessels [82]. In healthy participants, an increased number of neutrophils along with amplified concentrations of IL-6 and methyl-histamine were detected in sputum specimens collected at 6 and 24 hours post-exposure to DE (300μg.m^{-3} of PM_{10}) [83]. Dissimilar inflammatory responses were observed in mild allergic asthmatic and healthy subjects exposed to DE (108μg.m^{-3} of PM_{10}). Six hours after DE exposure, healthy subjects were diagnosed with neutrophilia and lymphocytosis in company with an increased level of IL-8 in BALF. In contrast, an increased expression of IL-10 in the bronchial epithelium was seen in asthmatics [84]. In healthy subjects, a significant increase in the count of eosinophils was discovered in BALF performed 6 hours after exposure to DE (270μg.m^{-3} of PM_{10})

[85]. One-hour exposure to DE (300µg.m^{-3} of PM_{10}) induced a significant increase in the expression of IL-13 in the bronchial epithelial cells at 6 hours post- exposure without considerable changes in IL-10 and IL-18 expression [86].

It has been shown that DEPs can induce sensitization to a neoallergen, which did not arise with exposure to the neoantigen alone in allergic subjects, suggesting the important role of DEPs in exaggerating the sensitization to allergens [87]. Numerous human nasal studies by Diaz-Sanchez *et al.* have demonstrated that exposure to mixed DEPs and allergen stimulate a Th2-type immune response, suggesting that DEPs can cooperate with allergen to promote allergic responses [88, 89]. As compared with allergen alone, intranasal challenge with combined DEPs and allergen potentiated the production of allergen-specific IgE in sensitized subjects [90]. Mixture of DEPs and allergen synergistically increased the expression of IL-4, IL-5, IL-6, IL-10 and IL-13 but resulted in reduced expression of IFN-γ and IL-2 (Th1 signature cytokines). Nasal instillation with DEPs plus allergen promoted a 16-fold increase in allergen-specific IgE production which suggests that DEPs can boost B-cell differentiation, and may significantly contribute to the increased prevalence of allergic airway diseases [91]. This synergistic interaction between DEPs and allergen exposure may explain at least part of the rising trends in allergic respiratory diseases.

1.7 T-helper cells differentiation in inflammatory responses

CD4+ T-cells play an essential role in adaptive immune responses. Once a pathogen is identified, it will be processed and presented to T cells. Following the activation, naive CD4+ T-cells differentiate into one of the sub-types of T-helper cells (Th1, Th2, Th9, Th17, or Th22), depending on the nature of antigen and the cytokines present in the surrounding milieu [92].

Th1 cells are responsible for immune responses against intracellular viruses and bacteria and secrete IL-2, IL-12, IFN-γ and TNF-β. Th2 cells are more effective against extracellular bacteria, helminth parasites, toxins, noxious environmental xenobiotics, and irritants [4]. They secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and induce eosinophil activation and differentiation; Th2 cells are more proficient B-cell helpers and can stimulate IgE production. Th9 cells are very similar to Th2 cells and there is a great plasticity between the two cell-types. Th9 cells secrete high levels of IL-9 but not IL-4, IL-5, or IL-13. They contribute to immune defense against helminth infections, and also have effects in development of chronic allergic inflammation and airway remodeling. Th22 cells largely secrete IL-22, IL-13, and TNF-α and are involved in the pathogenesis of inflammatory skin disorders such as atopic dermatitis. Th17 cells recruit neutrophils into the airways through IL-17 expression against extracellular bacteria and fungi [92-95]. Recently a novel dual Th2/Th17 cells are identified in severe neutrophilic asthma that can produce both IL-4 and IL-17A [96, 97].

1.7.1 Th2 cytokines

Allergic asthma is the result of an inflammatory process mediated by allergen-specific Th2 cytokines, including IL-4, IL-5, and IL-13. Th2 cells are enriched in the bronchial mucosa of atopic asthmatic patients and induce IgE expression and mast-cell expansion by IL-4 and promote the proliferation, differentiation, and activation of eosinophils by IL-5. Diesel exhaust is believed to be involved in the increased frequency of asthma and respiratory allergic diseases. Mixing allergens with DEP in *in vitro* studies and mice models have confirmed that DEP augments sensitization to allergens [19, 98-100].

1.7.2 IL-4 is a key cytokine in atopy

IL-4 is known to be an important cytokine in the development of allergic inflammation; it provides the first signal that initiates B-cell class switching to IgE production [101]. IL-4 can further enhance IgE-mediated immune response by up-regulating the expression of low-affinity IgE receptor (FcεRII/CD23) on B-lymphocytes and macrophages and high-affinity IgE receptor (FcεRI) on mast cells [102, 103]. IL-4 induces the differentiation of naive T lymphocytes into Th2 cells which secrete more IL-4, IL-5, and IL-13, maintaining a suitable environment for further Th2 cells differentiation [104, 105]. IL-4 can also stimulate the expression of VCAM-1 on endothelial cells, which leads to enhanced migration of T-cells, eosinophils, macrophages and mast cells to inflamed tissue [106]. IL-4 contributes to airway obstruction in asthma via the induction of mucus hypersecretion in mice and human cell lines, and increases the release of several pro-inflammatory cytokines such as IL-6, GM-CSF and eotaxin from human lung fibroblasts [107]. IL-4 is a major factor in the recruitment and activation of inflammatory cells that may contribute to inflammation and lung remodeling in chronic asthma [108].

1.8 Mast cells play a central role in the pathogenesis of allergic inflammation

Mast cells are bone marrow-derived tissue cells of the immune system and play a pivotal role in the pathogenesis of allergic reactions and inflammation. Mast cells originate from CD34+, CD117+ (c-kit) precursor cells in the bone marrow. Immature mast cells are released into the peripheral blood circulation and then migrate into their destination tissue where they are differentiated under the influence of the local cytokine milieu, mainly stem cell factor (SCF) which is a major chemotactic factor for mast cells and their progenitors [109]. SCF also elicits

cell-cell and cell-substratum adhesion, facilitates the proliferation, and sustains the survival, differentiation, and maturation of mast cells [110].

Mast cells are found resident throughout the connective tissues of the body, particularly near surfaces that are in contact with external environment such as blood vessels and nerves, below the skin surface and throughout the respiratory system [111]. Mast cells are known to play a significant role in both, early and late phase of the allergic reaction through the allergen and IgE-dependent release of potent mediators that may contribute to chronic inflammation. Upon stimulation by an allergen that binds to the IgE attached to their FcεRI receptors, mast cells release numerous pre-formed vasoactive and bronchospastic mediators (leukotriene C4 (LTC4), prostaglandin D2 (PGD2), heparin and histamine), tryptase and chymase, interleukins (IL-4, IL-5, IL-6, and TNF-$\boldsymbol{\alpha}$), stored in granules found throughout the cytoplasm of the cell [112]. Histological evidence for mast cell activation in bronchial tissues and the presence of elevated concentrations of histamine, tryptase, and PGD2, have been found in BALF and sputum samples from asthma patients [113].

In humans, two common phenotypes of mast cells are identified by their granular serine proteases protease. Tryptase is the most plentiful granule constituent in mature mast cells. Chymase is present in moderate quantity in a subtype of mast cells (MCTC) which predominate in connective tissue, while the other main subpopulation of mast cells (MCT) mostly found at mucosal surfaces such as bronchial lamina propria, in health, and the nasal and bronchial epithelium in rhinitis and asthma [114-116]. The number of tissue mast cells in normal condition is steady, but rises when disturbed by pathophysiologic conditions in inflamed tissues in allergic asthma and rhinitis [117, 118].

1.9 Eosinophils

The key inflammatory cells involved in asthma are lymphocytes, eosinophils, mast cells, antigen presenting cells (APCs) and neutrophils (in a fraction of asthmatic patients). The Th2 cytokines IL-4 and IL-13 increase the expression of VCAM-1 by the endothelial cells lining on mucosal blood vessels, which will facilitate eosinophil attachment to the surface of the endothelium and migration to adjacent inflamed airway tissue. High levels of IL-5, which is the major growth factor for the maturation, differentiation, survival and function of eosinophils, were found in the blood and bronchoalveolar lavage fluid (BALF) of patients with atopic asthma [119].

Eosinophils are pro-inflammatory white blood cells that are differentiated from myeloid progenitors in the bone marrow; mature eosinophils travel through blood vessels to the parasitic or allergic-affected areas in the body. Accumulation of eosinophils to sites of inflammation is now known as one of the clear signs of allergic inflammation and their increased number is directly associated with disease severity. They were always considered as end stage cells that were recruited to the lung by T cells; however, recent studies suggest that eosinophils play a more active role in the development of allergic airway inflammation than it was initially believed [120].

This has led to the hypothesis stating that eosinophils are the central effector cells, responsible for persistent airway inflammation. In lung tissue specimens, large numbers of eosinophils have been found, infiltrating the walls of airways in severe and mild asthma [121]. Upon activation, eosinophils are able to secret Th2 (IL-4, IL-5, IL-10, IL-13) and other cytokines, chemokines, eosinophil peroxidase and lipid mediators, which cause bronchoconstriction, vasodilatation and mucus hypersecretion. Activated eosinophils release toxic granule proteins capable of causing

airway tissue damage, including major basic protein (MBP), eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), which damage nerves and epithelial cells [122]. ECP also known as ribonuclease (RNase)-3, is a strong cytotoxic protein with bactericidal and antiviral properties. High levels of ECP are considered as a clinical indicator of active atopic asthma, it reflects the intensity of ongoing eosinophilic inflammation of the airways in allergic diseases [123].

1.10 Hypotheses and aims

1.10.1 Hypotheses: Co-exposure to DE (300 $\mu g.m^{-3}$ of $PM_{2.5}$) and common aeroallergens synergistically promote a Th2 immune response in atopic human lung with recruitment of innate and adaptive immune cells to the bronchial mucosal tissue

1.10.2 Specific aims

Aim 1: *Demonstrate that inhalation of DE followed by segmental allergen provocation will induce a shift towards a Th2 polarized immune response with increased IL-4 expression, CD4+ recruitment in the allergic airway submucosa.*

Aim 2: *Identify that breathing DE will intensify allergen-induced eosinophilic activation and infiltration along with an increase in the number of activated submucosal mast cells.*

Aim 3: *Demonstrate that co-exposure to DE and allergen will induce neutrophilia in the submucosa.*

Aim 4: *Demonstrate that dual exposure to DE and allergen will increase the number of plasma cells (CD138+) in the submucosa.*

Chapter 2: Experimental design and methods

2.1 Subject recruitment

Twelve atopic subjects (19–49 years old) were screened, informed of the protocol, procedures, and potential risk, and agreed to participate in the study (ClinicalTrials.gov identifier: NCT01792232). The UBC Research Ethics Board approved the study protocol and informed consent form. Allergic sensitization to house dust mite (HDM), birch and Pacific grasses were diagnosed by skin prick test. Birch-sensitive subjects were not studied in the birch season (February-April) and grass-sensitive subjects were not studied in the Pacific grasses season (May-August).

2.2 Exposure protocol

Each subject was exposed for 120 min to filtered air (FA) or freshly generated diesel exhaust (DE $PM_{2.5}$ 300µg.m^{-3}; Table 2.1) in a double-blinded crossover experiment in two separate occasions randomized and counter-balanced to order, with a four-week washout period between each condition. In this study design, each subject serves as his/her own control.

Table 2.1 Inhaled exposure characteristics.

Condition	$PM_{2.5}$ **(µg/m³)**	Particle Number **(#/cm³)**	CO **(ppm)**	NO **(ppb)**	NO_x **(ppb)**	NO_2 **(ppb)**	NO_2/PM# **(µg/#)**
FA	8.2 (6.9)	1750.4 (235.1)	2.8 (0.1)	25.3 (5.0)	71.1 (9.8)	45.9 (7.7)	4.9×10^{-9}
DE	302.0 (30.5)	5.4 × 105 (6.4 × 104)	14.1 (2.0)	8665.5 (1287.1)	9185.3 (1366.1)	519.7 (118.6)	1.8×10^{-9}

FA=Filtered Air; DE=Diesel Exhaust; Values are presented as mean (SD).

During the two-hour exposure, the subject was asked to exercise on a bicycle ergometer for a total of 30 min (2 × 15 min at ~60 rpm cadence and ~25 W of resistance) to increase ventilation-heart rate and mimic modest intermittent activity. Inside the exposure chamber, subjects' vital signs (blood pressure, heart rate and arterial oxygen saturation) were monitored using the pulse oximeter for every 20 min. Lung function was measured using spirometry test (FEV_1) pre-exposure and for every hour of exposure. (See figure 2.1)

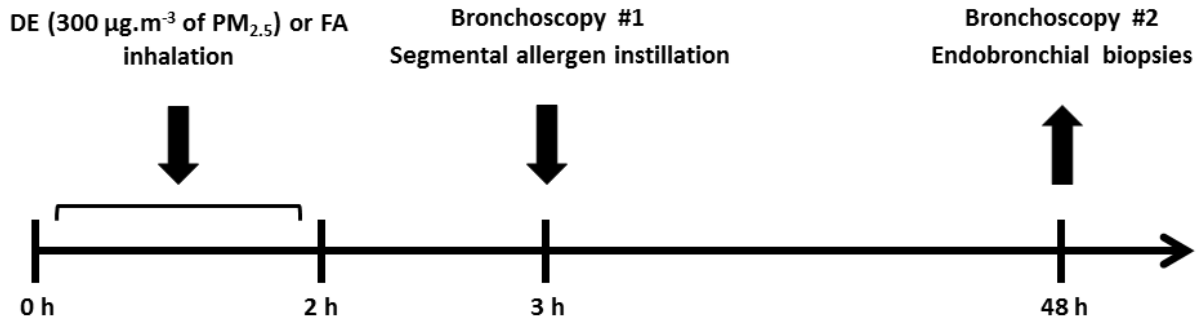

Figure 2.1 Schematic of exposure protocol. Study subjects were exposed to DE (300 µg.m^{-3} of $PM_{2.5}$) or FA (filtered air) for 2 h. One hour post-exposure, a segmental allergen challenge was performed with allergen or saline vehicle in the right upper and middle lobe or left lingular lobe. Forty-eight hours post-exposure endobronchial biopsies were obtained via bronchoscopy. The process was repeated following a washout period of 1 month with exposure conditions reversed compared to the first visit.

2.2.1 Bronchoscopy #1: segmental allergen challenge

One hour following each exposure to DE or FA, segmental allergen challenge (SAC) was performed by Dr. Chris Carlsten through standard fiberoptic bronchoscopy procedure. SAC was performed by administration of 5 mL solution of allergen extract (in a concentration 10-fold lower than that minimal dose producing a positive wheal ≥3 mm) in right lung (the right upper lobe and right middle lobe bronchial segments), and 5 mL diluents control was instilled in left lung (the left upper lobe and lingular segments). This order was reversed during the second exposure condition to provoke both sides of the lung.

2.2.1 Bronchoscopy #2: tissue sampling

The second bronchoscopy was done 48 h post-allergen challenge, endobronchial biopsy specimens (size ≤2 mm) were obtained from the same segments exposed to allergen or saline.

Following an approximately 1 month washout period, subjects returned and received the second two-hour exposure, followed by a bronchoscopy during which allergen and saline were administered to opposite lungs and different segments than those during the first exposure. Thus, endobronchial biopsies for each of the 4 different crossover conditions was created: 1) FAS: filtered air + saline, 2) DES: DE + saline, 3) FAA: filtered air + allergen and 4) DEA: DE + allergen.

2.3 Immunohistochemical (IHC) analysis procedure

Immunohistochemistry (IHC) merges anatomical, immunological and biochemical techniques to discover discrete tissue components by the interaction of target antigens with specific antibodies tagged with a detectable label. IHC allows visualizing the distribution and localization

of specific cellular constituents (cell membrane, cytoplasmic and nuclear) within cells and in the proper tissue context. This technique was used to determine the number of T-helper cells (CD4+), plasma cells (CD138+), neutrophils (elastase+), activated eosinophils (EG2+) and tryptase-positive (AA1+) mast cells in the lamina propria, and also to look at expression of a key Th2 cytokine, IL-4, in endobronchial biopsies.

2.4 Bronchial biopsies processing

The endobronchial mucosal biopsy specimens obtained at bronchoscopy (48 hours post-exposure) were immediately added to ice-cold acetone containing the protease inhibitors iodoacetamide (20 mM) and phenylmethylsulfonylfluoride (PMSF; 2 mM, Sigma, Oakville, ON) and fixed at –20°C overnight (16-24h). Next day, biopsies were transferred to fresh acetone and then to methyl benzoate (Sigma, Oakville, ON) for 15 min each at room temperature, before infiltration with glycol methacrylate (GMA) resin as previously described by Britten *et al.* [124].

2.5 Fixing media for IHC analysis: glycol methacrylate acrylic resin (GMA) embedding

Glycol methacrylate acrylic resin (GMA) is a hydrophilic plastic resin that was used in biopsy fixation and it provides a number of advantages over conventional histology methods such as frozen and paraffin-embedding techniques. Acetone is a non-cross-linking fixative solution that, with protease inhibitors and processing at a low temperature, ensures antigen preservation for immunohistochemistry. Frozen sections offer desirable antigens conservation but very poor morphology, compared with GMA samples. Paraffin-embedded sections demonstrate acceptable morphology but due to harsh fixation in formalin solution, most of the antigen epitopes in tissue will be compromised. As with paraffin embedding, GMA-embedded tissue

samples can be stored safely for long periods. GMA facilitates the cutting of very thin (2 µm) sections, thus sequential sections can be cut through one single cell, permitting the detection and multiple-antigen labeling to a single cell. The ability to derive large numbers of sections from small biopsies is particularly advantageous for clinical human tissue samples from sites such as lung and kidney where tissue availability is very limited due to ethical issues, experimental costs and subject discomfort [125]. However, there are drawbacks to GMA. GMA embedding is more expensive, time-consuming and less familiar to the immunology community. GMA is also not suitable for DNA/RNA *in situ* hybridization.

2.6 Glycol methacrylate acrylic resin (GMA) embedding

For embedding biopsies in GMA, JB-4 Embedding Kit (Polysciences, Warrington, PA) was used with some modification to its original manufacturer's instructions described in details by Wilson *et al.* [126]. For polymerization of GMA resin, benzoyl peroxide which is a plasticizer catalyst was added to the airtight embedding capsule containing tissue sample and was kept for 48 hours at 4° C. Polymerized resin blocks were stored desiccated at –20° C freezer until used for IHC staining with elected primary antibodies.

2.7 Biopsy quality evaluation

GMA blocks containing each biopsy were removed from embedding capsules and excess resin was trimmed away to form a trapezium shape around the tissue. Sections were cut at 2 µm using ultra-microtome (Leica EM UC6) and floated out onto distilled water (dH_2O) and picked up onto 10% poly-l-lysine (PLL)-coated slides (Fisher Scientific, Ottawa, ON). Slides were left on hot plate to completely dry out, then one drop of toluidine blue stain was added on tissue section

for 2 min. After washing excess stain off with dH_2O, slides were then blotted dry and mounted in DPX (Sigma, Oakville, ON) and were examined under light microscope to check biopsy quality. In order to qualify for immunohistochemical analysis in the submucosa, tissue section had to have a minimum of 0.46 mm^2 of submucosal tissue (lamina propria), excluding smooth muscle, glands and crush artifact [127]. If the section found to be of poor standard, further sections were cut from deeper levels within the biopsy and reassessed as described above. If no such level with acceptable histological standard was found in the biopsy, then it was excluded from further immunohistochemical analysis.

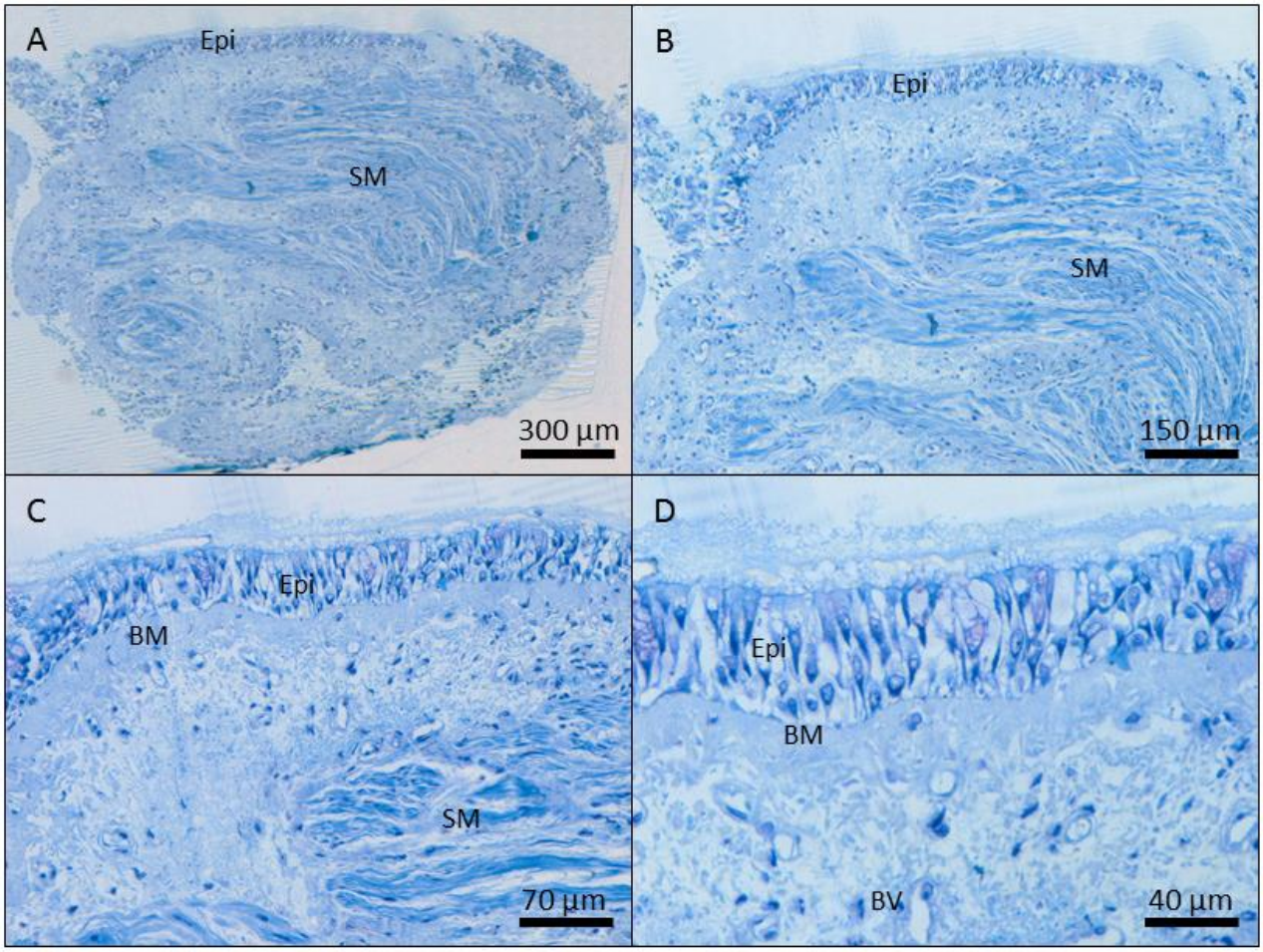

Figure 2.2 Histologic structure of a representative GMA embedded endobronchial biopsy that was cut 2 µm with toluidine blue staining. **A.** Representative 5X image of an entire section from an endobronchial biopsy. **B.** Representative 10X image of the endobronchial biopsy with a thick layer of SM. **C**. Representative 20X image of the endobronchial biopsy. **D.** Representative 40X image of the endobronchial biopsy with pseudostratified columnar epithelium supported on the basal side by BM. Epi=epithelium, BM=basement membrane, SM=smooth muscle, BV=blood vessel.

2.8 Titration of primary antibodies

The optimum titer or dilution is the concentration that gives the best staining with minimum background or non-specific binding (greatest signal to noise ratio). Before using Abs on test sections, the optimal antibody concentration was determined for each by using a series of dilutions in a titration experiment. Primarily, six double serial dilutions were tested: 1:25, 1:50, 1:100, 1:200, 1:400, and 1:800. Commercial Abs from reliable companies are generally very consistent, with small batch-to-batch variation, and, therefore, once a working dilution has been established, new batches only need confirming by using the current working dilution and one either side. Checkerboard titrations were run for titration of second- and third-stage Abs, in which the concentration of two Abs were different against each other [126]. Mouse anti-human monoclonal antibodies were used for immunostaining of tryptase (AA1), ECP, CD4, IL-4, CD138 and neutrophil elastase (NE) in endobronchial tissue biopsies. (See Table 2.2)

Table 2.2. List of monoclonal primary antibodies were used for immunohistochemistry staining.

Antibody (clone)	Marker	Cell	Concentration ($\mu g.mL^{-1}$)	Catalog No.	Supplier
AA1	Tryptase	Mast cells	0.1	ab2378	Abcam Inc.
EG2 (614)	ECP	Eosinophils	0.07	514-121	Diagnostics
IL-4 (4D9)	IL-4	Th2, mast cells	20.0	211-44-134AX	Amsbio Ltd.
CD4 (4B12)	CD4	Helper T cells	8.0	M731001-2	Dako Canada
CD138 (B-A38)	Syndecan-1	Plasma cells	1.0	MCA2459GA	AbD Serotec
NE (NP57)	Elastase 2	Neutrophils	0.1	M075201-2	Dako Canada
IgG1 (ICIGG1)	—	Isotype Control	20.0	ab91353	Abcam Inc.

2.9 IHC positive and negative controls

IHC experiments must include appropriate positive and negative controls to support the validity of staining and correct interpretation of immunostaining results. A positive control was included to ensure that the technique is working; negative controls to ensure there is no non-specific staining. Tonsil tissue specimens removed from patients (kindly provided by Dr. Andrew Thamboo, Dept. of Otolaryngology (ENT) at St. Paul's Hospital) was used as positive control, and staining with matched isotype control was used as negative control (Figure 2.3).

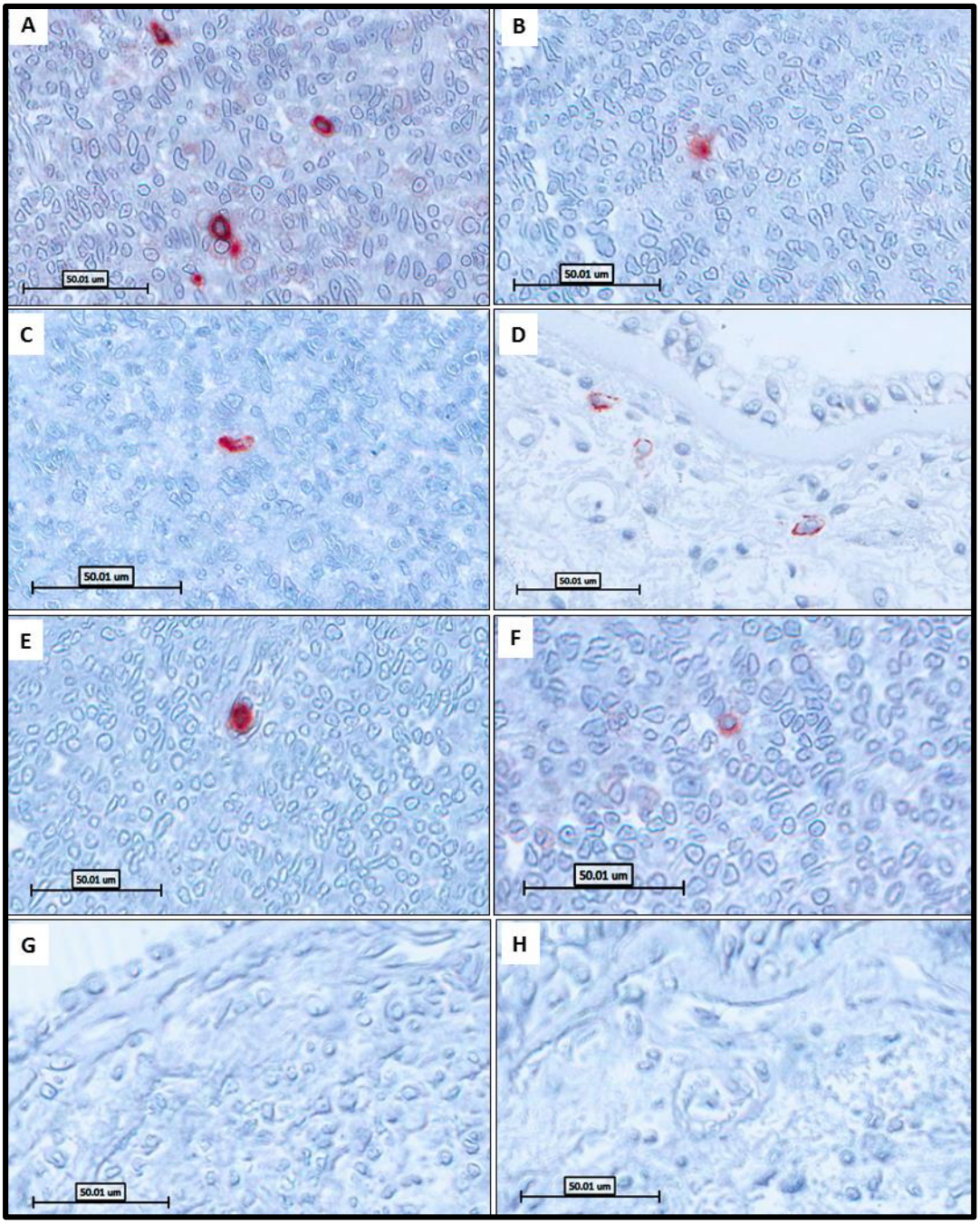

Figure 2.3 Immunohistochemical staining of positive and negative controls. A) Representative 40X image of positive staining using mAb AA1 for tryptase in human tonsil tissue; B) Representative 40X image of positive staining using mAb EG2 for ECP in human tonsil tissue; C) Representative 40X image of positive staining using mAb NP57 for neutrophil elastase in human tonsil tissue; D) Representative 40X image of positive staining using mAb B-A38 for CD138 in human lung tissue; E) Representative 40X image of positive staining using mAb 4B12 for CD4 in human tonsil tissue; F) Representative 40X image of positive staining using mAb 4D9 for IL-4 in human tonsil tissue; G) Representative 40X image of isotype control using mAb mouse IgG1 (0.07 µg.mL−1) in human lung tissue; H) Representative 40X image of isotype control staining using mAb mouse IgG1 (20.0 µg.mL−1) in human lung tissue.

2.10 Preparing GMA sections for IHC

Sections were cut at 2 µm using microtome and floated out onto a water bath containing ammonia (1ml ammonia in 500ml distilled water) for 1-2 min (ammonia helps antigenicity and provides better antibody staining). Sections were picked up on 10% poly-l-lysine (PLL)-coated slides (Fisher Scientific, Ottawa, ON) and dried out for at least 1 h at room temperature. Two sections were placed on each slide. Slides were used for IHC analysis immediately or were wrapped in aluminum foil and stored at –20°C; all of them were stained within no longer than 2 weeks [128].

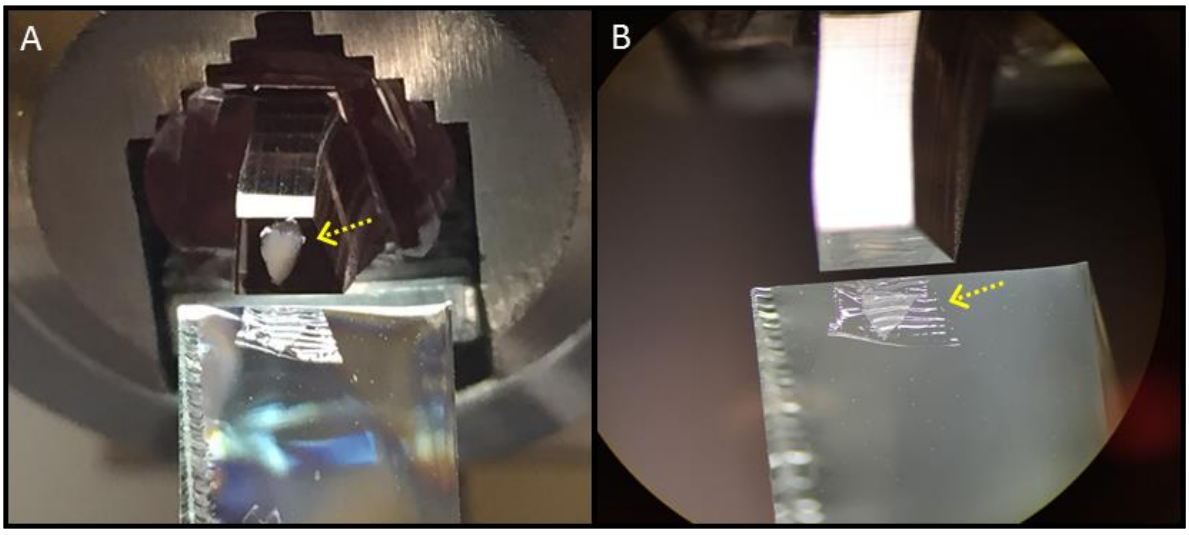

Figure 2.4 Representative image of sectioning a GMA block using an ultramicrotome with glass knife. **A** dashed yellow arrow points to small endobronchial biopsy inside a trimmed GMA block containing. **B** dashed yellow arrow shows a GMA section that was cut 2 µm.

2.11 IHC staining procedure

If slides were stored at –20°C, they were removed from the freezer, unwrapped, and laid out, to allow the condensation to evaporate. Prior to start of staining, a circle was drawn around tissue sections using a PAP pen (Dako, Burlington, Ontario), so that they were visualized during the

IHC procedure. The pen also provides a hydrophobic barrier that keeps staining reagents localized on the tissue sections, and allows use of less Abs and detection reagents per sections. Tissue sections were not allowed to dry out throughout the procedure and the staining tray was covered during the blocking, Ab incubation, and substrate stages.

2.11.1 Blocking steps to preventing non-specific staining

2.11.1.1 Blocking endogenous peroxidase activity

In lung tissue, a number of cells e.g. macrophages, mast cells, neutrophils and eosinophils contain endogenous peroxidase [129]. With the Avidin Biotin Complex (ABC) method that we employed for detecting positive signal, a conjugated horseradish peroxidase (HRP) was used to visualize epitope-antibody interactions. Endogenous peroxidases interfere with ABC detection system, due to reaction with the substrate solution (hydrogen peroxide and chromogen, e.g. AEC or DAB), resulting in non-specific background staining. To quench the endogenous peroxidase activity, the sections were incubated with endogenous peroxidase inhibitor solution: 10 mL 0.1% aqueous sodium (Na) azide plus 100 µL 30% hydrogen peroxide (H_2O_2) for 30 min. Pre-treatment of lung sections with freshly prepared peroxidase inhibitor solution results in the irreversible inactivation of endogenous peroxidases. After that, sections were washed thoroughly with Tris-buffered saline (TBS) for three cycles of five minutes each.

2.11.1.2 Blocking endogenous biotin activity

Biotin, also known as vitamin B7/ Vitamin H, is widely distributed in human tissues including lung, kidney, liver and acts as a coenzyme in reactions catalyzed by biotin-binding enzymes such as carboxylase, transcarboxylases, and decarboxylases. Most of these enzymes are present in mitochondria [130]. Endogenous biotin interacts with avidin conjugates employed in biotin/avidin detection systems (ABC method) resulting in false-positive signals [131]. Avidin/Biotin Blocking Kit (Vector Laboratories, Burlington, Ontario) was used to block all endogenous biotin, biotin receptors, and avidin-binding sites present in lung tissue sections. Avidin block (solution A) was applied for 20 min, washed briefly three times with TBS for 2 minutes per wash. Biotin block (solution B) was then added and left for a further 20 minutes, followed by a repeat brief wash. Culture-medium-blocking solution: 20% fetal calf serum plus 1% bovine serum albumin in Dulbecco's modified essential medium (Sigma, Oakville, ON), then was applied for 30 min. The culture medium was removed by gentle drainage and the primary antibody (150 µl/slide) were added to each section at the proper concentration. Coverslips were placed over the sections to prevent evaporation and equal antibody distribution. Slides were incubated overnight at room temperature (RT) with monoclonal primary antibody.

2.11.2 Avidin-biotin-peroxidase complex (ABC) staining system

The ABC method is the most commonly used IHC method that allows for the amplification of detection due to the strong affinity of avidin for the biotin (over one million times higher than an antibody for most antigens) [132]. It involves a biotin-labeled secondary antibody that serves as a bridge between tissue-bound primary antibody and an avidin conjugated enzyme such as HRP [133]. (See Figure 2.3)

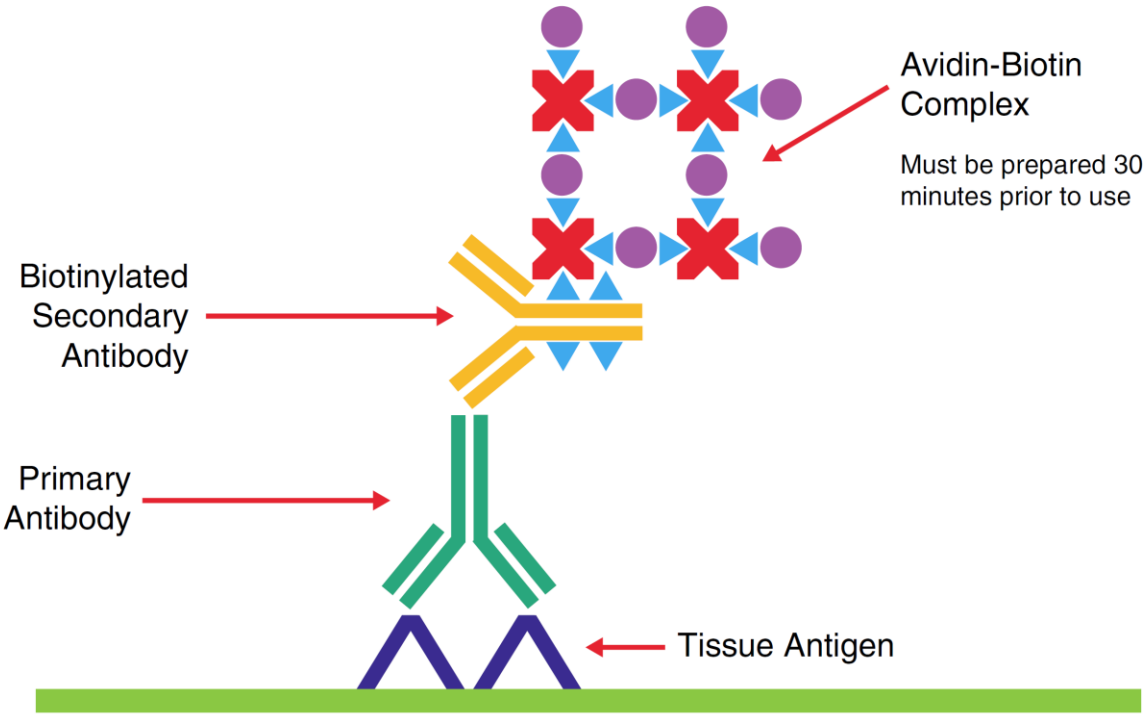

Figure 2.5 Avidin-biotin complex (ABC) method. (Image from Dako IHC guidebook 5th edition accessed from http://www.dako.com/08002_ihc_staining_methods_5ed.pdf)

Slides were rinsed three times in TBS for 5 min for removing any extra unbound primary antibody. The second stage biotinylated antibody (F(ab')2 fragment) rabbit anti-mouse (Dako Canada, Mississauga, Ontario) was then applied for 2 hours. To eliminate any excess unbound secondary antibody, the slides were drained and washed by TBS for three cycles of five minutes each. The ABC solution was prepared 30 min prior to adding to slides using VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlington, Ontario), then was applied to the sections and incubated for 2 hours. The slides were drained and washed three times in TBS for 5 min prior to addition of the chromogen.

The antigenic sites where primary antibody binds to tissue were visualized by the application of AEC (3-amino-9-ethylcarbazole). AEC is a chromogenic substrate that reacts with the peroxidase enzyme (HRP) in the presence of the substrate reagent (H_2O_2) and upon being oxidized form a colored insoluble precipitate. AEC forms a rose red end product, which when used with Mayer's hematoxylin, provides valuable contrast with the dark purple staining nuclei, and is a good choice for assessing the number of cells expressing the antigen of interest.

AEC were applied on sections for 20 min and DAB for 10 min at RT, before rinsing in TBS and then running tap water for 5 min. Mayer's hematoxylin solution (Dako, Burlington, Ontario) was used as the counterstain, since it is a non-alcohol based dye compatible with AEC chromogen, which is alcohol soluble. The slides were submerged in hematoxylin solution for 2 min, followed by bluing in running tap water. It stains the nuclei of cells blue to bluish-purple. Coverslips were immediately applied on sections with Crystal mount (Sigma, Oakville, ON), an aqueous based permanent mounting medium. Slides were then allowed to dry at RT for at least 2 hours prior to scanning and analysis.

2.12 Quantification of immunohistochemistry

The immunostained slides were sent to the UBC Centre for Heart Lung Innovation (Graphic and Imaging core, St. Paul's Hospital) to be scanned by Aperio ScanScope XT (Aperio Technologies, Vista, CA) at 40x magnification. Morphometric and immunohistochemical analysis was performed on the generated high-resolution digital images (0.25 microns/pixel) using Aperio ImageScope (version 11.2.0.780). The incorporated Positive Pixel Count (PPC) algorithm (version 9.1) was used to quantify inflammatory cells that stained positive (positive pixels) for

each antibody of interest in the bronchial submucosa, excluding all areas with smooth muscle, mucosal glands, blood vessels, and areas with artifact such as torn, crushed or folded tissue.

The PPC algorithm counts pixels in the selected area in accordance with hue-saturation-intensity (HSI) color model [134, 135]. The HSI model describes a color based on its hue (pure color), saturation (purity or amount of pure color diluted with white light) and intensity (brightness).

To quantify AEC-positive pixels, a hue value of 0.0 (red) and hue width of 0.5 was used, and all the three intensity ranges (weak, positive, and strong) of staining were considered as positive. A color saturation threshold of 0.1 was used except when it was increased to 0.15 for analyzing sections with non-specific background staining.

The number of positive pixels were divided by the total number of pixels (positive and negative) in the analyzed area, and multiplied by 100, to calculate the percentage of positive pixels.

$$\frac{\text{Number of Positive pixels}}{\text{Total number of Positive pixels} + \text{Negative pixels}} \times 100 \left(\% \text{ Positivity }\right)$$

Mark-up images were generated by ImageScope software which give a visual representation of the numerical results will be reviewed to ensure that automated image analysis was performed accurately. (See Figure 2.4)

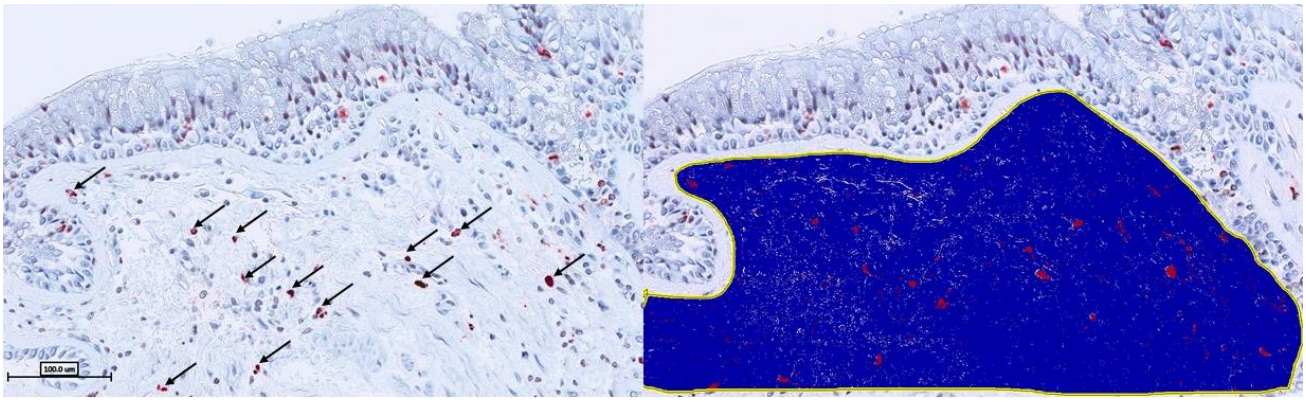

Figure 2.6 Demonstration of image analysis using Aperio® ImageScope™ software. A positive pixel count algorithm was used to quantify positive staining in the submucosa (blue region) of bronchial biopsies for tryptase, ECP, NE, CD4, CD138 and IL-4. The airway epithelium was not examined and positive staining in the epithelium was excluded from analysis. **A**. Representative image of tryptase positive staining from a subject exposed to FAA. Black arrows denote positive staining in submucosa area that are selected by the positive pixel count. **B**. Image from a with submucosa region selected by manual trace followed by positive pixel count recognition of tryptase stain (red colour) within submucosa region (blue colour).

2.13 Statistical analysis

One-way repeated measures ANOVA with Bonferroni multiple comparison post hoc tests and Pearson correlation coefficients matrix were performed using GraphPad Prism® 6 software (GraphPad Software Inc., La Jolla, CA). Combined Robust regression and Outlier removal method (ROUT test) was used and an outlier in CD138 dataset was identified and removed. A p-value of <0.05 was considered statistically significant. Error bars shown represent the standard error of mean (±SEM).

Chapter 3: Results[1]

3.1 Subject characteristics

Study subject gender, age, height, weight, body mass index (BMI), forced expiratory volume in 1 second (FEV1), methacholine PC20, and allergen used for segmental allergen challenge are described in Table 3.1. The study involved samples from 7 female subjects and 5 male subjects. Samples from all 12 subjects were used for IL-4 immunostaining and morphometric analysis. For tryptase, eosinophil cationic protein (ECP), CD4, CD138 and neutrophil elastase (NE) staining, two bronchial biopsies did not contain sufficient submucosa area, resulting in only 10 subjects available for comparison across the four experimental conditions for these endpoints.

[1] A version of chapter 3 has been published. Hosseini A, Hirota JA, Hackett TL, McNagny KM, Wilson SJ, Carlsten C. (2016) Morphometric analysis of inflammation in bronchial biopsies following exposure to inhaled diesel exhaust and allergen challenge in atopic subjects. Particle and Fibre Toxicology. 13(1):2.

Table 3.1 Subject characteristics.

Subject	**Gender**	**Age** (years)	**Height** (cm)	**Weight** (kg)	**BMI**	**FEV_1** (% of pred)	**Methacholine PC_{20}** (mg/mL)	**Positive SPT**
1	F	20	158	54.6	22	104	13.85	HDM
2	F	31	173	70	24	113	>16	HDM
3*	M	32	161	68	26	123	30.74	Pacific Grasses
4	F	34	157	55	22	79	0.23	Pacific Grasses
5	M	27	178	70	22	105	>128	HDM
6	F	25	173	73	25	117	>128	HDM
7*	M	27	186	85	25	107	87.64	Pacific Grasses
8	F	46	165	65	24	63	>128	HDM
9*	F	31	146	50	23	103	0.26	Birch
10	M	28	176	90	29	100	19.12	Pacific Grasses
11*	M	23	169	83	29	104	2.41	Pacific Grasses
12	F	23	172	96	32	101	54.32	HDM
Mean (SD)		**29 (7)**	**168 (11)**	**72 (15)**	**25 (3)**	**102 (16)**		

M=male; F=female; BMI=body mass index; FEV1=forced expiratory volume in one second; % of pred=percentage of predicted; PC20=provocative concentration causing a 20% fall in FEV1; SPT=skin prick test; HDM=house dust mite allergen; *=previous smoker.

3.2 Submucosal immune responses induced by single exposure or co-exposure

Tissue sections were immunostained for tryptase, eosinophil cationic protein (ECP), neutrophil elastase (NE), CD138, CD4 and interleukin (IL)-4. The percent positivity of positive cells were quantified in the bronchial submucosa by Aperio ImageScope Software. (See Table 3.2)

Table 3.2 Percent positivity for inflammatory biomarkers' expression in the lung submucosa. Data are expressed as mean ± standard error of the mean (SEM).

Exposure condition	AA1	ECP	NE	CD138	CD4	IL-4
FAS	0.460 ± 0.053	0.308 ± 0.102	0.045 ± 0.014	0.017 ± 0.006	0.087 ± 0.018	0.127 ± 0.062
DES	0.681 ± 0.153	0.471 ± 0.175	0.077 ± 0.024	0.044 ± 0.024	0.130 ± 0.025	0.353 ± 0.088
FAA	0.646 ± 0.128	0.553 ± 0.109	0.229 ± 0.069	0.044 ± 0.008*	0.144 ± 0.041	0.426 ± 0.130
DEA	0.738 ± 0.159	0.487 ± 0.201	0.224 ± 0.047*	0.120 ± 0.031*, †, ‡	0.311 ± 0.060*	0.548 ± 0.143*

FAS=filtered air + saline; DES=DE + saline; FAA=filtered air + allergen; DEA=DE + allergen; AA1=tryptase; ECP=eosinophil cationic protein; NE=neutrophil elastase; *p<0.05, compared with FAS; †p<0.05, compared with DES; ‡p<0.05, compared with DEA.

3.2.1 Tryptase is unchanged by the combination of diesel exhaust and allergen (DEA)

Analysis of tryptase-positive staining in bronchial mucosa revealed distinct and strong granular cytoplasmic staining in cells with isolated observations of extracellular staining (Figure 3.1B). Quantification of the tryptase positively stained pixels, demonstrated no differences between any experimental conditions (Figure 3.1C) although there was a trend to increased staining in the DEA (diesel exhaust + allergen) samples.

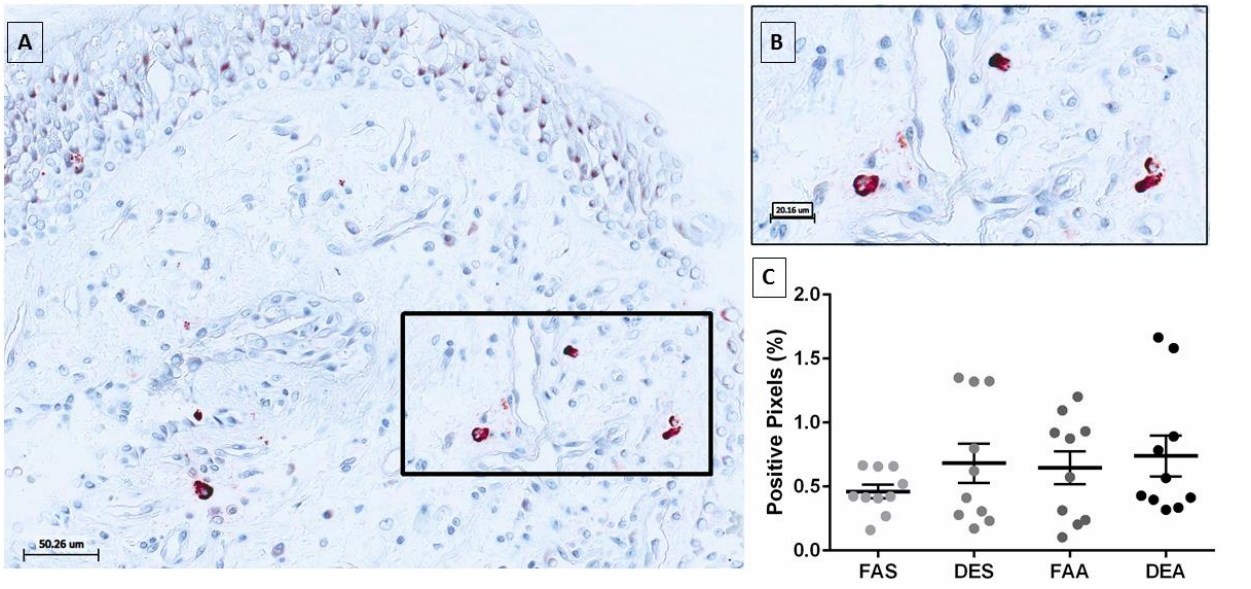

Figure 3.1 Immunohistochemical staining of tryptase-positive mast cells in human bronchial submucosa tissue. **A** Representative 20X image of positive staining using mAb AA1 for tryptase with positive staining in red with Mayer's hematoxylin counterstain in blue from a subject exposed to FAA. **B** Zoom region (40X) highlighted in black box of panel A. **C** Positive pixel count quantification of submucosa region for tryptase staining. Data are expressed as mean ± SEM. n = 10 for each experimental condition.

3.2.2 ECP is unaffected by the combination of diesel exhaust and allergen (DEA)

Analysis of ECP-positive staining in bronchial mucosa revealed distinct cytoplasmic localization in cells with pervasive observations of extracellular staining suggesting eosinophil degranulation (Figure 3.2B). Quantification of the ECP-positive pixels, demonstrated no differences between experimental conditions (Figure 3.2C).

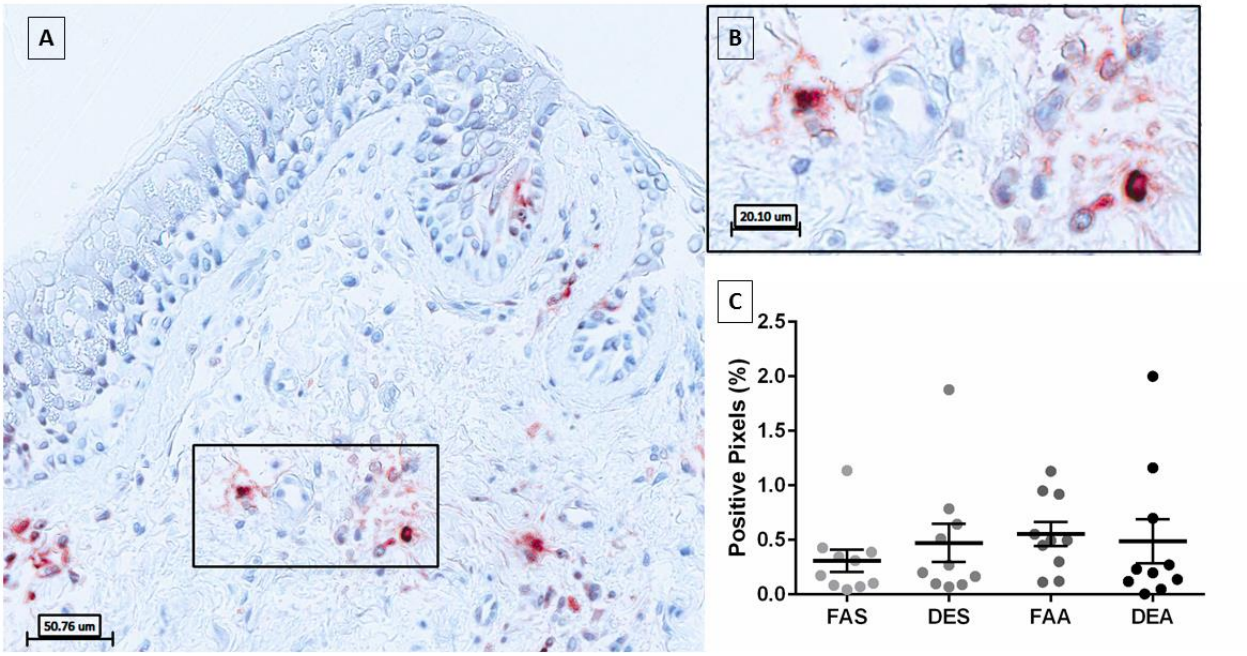

Figure 3.2 Immunohistochemical staining of ECP-positive eosinophils in human bronchial submucosa tissue. **A** Representative 20X image of positive staining using mAb EG2 for ECP with positive staining in red with Mayer's hematoxylin counterstain in blue from a subject exposed to FAA. **B** Zoom region (40X) highlighted in black box of panel A. **C** Positive pixel count quantification of submucosa region for ECP staining. Data are expressed as mean ± SEM. n =10 for each experimental condition

3.2.3 Neutrophil elastase is elevated by DEA

Analysis of NE-positive staining in bronchial mucosa revealed distinct immunohistochemical localization of elastase in neutrophils with no observations of extracellular staining (Figure 3.3B). Quantification of the NE-stained pixels demonstrated a significant increase ($p=0.031$) in staining after DEA (0.224 ± 0.047) relative to FAS (0.045 ± 0.014). There were no significant differences between FAS vs. DES (0.077 ± 0.024, $p>0.999$) or FAA (0.229 ± 0.069, $p=0.175$) (Figure 3.3C).

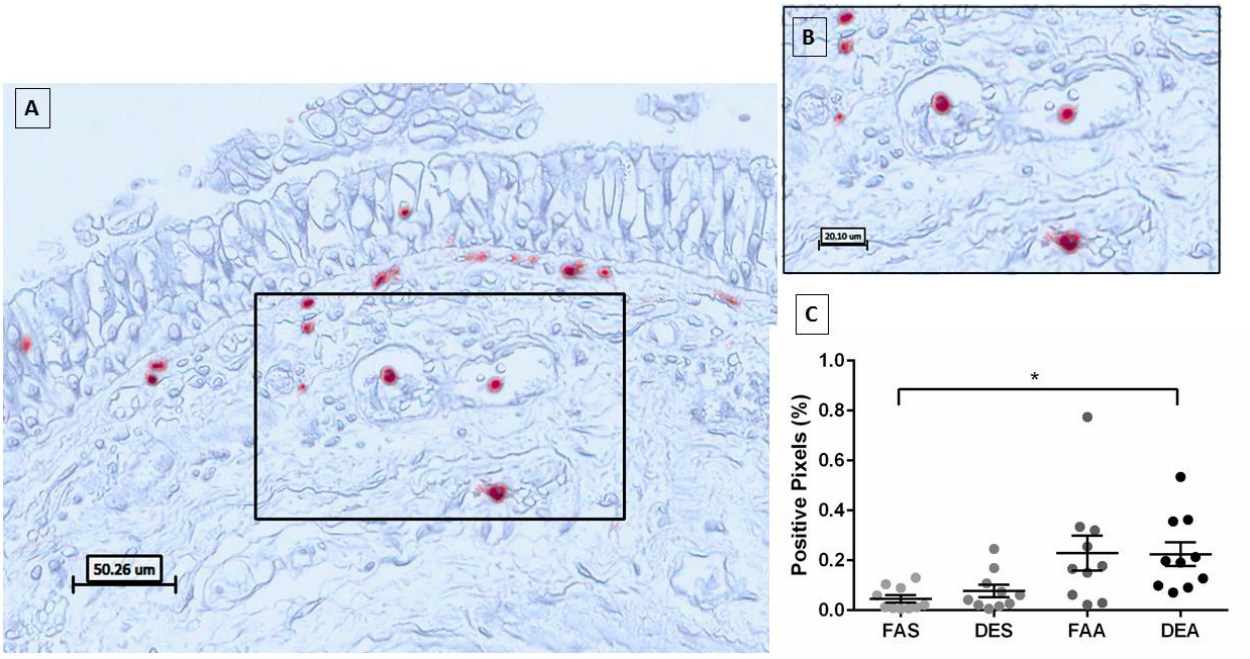

Figure 3.3 Immunohistochemical staining of elastase-positive neutrophils in human bronchial submucosa tissue. **A** Representative 20X image of positive staining using mAb NP57 for NE with positive staining in red with Mayer's hematoxylin counterstain in blue from a subject exposed to FAA. **B** Zoom region (40X) as highlighted in black box of panel A. **C** Positive pixel count quantification of submucosa region for NE staining. Data are expressed as mean ± SEM. n = 10 for each experimental condition. *$p < 0.05$

3.2.4 CD138 positive cells are elevated by FAA and DEA

Analysis of CD138-positive staining in bronchial mucosa revealed distinct membrane staining in plasma cells (Figure 3.4B). Quantification of the CD138-positive pixels demonstrated a significant increase in staining after DEA (0.120 ± 0.031) relative to FAS (0.017 ± 0.006, p=0.015), DES (0.044 ± 0.024, p=0.040), and FAA (0.044 ± 0.008, p=0.037). CD138-positive staining in FAA was elevated relative to FAS (0.017 ± 0.006; p=0.049) (Figure 3.4C).

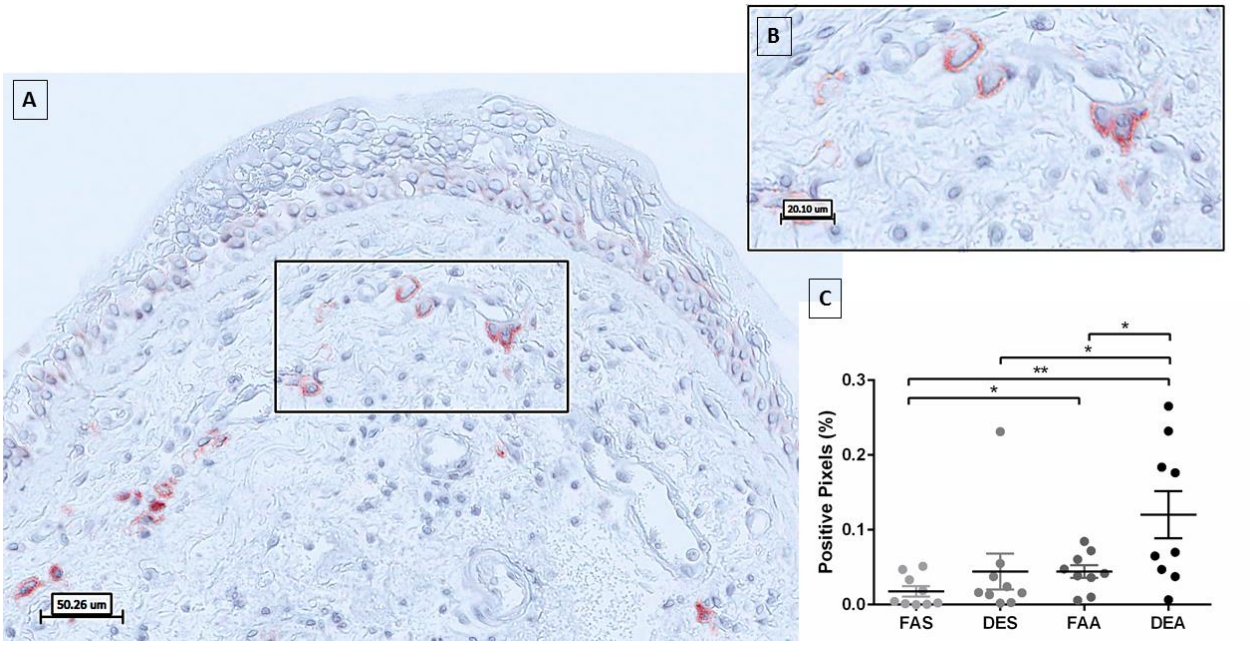

Figure 3.4 Immunohistochemical staining of CD138-positive plasma cells in human bronchial submucosa tissue. **A** Representative 20X image of positive staining using mAb B-A38 for CD138 with positive staining in red with Mayer's hematoxylin counterstain in blue from a subject exposed to FAA. **B** Zoom region (40X) highlighted in black box of panel A. **C** Positive pixel count quantification of submucosa region for CD138 staining. Data are expressed as mean ± SEM. n = 9 for each experimental condition. *p < 0.05; **p < 0.01

3.2.5 CD4 positive cells are significantly elevated by DEA

Analysis of CD4-positive staining in bronchial mucosa revealed distinct membrane staining in T cells (Figure 3.5B). Quantification of the CD4-positive pixels demonstrated a significant ($p=0.035$) increase in staining after DEA (0.311 ± 0.060) relative to FAS (filtered air + saline [control (vehicle) for allergen]; 0.087 ± 0.018).

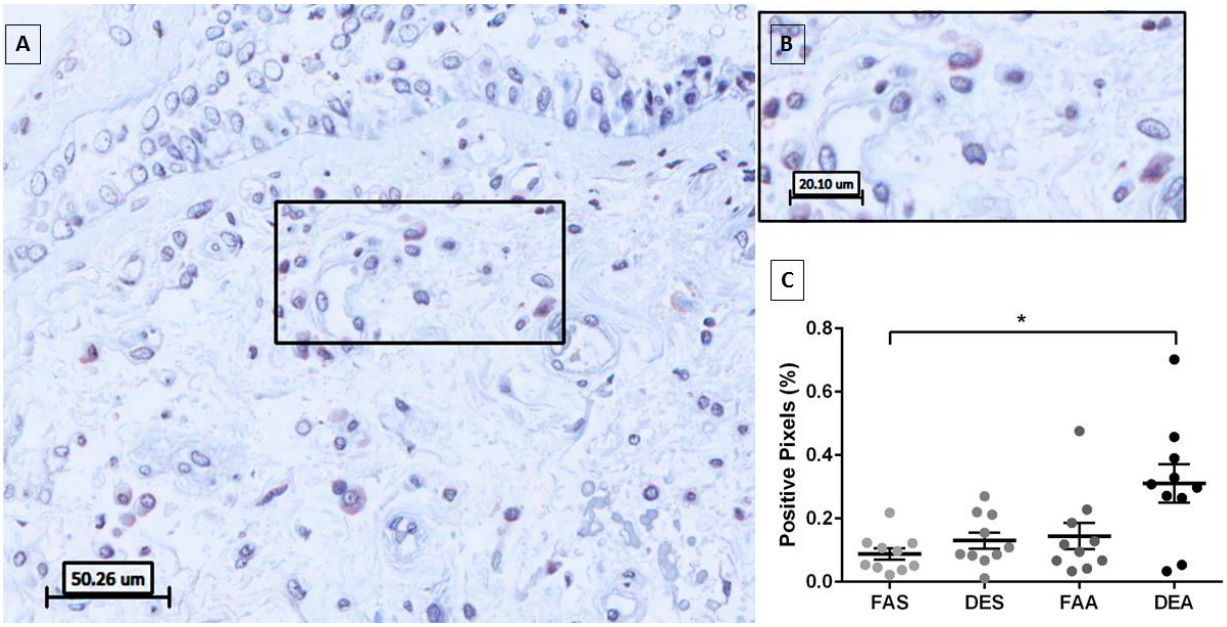

Figure 3.5 Immunohistochemical staining of CD4-positive T cells in human bronchial submucosa tissue. **A** Representative 20X image of positive staining using mAb 4B12 for CD4 with positive staining in red with Mayer's hematoxylin counterstain in blue from a subject exposed to FAA. **B** Zoom region (40X) highlighted in black box of panel A. **C** Positive pixel count quantification of submucosa region for CD4 staining. Data are expressed as mean ± SEM. n = 10 for each experimental condition. *$p < 0.05$

3.2.6 IL-4 expression is elevated by DEA

Analysis of IL-4-positive staining in bronchial mucosa revealed distinct immunohistochemical localization of IL-4 in cells with no observations of extracellular staining (Figure 3.6B). Quantification of the IL-4-stained pixels, demonstrated a significant increase ($p=0.034$) in staining for DEA samples (0.548 ± 0.143) relative to FAS samples (0.127 ± 0.062). There were no significant differences between FAS vs. DES (0.353 ± 0.088, $p=0.086$) or FAA (0.426 ± 0.130, $p=0.150$).

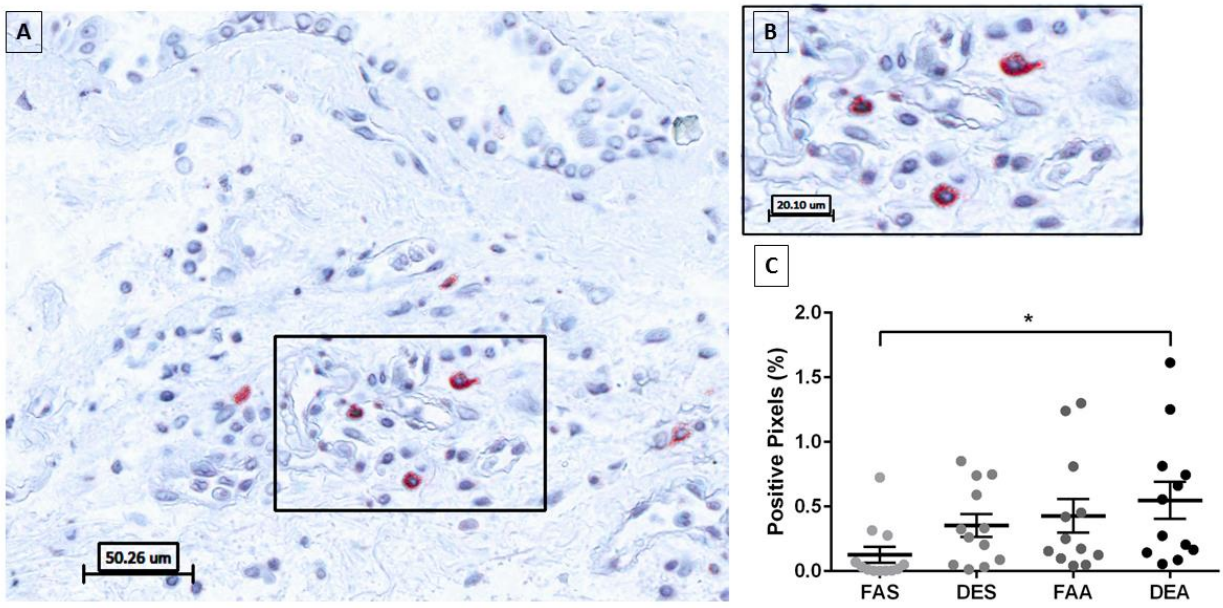

Figure 3.6 Immunohistochemical staining of IL-4-positive cells in human bronchial submucosa tissue. **A** Representative 20X image of positive staining using mAb 4D9 for IL-4 with positive staining in red with Mayer's hematoxylin counterstain in blue from a subject exposed to FAA. **B** Zoom region (40X) highlighted in black box of panel A. **C** Positive pixel count quantification of submucosa region for IL-4 staining. Data are expressed as mean ± SEM. n = 12 for each experimental condition. *$p < 0.05$

3.2.7 Correlation coefficient analysis

Pearson correlation coefficients matrix was performed for each of the endpoints versus each other. No endpoints were significantly correlated under DEA, suggesting distinctive biology attributable to the various endpoints induced by DEA. NE and CD138 were positively correlated (r=0.76, p<0.01) after DES. (Table 3.3)

Table 3.3 Pearson correlation coefficients matrix for inflammatory biomarkers' expression in the lung submucosa measured after FAS (filtered air + saline) condition (normal font), and DES (diesel exhaust + saline) condition (italics).

Variable	AA1	ECP	CD4	IL-4	CD138	NE
AA1	**1**	0.15 (p=0.67)	0.21 (p=0.55)	0.23 (p=0.51)	-0.07 (p=0.84)	0.05 (p=0.88)
ECP	*0.02 (p=0.94)*	**1**	0.02 (p=0.95)	-0.26 (p=0.45)	0.08 (p=0.80)	-0.18 (p=0.60)
CD4	*0.20 (p=0.56)*	*0.37 (p=0.29)*	**1**	-0.17 (p=0.63)	0.23 (p=0.51)	0.38 (p=0.27)
IL-4	*0.46 (p=0.17)*	*-0.31 (p=0.37)*	*-0.15 (p=0.67)*	**1**	0.53 (p=0.88)	0.37 (p=0.28)
CD138	*-0.09 (p=0.79)*	*0.04 (p=0.90)*	*0.56 (p=0.09)*	*-0.25 (p=0.48)*	**1**	0.13 (p=0.18)
NE	*-0.01 (p=0.97)*	*0.20 (p=0.56)*	*0.32 (p=0.36)*	*-0.01 (p=0.96)*	*0.76* ***(p=0.01)****	**1**

AA1=tryptase; ECP=eosinophil cationic protein; NE=neutrophil elastase; *=p<0.05.

After FAA, tryptase and CD138 were positively correlated (r=0.64, p<0.04) and ECP and IL-4 were also positively correlated (r=0.61, p<0.05). (Table 3.4)

Table 3.4 Pearson correlation coefficients matrix for inflammatory biomarkers' expression in the lung submucosa measured after FAA (filtered air + allergen) condition (normal font) and DEA (diesel exhaust + allergen) condition (italics).

Variable	AA1	ECP	CD4	IL-4	CD138	NE
AA1	**1**	0.07 (p=0.84)	-0.19 (p=0.58)	-0.30 (p=0.40)	0.64 **(p=0.04)***	-0.005 (p=0.99)
ECP	*0.07 (p=0.84)*	**1**	-0.26 (p=0.45)	0.61 **(p=0.05)***	-0.38 (p=0.27)	0.32 (p=0.35)
CD4	*-0.44 (p=0.20)*	*-0.42 (p=0.22)*	**1**	-0.31 (p=0.36)	-0.06 (p=0.86)	-0.09 (p=0.80)
IL-4	*0.33 (p=0.34)*	*-0.23 (p=0.51)*	*0.23 (p=0.51)*	**1**	-0.34 (p=0.33)	-0.23 (p=0.51)
CD138	*-0.25 (p=0.48)*	*-0.37 (p=0.28)*	*0.54 (p=0.10)*	*0.19 (p=0.58)*	**1**	-0.33 (p=0.34)
NE	*0.14 (p=0.68)*	*-0.21 (p=0.55)*	*0.38 (p=0.27)*	*0.16 (p=0.65)*	*-0.13 (p=0.72)*	**1**

AA1=tryptase; ECP=eosinophil cationic protein; NE=neutrophil elastase; *=p<0.05.

Chapter 4: Discussion

4.1 Overview

To our knowledge this is the first blinded human crossover study using controlled exposures to a combination of DE and allergens to investigate the lower airway inflammatory responses within bronchial biopsies in atopic individuals. Considerable evidence suggests that that the effects of DE and allergens are synergistic [136], but this evidence comes primarily from human nasal (upper airway) models or pre-clinical (animal or *in vitro*) models. ***We demonstrate that combination exposure of DE followed by allergen results in augmented CD4, IL-4, NE and CD138 (but not tryptase or ECP) in the submucosa, which is not observed with exposure to either allergen or DE alone.***

The concentration of DE 300 $\mu g.m^{-3}$ used in our study is most relevant in the context of occupational exposure in Western countries or the daily average level of PM in some developing countries in the world such as China and India [137, 138]. Our results suggest that single exposure models that have examined allergen and air pollution in isolation may underestimate the real-world impact of heterogeneous environmental exposures on airway inflammation indices in atopic humans. This has implications for health policy aimed at protecting air quality for vulnerable populations [139], as the additional vulnerability induced by dual exposures may need be taken into account. Furthermore, our data suggests that single exposure models in humans used for drug efficacy testing [140-142] may not reflect efficacy of therapy in real-world complex exposures.

4.2 Tryptase is a reliable biomarker of mast cells presence and activation

Mast cells are known to participate in allergic inflammation [143]. Mast cells originate from $CD34^+$, $CD117^+$ (c-kit) precursor cells in the bone marrow. Immature mast cells are released into the peripheral blood circulation and then migrate into their destination tissue where they are differentiated under the influence of the local cytokine milieu. The number of tissue mast cells in normal condition is steady, but mast cell recruitment increases when disturbed by pathophysiologic conditions in inflamed tissues in allergic asthma and rhinitis [117, 118]. Tryptase is the most plentiful granule constituent in mature and activated mast cells [144], thus we stained for tryptase as it is a reliable biomarker of mast cell presence and activation. Basophils are the only other cell type that express tryptase, but their frequency is known to be considerably lower than that of in mast cells [145] and tryptase levels in human basophils are less than 1% of those in tissue mast cells [146]. Our results demonstrate that 48 hours following co-exposure to DE and allergen, there was no elevations in tryptase positive cells in the submucosa. Previous studies have shown increases the number of mast cells in healthy volunteers at 6 hours [147] and 18 hours [148] post-exposure to DE. Mast cells are known to play a significant role in both, early and late phase of the allergic reaction through the allergen and IgE-dependent release of potent mediators that may contribute to allergic inflammation. To fully understand the role of mast cells in response to combined exposure to DE and allergen, additional studies looking at kinetics of activated mast cells in earlier time points are needed.

4.3 Eosinophils play significant role in the development of allergic airway inflammation

Eosinophils are another pro-inflammatory white blood cell type that differentiates from myeloid progenitors in the bone marrow; mature eosinophils travel through blood vessels to mucosal surfaces throughout the body [119]. Activated eosinophils release toxic granule proteins capable of causing airway tissue damage, including major basic protein (MBP), eosinophil peroxidase (EPO) and ECP, which damage nerves and epithelial cells [123]. ECP also known as ribonuclease (RNase)-3, is a strong cytotoxic protein with bactericidal and antiviral properties. Accumulation of eosinophils to sites of inflammation are now known as one of the clear signs of allergic inflammation and their increased number is directly associated with disease severity [120]. Eosinophils have historically been considered an effector cell in asthma recruited to the lung by T cells; however, recent studies suggest that eosinophils also play an active role in the development of allergic airway inflammation [121]. Animal toxicological studies have shown that co-administration of allergen and DE enhances cellular inflammation characterized by an increase in airway eosinophils, neutrophils and mast cells. We examined ECP as a selective marker for eosinophils, as this protein is largely restricted to these cells. There is evidence that ECP can be detected in neutrophils [149, 150], but rarely and weakly [151]. We appreciate that MBP or EPO may also be used to quantify eosinophils in tissue, but ECP staining allowed us to compare our results with other DE controlled exposure studies [152, 153].

High levels of ECP are considered as a clinical indicator of active atopic asthma and reflect the intensity of ongoing eosinophilic inflammation of the airways in allergic diseases [154]. Our data demonstrate that in atopic individuals, combination exposure (DE followed by allergen) does

not impact ECP expression in bronchial submucosa. This is consistent with a previous controlled DE exposure study that showed no significant increase in the count of tissue eosinophils at 18 hours post-exposure [148], although studies examining bronchoalveolar lavage [85] and induced sputum [153] have shown increases at 6 hours post-exposure. Further studies examining the time-course of both the tissue and luminal lung compartments will be required to fully understand the kinetics and localization of eosinophils following combination exposures.

We chose a later time point 48 hrs for sampling tissue biopsies, thus there is potential that early inflammatory cell recruitment and activation was missed due to resolution of the inflammatory episode. A previous study utilizing segmental allergen challenge was aimed to elucidate the time course of inflammatory events in allergic airway diseases. They have shown that the level of tryptase increases immediately within 12 min following the segmental allergen broncho-provocation with ragweed in bronchoalveolar lavage (BAL) fluid from allergic rhinitis patients, but no elevation was observed in 48 hours. In contrast, ECP levels in lavage samples only increased in late response (48 hours) but not within minutes post allergen challenge [155]. Their findings may suggest that in our tissue biopsies, ECP was released from the tissue eosinophils and may be measureable in BAL samples at 48 hours [156]. A limitation of our study is that the single 48-hr time-point following exposure that may have missed components of the inflammatory response that occurred at earlier time-points.

4.4 Neutrophils accumulation is induced by co-exposure to DEP and allergen

Neutrophilic inflammation is associated with progression and the development of chronic respiratory diseases, such as severe asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and idiopathic pulmonary fibrosis (IPF) [157]. Neutrophils are granulocytes and one of the first responders to the environmental insults and migrate to the site. Neutrophil Elastase (NE) is a serine protease that is expressed in bone marrow precursor cells [158, 159]. Granular neutrophil elastase is an essential effector of the innate immune responses, with resilient antimicrobial activity [160]. In neutrophils, NE contributes mainly to digestion of ingested foreign particles, chemotaxis, infiltration and tissue remodeling by degrading connective tissue proteins such as elastin and collagens [161]. Exposure to DEP is associated with accumulation of neutrophils and it has been shown that DEP can activate neutrophils and augment the expression of NE, IL-8, leukotriene B4 (LTB4) and matrix metalloproteinase 9 (MMP-9) [162, 163]. Thus, DEP exposure through activating neutrophils and releasing their harmful mediators can contribute to the pathogenesis of chronic inflammatory lung diseases. NE proteolytic activity initiates an inflammatory process leading to goblet cell metaplasia [164], hypersecretion in asthmatics [165], airway smooth muscle cell proliferation [166]. High levels of elastase has been reported in the sputum samples from asthmatics [167, 168] and patients with acute severe asthma [169].

We demonstrate that NE expression is increased in DEA vs. FAS ($p= 0.031$), suggesting neutrophilic inflammation is induced by combined exposure to DE and allergen. Our data are consistent with previous human controlled studies that reported a significant increase in the percentage of sputum neutrophils at 6 hours after exposure to DE, and a non-significant

increase at 24 hours post-exposure in healthy subjects [170]. Six hours post-exposure to DE, the immunostaining of bronchial biopsies presented an elevation in the number of submucosal neutrophils [147]. Also the percentage of neutrophils significantly increased in BAL after exposure to both filtered and unfiltered DE at 24 hours after in healthy subjects [171].

4.5 CD138 is specific marker for tissue plasma cells

CD138 (syndecan-1) is a cell surface proteoglycan and predominantly is expressed on mature plasma cells and weakly in epithelial cells. CD138 is highly sensitive and specific marker for identification of plasma cells and plasma cell differentiation. CD138 modulates cell growth, differentiation, adhesion and migration, thus plays important roles in the regulation of inflammatory responses [172].

Aggregation of $CD138^+$ plasma cells were identified in pulmonary fibrosis [173] and in the lung submucosa of severe asthmatics with increased inflammatory lymphocytes infiltrates [174]. Increased frequencies of $CD138^+$ IgE^+ cells were detected in the lamina propria of the nasal mucosal biopsies from allergic patients [175]. Also the number of $CD138^+$ IgE^+ cells was positively correlated with the IgE serum titres in atopic individuals [176]. Exposure to environmental allergens triggers Th2 cells differentiation and production of IL-4 and IL-13 which proliferate and differentiate B cells into plasma cells and switch to IgE synthesis [177]. It has been shown that inhalation allergen challenge significantly increases the number of $CD138^+$ IgE-secreting cells in murine lungs [178]. $CD138^+$ cells were detectable in bronchial biopsies but not in BAL at 24 hours after segmental allergen challenge in atopic asthmatics [179]. CD138 immunostaining is proven to be an excellent indicative of IgE^+ cells in the lung tissue [180].

We demonstrate that CD138 expression is increased in DEA vs. FAS, DES and FAA (p= 0.015, p=0.040, p=0.037, respectively), suggesting plasmacytosis is induced by DE and allergen. CD138 expression in FAA was elevated relative to FAS (p=0.049), suggesting the effect of allergen challenge in submucosal plasmacytosis. Our data are consistent with a previous human nasal model that have confirmed that co-administration of DE particles and allergen stimulate an increase in level of allergen specific IgE in nasal lavage samples [91].

4.6 CD4+ T helper cells drive the pathogenesis of allergic inflammation

$CD4^+$ T helper cells play an essential role in adaptive allergic immune responses. CD4 is a transmembrane glycoprotein selectively expressed on the surface of helper T-cells and plays an important role in the regulation of T-cell signalling and its functional consequences [181]. Following activation, naive $CD4^+$ T-cells differentiate into one of the sub-types of T helper cells: Th1 [182], Th2 [183], Th9 [184], Th17 [185], or Th22 [186], depending on the nature of antigen and the cytokines present in the surrounding milieu [93]. Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and induce eosinophil activation and differentiation; Th2 cells are more proficient B-cell helpers and can stimulate IgG_1 and IgE production. Thus, they are well positioned to play substantial role in the pathogenesis of allergic inflammation. In the present work, we explored whether co-exposure to DE and allergen induced recruitment of $CD4^+$ cells in human lung tissue. We demonstrate that the number of $CD4^+$ cells significantly increased only in DEA vs. FAS (p= 0.035), which suggest an interaction between DE and allergen. In a single exposure model in healthy subjects, it has been previously argued that DE does not alter the number of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes in the bronchial tissue at 18 hours post-exposure [187].

4.7 IL-4 is the signature cytokine of Th2 cells

IL-4 is known to be an important cytokine in the development of allergic inflammation; it provides the first signal that initiates B-cell class switching to IgE production [102]. IL-4 can further enhance IgE-mediated immune responses by up-regulating the expression of low-affinity IgE receptor (FcεRII/CD23) on B-lymphocytes and macrophages and the high-affinity IgE receptor (FcεRI) on mast cells [103, 104]. IL-4 induces the differentiation of naive T lymphocytes into Th2 cells which secrete more IL-4, IL-5, and IL-13, maintaining a suitable environment for further Th2 cells differentiation [105, 106]. IL-4 can also stimulate the expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, which leads to enhanced migration of T-cells, eosinophils, macrophages and mast cells to inflamed tissue [107]. To our knowledge there is no specific marker for Th2 cells but IL-4 is a signature cytokine of type 2 immunity. IL-4 is known as a positive feedback cytokine for Th2 cell differentiation that stimulates the differentiation of naive $CD4^+$ cells into IL-4-secreting Th2 cells [188, 189]. It has been shown that human mast cells are one of the major sources of IL-4 in the skin, nasal and bronchial tissue [190-192]. IL-4 is able to induce the development of Th2 cells; thus, this stored and preformed IL-4 within mast cell granules has an important influence during the initiation and maintenance of the allergic immunological response. Basophils [193], naive T cells [194] and innate lymphoid cells [195] are also immediate source of IL-4 upstream of Th2 cells differentiation. IL-4 contributes to airway obstruction in asthma via the induction of mucus hypersecretion in mice and human cell lines, and increases the release of several pro-inflammatory cytokines such as IL-6, GM-CSF and eotaxin from human lung fibroblasts [108]. IL-

4 is a major factor in the recruitment and activation of inflammatory cells that may contribute to inflammation and lung remodeling in chronic asthma [196].

We demonstrate that IL-4 expression is increased in DEA vs. FAS ($p= 0.034$), suggesting a Th2 immune response is induced by DE and allergen that is not observed with isolated (single) exposures. Our data are consistent with *in vivo* animal, *in vitro,* and human studies that have confirmed DE has strong pro-Th2 effect [91, 197, 198]. Human nasal challenge studies have shown that co-administration of DE particles and allergen stimulate a Th2 immune response in nasal wash samples 4 days post-challenge [21, 88, 90]. There is one conflicting study that has shown no differences in expression of IL-4 in the bronchial submucosa but its authors mention that the bronchial tissue in their study was assessed at a single time point 6 hours post-DE exposure [199] while most of the cytokine changes observed in previous human nasal studies and animal exposure studies were found 24 to 48 hours post-exposure [91, 200] A previous study also evaluated the effects of diesel exhaust inhalation in enhancing allergic immunologic responses in lower airways [201]. Consistent with our results, they similarly found an increase in the IL-4 level (by 1.7-fold, which was close to statistical significance); they also found a non-significant elevation in the number of eosinophils in induced sputum due to DE exposure. However, there are some fundamental differences between their model and our current study a) their allergen challenge was performed by inhalation but we challenged our subjects segmentally, with saline control simultaneously, which confers some advantages and limitations; b) the diesel exhaust concentration that we used was ~300 $\mu g.m^{-3}$ $PM_{2.5}$ while theirs was ~100 $\mu g.m^{-3}$; c) they analyzed sputum and blood that were acquired 22 hour post-exposure while we analyzed endobronchial biopsies that were obtained 48 hours post-exposure.

Chapter 5: Conclusions

In conclusion, we have demonstrated for the first time that acute exposure to DE followed by segmental allergen challenge increases the number of submucosal CD4 cells, CD138-positive plasma cells and expression of neutrophil elastase and IL-4 in the submucosa of atopic human subjects. Our study design and results suggest that experimental data from complex exposures can capture real-world exposures that enlighten our understanding of biology and maybe inform those concerned with public health and policy based on complex exposures.

5.1 Strengths and limitations

A primary strength of our study is that it is a double-blinded cross-over study that fundamentally eliminates typical confounding covariates, since each subject serves as his/her own control. Our study does have limitations, however. One limitation is generalizability. For example, given the specific gap (one hour) between inhalation exposure and segmental allergen, it is difficult to know whether similar findings would occur with simultaneous exposure or some other gap, but we effectively consider this “co-exposure” given that the particles from DE will remain in the airways for hours after inhalation and thus then be present when the allergen is inhaled (though, admittedly, the exact dynamics therein are unknown and an important future direction for our work). Another concern is whether airway changes relevant to our hypotheses can be induced by the bronchoscopy procedure itself. Investigative bronchoscopy and bronchial provocation challenge are commonly used techniques in airway inflammation research [202]. While FEV1 and PEFR dropped due to bronchoscopy with lavage and biopsies, both returned to baseline within 2 to 24 hours [203] [204]. Accordingly, we doubt that significant inflammation from these procedures persists through 48 hours.

5.2 Future directions

This thesis studied the effects of acute exposure to diesel exhaust and allergens, thus it would be interesting if future studies discover pulmonary effects of the chronic exposures in a human model. Long term exposure studies would be able to capture more details of the airways inflammation and its progression for a longer period of time. It is critical to identify the combined effects of various air pollutants and how they can modify pulmonary immune responses. More research is needed to further address the effect of complex exposures on human health. Future studies may focus on assessing the effects of air pollution exposure on most vulnerable populations including severe asthmatics and COPD patients.

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