THE EFFECTS OF DIESEL EXHAUST ON NEUTROPHIL FUNCTIONS IN NEVER-SMOKERS, EX-SMOKERS, AND COPD PATIENTS

by

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B.Kin, University of Toronto, 2016

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

June 2018

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The effects of diesel exhaust on neutrophil functions in never-smokers, ex-smokers and COPD patients

submitted by Denise Wooding in partial fulfillment of the requirement for

the degree of Master of Science

in Experimental Medicine

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Abstract

Introduction: Traffic-related air pollution (TRAP) is associated with COPD epidemiologically, however the mechanism of this interaction remains unclear. Neutrophils, a key inflammatory cell in COPD, migrate to the lungs following TRAP exposure, but their functional role in this response is poorly understood. The aim of this study is to elucidate the effects of TRAP exposure on neutrophil function, using the model of diesel exhaust (DE).

Methods: *In vitro:* Isolated peripheral blood neutrophils were incubated with diesel exhaust particles (DEPs) before quantifying activation marker expression, oxidative burst, and neutrophil extracellular traps (NETs). *In vivo:* Subjects from three groups (never-smokers, ex-smokers, and mild-moderate COPD) participated in a randomized double-blind controlled human exposure crossover study. Subjects were exposed on one occasion to filtered air and on another occasion to diluted DE. Blood and bronchoalveolar lavage (BAL) samples were used to assess the effect of DE on the number and function of pulmonary and systemic neutrophils.

Results: DEPs increased CD66b, oxidative burst, and NET formation *in vitro.* Controlled human exposure to DE reduced the proportion of circulating band cells, increased NET formation in BAL, and resulted in lymphocytic but not neutrophilic inflammation. The effect of DE on band cells and peripheral CD182 expression was distinct in the COPD group. There was no effect on oxidative burst or activation marker expression in pulmonary neutrophils.

Conclusion: Diesel exhaust increases some, but not all, neutrophil effector activities *in vitro* and *in vivo*. Two potential mechanisms for susceptibility in COPD patients were identified. These results demonstrate a functional role for neutrophils in the inflammatory response to diesel exhaust. The potential for DE-induced neutrophil activity to promote lung tissue damage and clinical features of COPD may be an area of future investigation.

Lay Summary

Air pollution is responsible for 4.2 million deaths per year. Air pollution might be able to cause or worsen a disease called chronic obstructive pulmonary disease (COPD), which is historically most related to cigarette smoking. However, we do not fully understand how air pollution exposure would cause this to happen. This study investigated the effects of diesel exhaust, a model of traffic-related air pollution, on the body's first-responder immune cells, called neutrophils. Neutrophils were stimulated with diesel exhaust particles in test tubes to see how their behavior was affected. Then, a controlled human exposure study was performed, where volunteers were exposed to diluted diesel exhaust reflective of the air pollution in polluted urban cities, and neutrophils from blood and lung samples were tested. Overall, neutrophils changed some of their behaviors after diesel exhaust exposure, which may help explain how the body's reaction to air pollution could eventually result in COPD.

Preface

The work presented herein was part of a controlled human exposure study performed at Vancouver General Hospital in the Air Pollution Exposure Laboratory. Biological samples obtained from the study were processed in the Jack Bell Research Centre. The controlled human exposure study was designed by the principle investigator Dr. Christopher Carlsten, and approved by the Vancouver Coastal Health Research Institute and UBC Clinical Research Ethics Board (#H14-00821). The study is registered at clinicaltrails.gov with the identifier NCT02236039.

This thesis is the original intellectual product of the author, D. Wooding, who was responsible for the major concept development, data collection and analysis as it pertains to the neutrophil assays described. Dr. O. Peña was involved in the early stages of concept development. Danay Maestre-Batlle assisted with flow cytometric data collection, and Wayne Tse assisted with acquiring fluorescence microscopy images. Min Hyung Ryu was the lead on the human exposure study and was responsible for subject recruitment, planning, and patient ushering, and contributed thesis edits along with Ryan Huff. The supervisory author on this project, Dr. C. Carlsten, designed the overall human exposure study and performed the bronchoscopy procedures, which formed the basis of this work, supported concept development throughout the duration of this research, and contributed thesis edits.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	analysis of variance
ATS	American Thoracic Society
BAL	bronchoalveolar lavage
CD	cluster of differentiation
CO	carbon monoxide
CO_2	carbon dioxide
COPA	COPD Originates in Polluted Air study
COPD	chronic obstructive pulmonary disease
DALY	disability-adjusted life years
DCF	2'7'-dichlorofluorescein
DE	diesel exhaust
DEPs	diesel exhaust particles
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
FA	filtered air
FBS	fetal bovine serum
FEV_1	forced expiratory volume in one second
FEF_{25-75}	forced expiratory flow between 25% and 75% vital capacity
fMLP	N-Formylmethionine-leucyl-phenylalanine
FVC	forced vital capacity
FVS	fixable viability stain
G-CSF	granulocyte colony stimulating factor
GOLD	global initiative for chronic obstructive lung disease
GRO-α	growth-related oncogene-alpha
GTP	guanosine triphosphate
H_2DCFDA	2'7'-dichlordihydrofluorescein diacetate
H_2O_2	hydrogen peroxide
H3cit	citrullinated histone H3
HAC	heterocyclic aromatic hydrocarbons
HEPA	high-efficiency particulate air
HOCl	hypochlorous acid
ICAM	intercellular adhesion molecule
IL	interleukin
LPS	lipopolysaccharide
MFI	median fluorescence intensity
MMP	matrix metalloprotease
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NO	nitric oxide
NO_2	nitrogen dioxide
NO_x	nitrogen oxide

NPAH	nitropolyaromatic hydrocarbons
O_2^-	superoxide
PAD4	protein arginine deiminase 4
PAH	polyaromatic hydrocarbons
PBS	phosphate-buffered saline
PKC	protein kinase C
PM	particulate matter
$PM_{2.5}$	particulate matter sized 2.5 microns in diameter or less
PM_{10}	particulate matter sized 10 microns in diameter or less
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear cell
ppb	parts per billion
ppm	parts per million
rcf	relative centrifugal force
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SO_2	sulfur dioxide
TBS	tris-buffered saline
TNF	tumor necrosis factor
TRAP	traffic-related air pollution
TVOC	total volatile organic compounds
WHO	world health organization

Acknowledgements

This thesis would not have been possible without the help of many incredible people around me. I would like to thank my supervisor, Dr. Christopher Carlsten, for supporting my goals and challenging me to grow. I could not have asked for a better mentor to look up to, and you helped make my experience in graduate studies a truly enjoyable one.

To the Carlsten lab team – students, staff, and volunteers – my respect for you as colleagues is rivalled only by my appreciation for you as friends. Thank you for making this lab a happy place. I would like to extend a very special thanks to Min Hyung Ryu for mentoring me and for all of your hard work with the COPA study.

Dedication

To my family, for being a constant source of strength through all of life's challenges.

Chapter 1: Introduction

1.1 Purpose and Objectives

Ambient air pollution is responsible for 4.2 million deaths every year. A major proportion of this burden can be attributed to respiratory diseases, including chronic obstructive pulmonary disease (COPD). A growing body of evidence now suggests that air pollution contributes to COPD hospitalizations and mortality, and potentially even COPD incidence. However, the mechanisms through which air pollution exposure results in the observed negative effects are not well understood and cannot be assumed the same as those associated with cigarette smoking, given differences in constituents and in inhalation delivery. Neutrophils are consistently shown to migrate to the lungs following acute exposure to diesel exhaust (DE), a model of traffic-related air pollution (TRAP). However, the effects of DE on neutrophil functional activity are not known. Given that neutrophils are central to COPD pathophysiology, and are capable of perpetuating inflammation and damaging self-tissues through their effector mechanisms, further insight to their functional role in response to DE would add insight to the potential mechanistic link between air pollution and COPD. Controlled human exposure studies to DE, a paradigmatic model of TRAP, are helpful for identifying inflammatory pathways activated by acute exposure, in an effort to provide biological plausibility to observed epidemiological evidence. There is increasing need to understand the effects of these exposures in at-risk populations, such as the elderly, ex-smokers and COPD patients, as these individuals significantly contribute to the burden of air pollution-related disease, but have not been the focus of investigation in a controlled human air pollution exposure study to DE. The overarching aim of this thesis is to describe the effects of DE on neutrophil function, in order to better understand the connection between TRAP and respiratory disease including COPD. The three principal objectives were:

1) To measure the effects of diesel exhaust particles (DEPs) on neutrophil function *in vitro*; 2) To assess the effects of an acute human exposure to DE on the level of neutrophils in the systemic and lung compartments; and, 3) To determine whether acute human exposure to DE alters neutrophil function within the lung.

1.2 Methods

Neutrophils were isolated from peripheral blood, and stimulated *in vitro* with DEPs to quantify the neutrophil functional response, measured by changes in surface marker expression, oxidative burst, and the release of neutrophil extracellular traps (NETs). Then, a controlled human exposure study was performed, which included eight individuals aged 40-80, categorized as never-smokers, ex-smokers without COPD, or ex-smokers with mild-moderate COPD. Subjects were exposed for 2 hours to filtered air (FA) on one occasion, and on another occasion, to diluted DE (300 $\mu g/m^3$ of particulate matter sized 2.5 microns or less, $PM_{2.5}$; representative of a polluted urban city such as Beijing). Blood samples were obtained before and after (0h and 24h post-exposure) and bronchoalveolar lavage (BAL) was obtained by bronchoscopy performed 24h post-exposure. Neutrophil transit through the blood stream, migration to the lung, and activity within the lung were assessed. The effect of exposure on specified endpoints was quantified using a linear mixed effects model, with the subject as the random effect, such that each individual was compared against themselves in the FA versus DE exposures. Additionally, subject group (never-smoker, ex-smoker, or COPD) was assessed as an effect modifier, to illuminate the potential susceptibility of at-risk groups to DE exposure.

1.3 Significance

Neutrophils are consistently implicated in the human inflammatory response to DE, demonstrated by their migration to the lung following acute exposure. However, their functional role has not previously been described in this context. Neutrophil effector functions are considered a double-edged sword, as they are indispensable for immunoprotection and yet known to contribute to pathological conditions such as COPD through promotion of pro-inflammatory signaling, mucus hypersecretion, tissue damage, and remodeling in the lung. A controlled human exposure study delineating the functional role of neutrophils adds to our understanding of the adverse effects of air pollution described in epidemiological work, particularly in the context of COPD. Overall, this study addresses a current call-to-action in the community, to both improve our understanding of the relationship between air pollution and COPD and also assess the potential susceptibility of at-risk groups in this setting.

Chapter 2: Background & Literature Review

2.1. Ambient Air Pollution and COPD

2.1.1. Global burden of air pollution

Ambient air pollution is responsible for 4.2 million deaths each year, and without aggressive intervention, this number is projected to increase 50% by 2020 (1). Additionally, over 80% of the world's population live in areas that fail to meet minimum air quality standards set by the World Health Organization (WHO) (2). In terms of disability-adjusted life years (DALYs), which can be thought of as 'healthy years of life lost', air pollution is responsible for about half of DALYs related to lower respiratory tract infections and COPD, and a quarter of DALYs associated with ischemic heart disease, and cancers of the trachea, bronchus and lungs (3). Although the economic burden is challenging to quantify, it is estimated that 3.5% of health care spending was allocated to diseases caused by air pollution in high income countries in 2013, and that pollution-related disease in the United States cost $53 billion in 2015 due to loss of productivity alone (3).

2.1.2. Air pollution and COPD exacerbation

A strong relationship between airborne pollutant levels and COPD hospital admissions has been demonstrated in studies across the world, suggesting a role for pollution in the acute worsening of existing respiratory disease. For example, a European study demonstrated that a 50$\mu g/m^3$ increase in the daily mean of each of SO_2, NO_2, ozone, and particulates was associated with a relative risk for COPD admissions of 1.02-1.04 (4). A similar American study identified increased risk of COPD hospital admissions in relation to ambient NO_2, SO_2, CO, and PM levels (5). Furthermore, increases in ambient SO_2 and black smoke increased the daily number of emergency room admissions for COPD in Barcelona and Spain (6). Similarly, a study from Hong

Kong estimated an increased relative risk for COPD hospital admissions between 1.007 and 1.034 for every 10μg/m^3 increase of SO_2, NO_2, ozone and $PM_{2.5}$ (7).

2.1.3. Air pollution as a potential cause of COPD

Whether ambient air pollution plays a causal role in the development of COPD is an ongoing issue of debate, but supporting evidence has drawn considerable attention (8, 9). There is some evidence that traffic-related or ambient air pollution exposure could lead to the development of COPD, but practical challenges have made it difficult to directly assess this (10). A Swiss cohort study followed over 4,700 women across a period of 9 years and determined that a rather modest increase of 7μg/m^3 in particulate matter sized 10 microns or less in diameter (PM_{10}) was associated with more rapid FEV_1 decline, decreased FVC, and an odds ratio for COPD of 1.33 (11). Women living near a major road also had significantly reduced lung function, and 1.8-fold increased risk of COPD (11). Ambient PM_{10} concentrations have since declined, and a follow-up of this same cohort demonstrated a subsequent improvement (i.e. decreased rate of decline) in annual FEV_1, FEV_1/FVC and FEF_{25-75} (12). A cohort study from Vancouver, BC followed individuals without known COPD (based on physician diagnosis in health records) during a one-year exposure period and four-year follow-up period (13). In this study, black carbon, a component TRAP, was strongly associated with COPD hospitalization and mortality during the follow-up period (13). These data are suggestive of a relationship between TRAP and COPD incidence, with the caveat that the potential for pre-existing COPD was not tested spirometrically. Given that reductions in lung growth can lead to COPD by virtue of lowering peak FEV_1 achieved around ages 18-20, exposures affecting the lung growth phase have a potential causative role in COPD (8). Notably, both urban and traffic-related air pollution during

childhood and early adolescence adversely affect lung development, resulting in reduced FEV_1. (14-16). Specifically, living near a major road was associated with an 81 mL deficit in FEV_1 growth over a period of 8 years (14). Contrarily, other cross-sectional studies have found no association between residential proximity to road traffic, or exposure to NO_2 or NO_x, with COPD (17, 18). To summarize, the relationship between TRAP and COPD incidence has been considered likely but suggestive, in part due to the limited number of studies, and inconsistent findings, indicating a need to improve our understanding of this connection (8).

2.1.4. Occupational diesel exhaust and COPD

There is some indirect evidence that occupational DE exposure increases the risk of COPD morbidity and mortality. For example, occupations where DE exposure is likely (i.e. construction, transportation, trucking) have an odds ratio of 1.2-1.3 for COPD (19). In a study examining workers from the US railroad industry, each additional year of work at the job was associated with an 2.5% increased risk of COPD mortality (20). Another case-control study estimated participants' occupational DE exposure using a job history questionnaire (i.e. self-reported weekly exposures) as well as an industrial hygienist's assessment of the workplace, and determined an odds ratio of 1.9 for developing COPD in individuals with any DE exposure compared to individuals without DE exposure at their workplace (21). The risk was higher for never-smokers, and for those with higher estimated exposure levels (21). One limitation of these studies is that DE exposure levels were not explicitly measured, and the potential for confounding factors (i.e. other work-related traits and exposures) cannot be ruled out. For example, there is substantial evidence linking occupational dusts and fumes with COPD

morbidity (22), and although DE may be grouped into this category, it is difficult to know the precise contribution of DE in particular.

Altogether, previous research supports the conclusion that air pollution plays an important role in decreasing pulmonary function. The specific connection to COPD is limited in that relatively few studies tested spirometry to define COPD (8). One challenge is that there is currently a lack of understanding of the mechanism(s) through which ambient air pollution may lead to COPD (8). One previous study compared particle deposition in the lungs of normal subjects and individuals with varying degrees of obstruction and/or COPD, and found that COPD patients displayed a marked increase in particulate matter deposition in the lung, which may help explain their susceptibility to these exposures (23). However, improved understanding of the biological processes potentially connecting air pollution and COPD would complement epidemiology, with the aim of uncovering why certain people are more susceptible to the adverse effects of air pollution.

2.2. Diesel Exhaust

Diesel engines are widely used for both on-road and off-road equipment (24). Heavy-duty machinery in the mining, construction, and railroad industries are often equipped with diesel engines, as well as large on-road vehicles such as buses and trucks, and some personal vehicles (24). Due it its compositional complexity, it is difficult to determine exactly how much DE contributes to the broader mixture of ambient air pollutants. Relative to gasoline engines, diesel engines emit relatively low levels of CO and CO_2, but can generate over 100 times the number of particles per distance traveled (25). Therefore, DE is estimated to contribute 90-100% of PM

emissions derived from motor vehicles (26, 27). More broadly, all types of fossil fuel combustion together are estimated to account for approximately 85% of airborne $PM_{2.5}$ and nearly all pollution from oxides, sulphur and nitrogen in middle- and high- income countries (3). $PM_{2.5}$ is capable of penetrating deeply into the lung, and chronic exposure is independently associated with respiratory, cardiovascular, and all-cause mortality (28). Although the particulate fraction of DE may be the most harmful, gaseous components such as CO, NO_2, and SO_2 have gained considerable attention, and are also associated with increases in cardiopulmonary mortality (29, 30) and airway inflammation (31). TRAP is fundamentally concerning because of its ubiquity, and the fact that high levels of emissions tend to occur in areas where human activity is most prominent (32).

2.2.1. Components

DE is a highly complex, heterogeneous mixture of compounds existing in solid, liquid and gaseous fractions (Figure 2.1) (33).

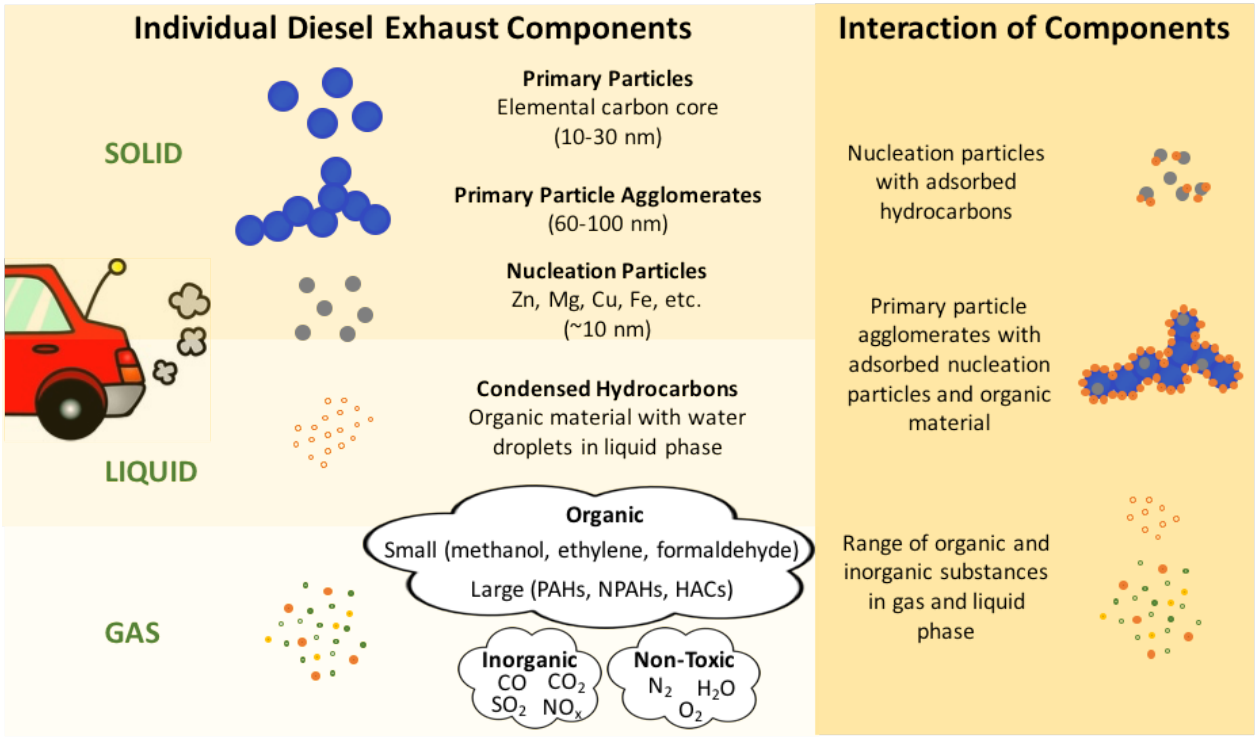

Figure 2.1. Schematic representation of major components of DE and how they interact. Diesel exhaust (DE) is composed of solid, liquid, and gaseous components which, upon emission, interact with each other to form agglomerates and newly formed compounds through atmospheric reactions. PAHs = polyaromatic hydrocarbons (PAHs); NPAHs = nitropolyaromatic hydrocarbons; HACs = heterocyclic aromatic hydrocarbons.

The solid fraction of DE is composed of particulates formed by incomplete burning of fossil fuel (34). Primary particles contain an elemental carbon core approximately 10-30nm in diameter, which can agglomerate to form larger 60-100nm particles (34). In addition to the elemental carbon present in these particles, metals and metal oxides derived from lubrication and fuel additives can form their own small particles (~10nm diameter) through nucleation processes, and can also adsorb onto the surfaces of primary particles (35).

Approximately 99% of the gas phase is composed of non-toxic gases such as nitrogen, oxygen and water vapor, with the remaining 1% consisting of a wider array of inorganic and organic

gases (36). The most notable harmful inorganic components of the gaseous fraction are NO_x, CO, CO_2 and SO_2 (37). The organic components, which include sulfur-containing compounds, consists of smaller organic molecules such as methanol, ethylene and formaldehyde, as well as larger more complex molecules such as benzene, naphthalene, pyrene, and their derivatives (commonly referred to collectively as polyaromatic hydrocarbons (PAHs), nitrated polyaromatic hydrocarbons (NPAHs) and heterocyclic aromatic compounds (HACs)) (37). These organic components can adsorb onto surfaces of primary particles, and can also condense to form particles that, in conjunction with water droplets, form the liquid fraction of DE (37). Therefore, particulate matter can be formed through two major mechanisms: directly, from incomplete burning of diesel fuel, as well indirectly through atmospheric reactions which cause gas-to-particle conversion (38).

Although elemental carbon is the most prevalent element found in the particle fraction of DE, it has limited ability to breakdown or dissolve in biological fluids such as lung-lining fluid (36). The toxic components which adsorb onto the particle surfaces (i.e. organic compounds and metals) are believed to be the major culprits in the toxicity of these particles, by chemically activating their surfaces, through the formation of quinones via partial oxidation of bound PAHs, and making them prone to participation in redox reactions within the airways (36).

2.2.2. Relevant exposure ranges

In Canada, daily averages of $PM_{2.5}$ levels in a selection of 8 cities ranges from 9.5 to 17.7 $\mu g/m^3$ (39). Nonetheless, hourly fluctuations can be large. For example, in the Lower Fraser Valley of British Columbia, daily mean PM_{10} concentrations range from 12.8 to 17.6 $\mu g/m^3$, but hourly

concentrations can exceed 200μg/m^3 (40). Particulate matter concentrations can also reach levels of 300μg/m^3 or more in the world's largest cities, during periods of heavy traffic, and in occupational settings (41-43). Therefore, acute exposure studies to DE can be titrated to levels of PM that reflect traffic-related and occupational exposures across a range of settings.

It is estimated that 897,000 Canadians are also exposed to DE at their workplace (44). Occupational exposures can be especially high in enclosed underground worksites where heavy machinery is used such as in construction or mining (24). In some instances, particulate matter exposures in the workplace can exceed 600μg/m^3 (43). Therefore, chronic occupational exposures to high levels of DE are a risk for many individuals.

2.2.3. Acute health effects

The acute health effects of DE and airborne particulate matter exposure has been examined largely through observational population studies and controlled human exposure studies (45). Slight increases in day-to-day ambient $PM_{2.5}$ measures are consistently associated with increases in hospital admissions, as well as all-cause, respiratory, and stroke-related mortality (46, 47). Relatively short-term exposures to DE have been associated with respiratory symptoms (48-51), low-grade pulmonary and peripheral inflammation (52-54), transient decreases in lung function (51, 55), and short-term cardiovascular dysfunction (56, 57).

2.2.4. Chronic health effects

Fine particulate matter, $PM_{2.5}$, can penetrate deep into to the lung and subsequently translocate into the bloodstream, causing both local and systemic effects (58). In 2015, ambient particulate

matter contributed to 69.7 million DALYs, making it the 5th leading global cause of DALYs (59). Long-term exposure to airborne particulate matter is linked to respiratory morbidity and mortality relating to asthma (60), COPD (61) and lung cancer (62), and is currently classified as a group I carcinogen by the International Agency for Research on Cancer (63). $PM_{2.5}$ is also independently related to cardiovascular mortality and cardiovascular diseases, including myocardial infarction, hypertension, and congestive heart failure (3). Chronic exposure to TRAP, in the form of living near a major road, increases the relative risk of all-cause and cardiopulmonary mortality by 1.41 and 1.95 respectively (64). Children and older adults, as well as individuals with pre-existing respiratory or cardiovascular disease, specific genetic polymorphisms, or low socio-economic status, are at increased risk of the detrimental effects of PM exposure (65).

2.3. COPD

2.3.1. Global and regional burden

COPD is a common, treatable, and preventable disease characterized by persistent respiratory symptoms and progressive airflow limitation that is not fully reversible (66). COPD is the 3rd leading cause of death worldwide (67) and contributed to an estimated 3.2 million deaths in 2015 (68). Globally, both COPD prevalence and mortality have increased in recent years (44.2% and 11.6%, respectively), though the age-adjusted rates have decreased (68). Therefore, overall population growth, and ageing of the global population, are outweighing downward trends in COPD prevalence and mortality (68). In Canada, an estimated 2.6 million individuals aged 35-79 have COPD, representing 17% of the adult population (69). Furthermore, the actual health

burden of COPD may be under-appreciated, as it also significantly increases the risk of mortality related to comorbidities such as lung cancer, ischemic heart disease, stroke and pneumonia (70).

2.3.2. Causes and risk factors

COPD is complex disease arising from a combination of genetic factors, environmental factors, and gene-environment interactions (71). COPD arises mainly from significant inhalation of noxious particles or gases which leads to respiratory symptoms and lung function decline (66). The most widely recognized risk factors for COPD are cigarette smoking and increasing age (72). Estimates show that approximately 50% of smokers may go on to develop COPD (73), and that an increase in 10 pack-years smoking history is associated with an odds ratio of approximately 1.2 for COPD (stage 2 or higher) (74). However, other exposures, including indoor and outdoor pollutants, contribute to substantial burden (See Figure 2.2 (68)). COPD burden attributable to non-smoking factors is most common in developing countries, as well as in women and younger individuals (8). These include indoor air pollution, which most commonly refers to wood and biomass burning for cooking, heating, and lighting in developing countries (75), and outdoor air pollution arising largely from fossil fuel combustion in developed, urban, and industrial areas (8). In Canada, approximately 27% of individuals with COPD are never-smokers (76). Non-smoking factors also contribute to COPD mortality, as it was estimated that pollution accounted for 51% of global COPD mortality risk in 2015 (61). Poor lung growth during childhood, often due to severe respiratory infections, can also cause COPD later in life by lowering the peak FEV_1 achieved during early adulthood, followed by a normal rate of age-related decline in lung function (77). The best known genetic factor in COPD is α-1 antitrypsin deficiency, which is present in approximately 1-3% of COPD patients (72).

This genetic deficiency increases the risk of developing COPD, and increases the severity of disease in individuals who are exposed to additional environmental factors such as cigarette smoke (78). The major biological role of α-1 antitrypsin is to inhibit the neutrophil-derived serine protease neutrophil elastase (NE), which degrades elastin as well as basement membrane and other extracellular matrix components (79). The observation that α-1 antitrypsin deficiency was associated with severe early-onset emphysema dates back to 1963, and is one of the first pieces of evidence drawing attention to the prominent role of neutrophils in COPD pathogenesis (80).

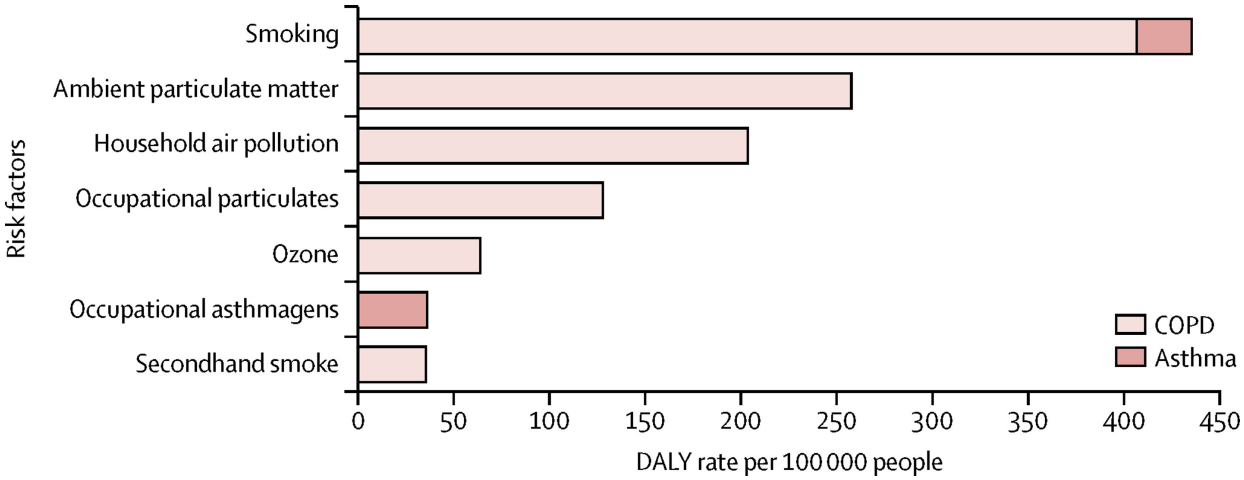

Figure 2.2. Age-standardized global disability-adjusted life years (DALYs) due to COPD and asthma attributable to seven inhalants. Figure obtained from *The Lancet Respiratory Medicine, 2017* (68).

2.3.3. Pathophysiology

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), airflow limitation in COPD is due to airway and/or alveolar abnormalities resulting from the inhalation of noxious particles and/or gases (66). Three major mechanisms contribute to the accelerated loss

of FEV_1 in COPD pathogenesis (81). The first is blocking of the small airways lumen (<2mm diameter) as a result of mucus hypersecretion and impaired mucus clearance, generally referred to as chronic obstructive bronchitis (82). The second is narrowing of the small airways due to scarring and inflammation, referred to as chronic bronchiolitis or small airways disease (83). The third is emphysematous loss of alveolar elasticity and alveolar attachments, which leads to airspace enlargement and reduces support and closure during expiration (84). COPD is widely recognized for its clinical heterogeneity and the relative contribution of these three features of COPD is highly variable across patients (66).

2.3.4. Airway abnormalities

A number of structural and functional abnormalities occur in COPD which contribute to symptoms and airflow limitation (66). Firstly, the small airways may undergo changes to their cellular composition, including increases in smooth muscle, epithelial metaplasia (replacement of columnar epithelium with squamous epithelium), and goblet cell hyperplasia (increase in mucus-secreting cells) (85). In addition, consistent exposure to irritants, inflammatory cytokines and growth factors stimulates the deposition of extracellular matrix components and leads to fibrosis of the small airways (86). Many of these changes are believed to be the result of an imbalance between proteases (i.e. matrix metalloproteases; MMPs, and NE), which degrade the lung tissue, and anti-proteases which counteract this effect (87). Aside from these changes in architecture, airway cell function is also altered in COPD. For example, mucus hypersecretion, impaired mucociliary clearance, and impaired epithelial barrier integrity contribute to the airflow limitation and susceptibility to infection in COPD. (88). Said abnormalities also reduce the airway lumen caliber, and restrict the capacity of the airways to increase their caliber during lung

inflation, which contributes to airflow obstruction to varying degrees in different COPD patients (89). Overall, the extent of airway wall thickening, fibrosis, and volume of mucous exudates are strongly associated with the degree of airflow obstruction and COPD progression (83, 90).

2.3.5. Alveolar abnormalities

Destruction of alveolar tissue characterises the emphysema component of COPD (87). In this process, alveolar walls are degraded, likely by high levels of proteases, leading to a loss of elasticity in the alveolar compartment, airspace enlargement, and loss of alveolar attachments (87). Overall, these changes reduce the elastic recoil force that normally aids in expiration, which reduces maximal expiratory flow and leads to hyperinflation of the lungs in COPD (88). Furthermore, emphysematous tissue damage reduces the area available for alveolar-capillary gas exchange, impairing the capacity of the respiratory system to remove CO_2 from, and deliver O_2 to the blood (84).

2.3.6. Role of inflammation in COPD

Noxious inhalants such a cigarette smoke and pollutants induce inflammation in the normal lung which, in certain susceptible individuals, leads to chronic inflammation characteristic of COPD (66). COPD severity is correlated with increasing airway wall thickening and accumulation of luminal mucous exudates (91), making both of these features central to disease progression. Concurrently, increases in the percentage of airways containing neutrophils, macrophages, CD4+, CD8+ and B lymphocytes, and lymphoid follicles are also correlated with disease progression (84). Therefore, the underlying mechanism for the progression of COPD appears to be closely related to infiltration of innate and adaptive immune cells, in coupling with a repair

process that thickens the airway walls and increases the volume of mucous exudates in the lumen. The specific mechanisms through which immune cells trigger structural and functional changes in the airway that lead to these characteristics are complex and not entirely understood. Some of the potential mechanistic inflammatory processes will be explored below, though the role of inflammation in COPD is an enormous topic and these examples should be considered far from exhaustive.

Accumulation of inflammatory cells contributes to the overall airway wall volume in COPD, but structural changes including epithelial metaplasia, increasing smooth muscle, and goblet cell hyperplasia, may also be influenced by inflammatory pathways in the airways (84). For example, inhaled insults such as cigarette smoke can activate resident alveolar macrophages to release transforming growth factor beta-1 (TGF-β1) which stimulates collagen deposition and is believed to contribute to small airways fibrosis (84). High levels of CD8+ T cells in the COPD airways also leads to increased release of granzyme B and perforin, which are capable of inducing apoptosis are in fact correlated with bronchial epithelial cell apoptosis in COPD (84). Proteases, released mainly by macrophages and neutrophils, are major contributors to emphysematous tissue destruction in COPD. For example, MMPs, including MMP-8, MMP-9, and NE are significantly increased in the sputum and BAL from COPD patients, and contribute to emphysematous tissue damage through degradation of collagen and elastin (84). Neutrophils in the airways also stimulate goblet cell degranulation, potentially through the release of NE and cathepsin G, and are proposed to contribute to mucus hypersecretion in this manner (84). Finally, increases in lymphoid follicles, containing B lymphocytes, dendritic cells, and T lymphocytes,

may perpetuate the inflammatory cycle in COPD, and contribute an autoimmune component through B-cell derived autoantibodies (84).

2.3.7. Symptoms and diagnosis

Chronic, progressive dyspnea is a hallmark symptom of COPD, and is a major cause of disability and anxiety associated with the disease (92). Overall, narrowing of the small airways with impaired ability to empty the lungs leads to hyperinflation, resulting in dyspnea during exertion and in more progressive disease, dyspnea at rest (81). Chronic cough that is sometimes accompanied by sputum production is also common (66). Some patients experience wheezing and chest tightness, and in more severe cases, fatigue, weight loss, anxiety and depression (93, 94). COPD may be suspected in individuals who experience the symptoms described above, and/or are subject to genetic (i.e. α_1- antitrypsin deficiency) or environmental (i.e. cigarette smoke, secondhand smoke, indoor or outdoor air pollution, occupational exposures) risk factors (66). A post-bronchodilator $FEV_1/FVC < 0.7$ (or, alternatively, such a ratio below the lower limit of normal) measured by spirometry is required to confirm this diagnosis (66).

2.3.8. Effects of smoking cessation

Smoking cessation is the most effective treatment for avoiding or reducing the progression of COPD in current smokers (95). Although smoking cessation improves respiratory symptoms and slows the rate of FEV_1 decline in individuals with and without COPD, airway inflammation generally persists in ex-smokers with COPD (95). For example, these patients continue to have elevated neutrophils in the sputum, and elevated eosinophils and IL-8 in both sputum and BAL relative to healthy controls (96). In smokers without COPD, quitting is generally more effective

in reducing airway inflammation. In other words, airway inflammation appears to be more reversible in so-called 'healthy smokers' than in COPD patients (97). However, certain features of inflammation do not completely normalize after quitting, even in this population. For example, sputum IL-8 levels are similar between smokers and ex-smokers (98), and higher than in non-smokers (99). A direct comparison of BAL in ex-smokers and smokers found that neutrophil numbers were lower in ex-smokers, but remained higher than non-smokers (100). Overall, these data are consistent with the notion that COPD patients experience limited or no inflammatory resolution after cessation, while asymptomatic smokers experience moderate but incomplete resolution (95).

2.4. Neutrophils

2.4.1. General characteristics

Neutrophils, or polymorphonuclear cells, are the most abundant leukocytes in the blood, and play a central role in the innate immune protection against infections (101). Neutrophils provide a crucial first line of cellular defense against invading pathogens, largely through release of toxic substances such as reactive oxygen species (ROS), proteases, antimicrobial peptides and NETs (102). Although they have a necessary protective role, neutrophils have also been associated with a number of chronic inflammatory conditions (103), since release of microbicidal products and high levels of ROS produced by neutrophils can cause host tissue damage (104). Recent work has demonstrated that neutrophils have other roles besides killing microorganisms, such as involvement in orchestrating and resolving the overall inflammatory response (102). In this manner, failure to terminate acute inflammatory reactions can also result in a pathogenic response that may be more damaging than protective (104).

2.4.2. Differentiation, maturation and recruitment

Neutrophils are derived from haematopoietic stem cells inside the bone marrow (101). During maturation, haematopoietic stem cells differentiate sequentially into myeloblasts, promyelocytes, myelocytes, band cells (or immature neutrophils), and finally polymorphonuclear cells (101). Concurrently, these precursors accumulate three subsets of granules containing proteases, antimicrobial proteins, adhesion molecules, and receptors, which are formed sequentially, beginning with primary granules, (i.e. azurophillic; contain NE, proteinase-3, and myeloperoxidase; MPO), followed by secondary granules (i.e. specific; contain collagenase, lactoferrin and lysozyme) and tertiary granules (i.e. gelatinase; contain gelatinase and lysozyme) (105). After the band cell stage, the nucleus undergoes segmentation, and the mature neutrophils undergo no further granule production (105). During infection, the bone marrow can be stimulated (primarily by the cytokine granulocyte colony stimulating factor; G-CSF), to increase the rate of neutrophil release and shorten the transit time within the bone marrow, in order to supply neutrophils to the circulating pool where they can migrate to the site of the insult (106).

2.4.3. Neutrophil functions

The presence of fully functional neutrophils is crucial for immunoprotection, as exemplified by increased risk and severity of infections, and often, premature mortality, in patients with neutrophil disorders such as neutropenia, chronic granulomatous disease, and leukocyte adhesion deficiency (107). However, it is equally important that neutrophil influx and clearance is carefully controlled to protect against neutrophil-mediated tissue damage (101). Neutrophils employ three primary effector mechanisms to kill microogranisms: oxidative burst, degranulation, and NETs.

2.4.3.1. Oxidative burst

When appropriately stimulated, neutrophils consume oxygen to produce large amounts of superoxide (O_2^-) in a process referred to as the oxidative (or respiratory) burst (108). The oxidative burst is produced by NADPH oxidase, a multicomponent protein complex that remains disassociated in resting cells, and is rapidly assembled at the plasma membrane when needed (109). The NADPH oxidase subunits are four oxidase-specific proteins ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$) and a GTPase (Rac1/2) (110). Given the toxicity of NADPH oxidase products, this mechanism is very tightly regulated and can be stimulated by phagocytosable particles (such as bacteria and yeast), certain chemotactic molecules (such as fMLP and IL-8), non-physiological stimuli (such as ionomycin and PMA), and bioactive lipids (109, 111, 112). The complex catalyzes the production of O_2^- from O_2, which can then dismutate to form hydrogen peroxide (H_2O_2) (113). Both O_2^- and H_2O_2 are involved in the antimicrobial system for phagocytic leukocytes, either by directly acting on microbes, or through reactions with other cell pathways to generate additional ROS (114).

2.4.3.2. Degranulation

Neutrophil granules contain a wide range of antimicrobial substances that can be delivered to the phagosome or exocytosed in a process called degranulation (115). Mature segmented neutrophils have a fourth class of protein reserves termed ‘secretory vesicles’, which contain a number of membrane-associated receptors that can be rapidly incorporated into the plasma membrane during the earliest stages of cell activation, including CD11b/CD18 (Mac-1) and CD16 (115). These molecules allow the neutrophil to establish firm contact with the endothelium, in order to extravasate from the circulation to the site of inflammation (115). Neutrophil granules and

secretory vesicles have an ordered propensity for mobilization, such that secretory vesicles are the most rapidly and most easily mobilized upon simulation, followed by tertiary granules, secondary granules, and finally primary granules (116). Certain granular contents work in concert with the NADPH oxidase; for example, MPO catalyzes the conversion of H_2O_2 from the NADPH oxidase complex, to produce a number of toxic oxidants including hypochlorous acid (HOCl) and reactive nitrogen species, which kill microorganisms through membrane attack (117). Other granular proteins, such as α-defensins, act as antimicrobial agents through formation of transmembrane pores (118), and have a secondary signaling role of attracting monocytes and T lymphocytes to the site of inflammation (119). Another major granular protein class is the serine proteases, which includes proteinase-3, cathepsin G and NE (115). These proteins exert antimicrobial activity, while they can also digest multiple components of the extracellular matrix including elastin, fibronectin and type IV collagen (115). Unrestrained neutrophil protease activity is believed to play a key role in tissue destruction in emphysema (120).

2.4.3.3. Neutrophil extracellular traps

The release of NETs was first described as a novel form of cell death termed 'NETosis', which was distinct from apoptosis or necrosis (121). During NETosis, neutrophils release webs of decondensed chromatin decorated with granule proteins and histones (122). These webs are capable of trapping and killing pathogens (123), but dysregulated levels have also been associated with tissue damage (124) and disease (125-128). Neutrophils can be stimulated to release NETs with various stimuli including ionomycin, PMA, IL-8, LPS, and TNF-α (122). Upon stimulation, the normally lobulated nucleus begins to diffuse, until the chromatin becomes

fully decondensed, interacts with cytoplasmic contents, and is eventually ejected from the cell in a web-like structure, studded with histones and granular proteins such as NE and MPO (123). The exact mechanistic events leading to NET formation remain to be fully elucidated, but more than one mechanism appears to exist and may be dependent on the stimulus and/or disease state (129, 130). For example, upon stimulation with the protein kinase C (PKC) activator phorbol myristate acetate (PMA), NET formation is dependent on the Raf-MEK-ERK pathway, and in this mechanism, NADPH oxidase assembly is an essential event (131). Contrarily, other studies have shown that NETs stimulated by ionomycin or uric acid do not require NADPH oxidase (129, 132). Another unclear mechanistic feature is the role of histone citrullination. The calcium-dependent protein, PAD4, facilitates chromatin decondensation by converting positively charged arginine residues to neutrally charged citrulline residues on histones (133). This process of histone citrullination has been referred to as a necessary step in NET formation (134). However, a more recent study determined that histone citrullination is a feature of ionomycin-induced, but not PMA-induced NET formation (133). Since then, *in vivo* NETs which lack citrullinated histones have been described in the context of COPD (130). Interestingly, the presence of citrullinated histones may have functional consequences, since the positive charge on histones is believed to assist in the bactericidal activity of histones in NETs (130). Therefore, histone citrullination during NET formation may assist in chromatin decondensation, but could also result in deficiencies in bacterial killing activity of histones (135).

2.5. Neutrophils in COPD

Neutrophilic inflammation is a key feature of COPD (66). Increased number of neutrophils are consistently found in the sputum (96, 136-138) and airway lavage fluid (139, 140) of COPD

patients, both during stable and exacerbated COPD. Neutrophils also have increased activation and functional activities in COPD. For example, neutrophils from COPD patients have increased neutrophil oxidative burst activity and Mac-1 (CD11b) expression compared to smokers without COPD (141). COPD patients have increased amounts of NETs in the airways, which correlates with airflow limitation (125, 130, 142). The amount of granule protein NE in the BAL is positively correlated with increasing COPD severity (143). NE is also believed to contribute to emphysematous tissue damage (144), stimulate mucus hypersecretion (145), and reduce ciliary beat frequency (146). Overall, there is a strong involvement for neutrophils in COPD, which is demonstrated by alterations in pulmonary neutrophil number and function in this population.

2.6. Neutrophils and Air Pollution

A number of controlled human exposure crossover studies have shown that neutrophils migrate to the lungs following an acute air pollution exposure. A summary of the neutrophilic effects (i.e. number of neutrophils, proportion of neutrophils, and neutrophil chemotactic signaling) from controlled acute human exposure crossover studies to DE, DEPs, or ambient particulate matter can be found in Table 2.1.

Table 2.1. The effect of DE on neutrophils from previous controlled human exposure crossover studies.

Participants	Exposure (Type, Dose, Duration)	Sample Timing	Effect
15 healthy	DE (300μg/m^3 PM_{10}), 1h	6h-post	Increased neutrophil number in bronchial epithelium, bronchial submucosa, blood and bronchial wash (52).
15 healthy	DE (300μg/m^3 PM_{10}), 1h	6h-post	Increased IL-8 transcription in bronchial tissue and BW, increased IL-8 and GRO-α expression in the bronchial epithelium (147).
15 healthy	DE (100μg/m^3 PM_{10}), 2h	18h-post	Increased neutrophil numbers, IL-8 and MPO concentration in bronchial wash (53).
25 healthy, 15 mild asthmatics	DE (100μg/m^3 PM_{10}), 2h	6h-post	Increased proportion of neutrophils and increased IL-8 in bronchial wash in healthy subjects (54).
15 healthy	DE (300μg/m^3 PM_{10}), 1h	6h- and 24h-post	Increased proportion of sputum neutrophils 6h post-exposure (148).
26 healthy	DE (300μg/m^3 $PM_{2.5}$), 2h	30h-post	No change in proportion of sputum neutrophils (149).
10 healthy	DEPs (200μg/m^3 PM_{10}), 2h	4h- and 24h-post	Increased proportion of sputum neutrophils and MPO 4h post-exposure (150).

Overall, these studies clearly and consistently demonstrate that neutrophils are involved in the inflammatory response to DE. However, the functional role of neutrophils in this response has never been investigated. The observed increases in MPO secreted in the sputum and bronchial wash (BW) from the studies above suggests that neutrophils may be activated by DE. However, the source of MPO in these studies cannot be determined, and although it is primarily derived from neutrophils, smaller quantities can also be found in monocytes and macrophages (151). Despite the fact that neutrophil activation has never been comprehensively studied in the context of DE, IL-8 and GRO-α, which are upregulated after acute DE exposure, have the ability to

increase neutrophil functional activity (152). Therefore, further investigation into the functional role of neutrophils in response to DE would improve our mechanistic understanding of how DE exposure leads to negative effects in the lung, as is observed epidemiologically.

2.7. Rationale

High levels of TRAP are associated with increased risk of developing COPD (11, 17, 153), and daily fluctuations in pollutant levels correspond with COPD hospitalization rates (5, 154, 155). However, the mechanism by which TRAP could potentially lead to the development or worsening of COPD is not fully understood. Controlled human exposure studies allow us to assess physiological changes induced by controlled, short-term exposures, which can lend insight to the potential mechanisms through which longer or repeated exposures may result in the observed epidemiological outcomes (156). Although controlled human exposure studies have consistently identified neutrophilic recruitment and the release of inflammatory mediators as a potential mechanism for their deleterious effects (52-54, 147, 150), further investigation into the functional role of neutrophils is needed to understand how they could potentially contribute to disease. Furthermore, no previous controlled human DE exposure study has included at-risk groups such as ex-smokers or COPD patients to identify the potential susceptibility of these individuals. Previous studies have demonstrated that increased neutrophil activity correlates with poor clinical outcomes in the context of COPD (125, 143), as well as results in emphysematous tissue damage (144), mucus hypersecretion (145, 157) and reduced ciliary activity (146). Altogether, these studies indicate increased activity of neutrophils could be a potential driving mechanism of DE-induced damage in the lung, that is exacerbated in patients with COPD.

Therefore, the aim of this thesis is to assess the following hypotheses, using *in vitro* and *in vivo* methodologies described in the next chapter.

2.8. Hypothesis and Objectives

2.8.1. Hypothesis

DEPs directly induce neutrophil activation *in vitro*. Acute human exposure to DE stimulates the release of neutrophils into the bloodstream, increases neutrophil recruitment to the lung, and enhances the functional activity of neutrophils in the lung.

2.8.2. Objective 1: To measure the effects of DEPs on neutrophil function *in vitro*

In this thesis, I first assessed whether DEPs could induce neutrophil activity in a controlled experimental setting. To achieve this, I isolated peripheral blood neutrophils and stimulated them with DEPs before measuring changes in the expression of functional surface activation markers, ROS production, and NET release.

2.8.3. Objective 2: To assess the effects of an acute human exposure to DE on neutrophil transit from bone marrow to the lung

There is an existing understanding that neutrophils appear in the lung following acute DE exposure, but the course of neutrophil transit beginning from release from the bone marrow, into the circulation, and eventually to the lung has never been evaluated in this context. I examined blood and BAL samples from an acute human exposure study to DE, which included never-smokers, ex-smokers, and COPD patients, to assess bone marrow stimulation (measuring

peripheral band cells as a surrogate), and neutrophil migration through the bloodstream to the lung. Additionally, I examined the susceptibility of the three subject groups to these effects.

2.8.4. Objective 3: To determine whether acute human exposure to DE alters neutrophil functional activity in the lung

I evaluated three features of neutrophil function in the BAL of individuals exposed to an acute bout of DE: 1) the expression of surface activation markers, 2) ROS production, and 3) release of NETs. Similar to Objective 2, I examined the susceptibility of the three subject groups to these effects.

Chapter 3: Methods

3.1. Determining the effects of diesel exhaust particles on peripheral neutrophil function *in vitro*

3.1.1. Peripheral blood neutrophil isolation

Peripheral blood was obtained from four consenting male volunteers (two healthy, and two mild asthmatics) with 9mL heparin tubes (Vacuette Heparin Tubes, Greiner Bio-One, Catalog #455051) and immediately placed on ice. Neutrophils were isolated using a magnetic bead-based negative selection kit (EasySep Direct Human Neutrophil Isolation Kit, StemCell, Catalog #19666) according to manufacturer's protocol. Briefly, 7-9 mL of blood was incubated with the isolation cocktail (containing monoclonal antibodies recognizing non-neutrophil surface markers, as well as an Fc receptor blocking antibody) and magnetic particles, for 5 min in a 50mL falcon tube. The sample was topped up with EasySep Buffer (StemCell, Catalog #20144) and placed into a magnet (The Big Easy EasySep Magnet, StemCell, Catalog #18001) for 5 min. The enriched sample was transferred to a new tube before adding fresh magnetic particles, incubating for 5 min, and placing into the magnet for a second 5-min separation. The sample was then transferred to a new tube once again, and immediately placed back in the magnet for a third 5-min separation. Isolated neutrophils were centrifuged at 300 rcf for 7 mins, then resuspended in cold phenol red-free RPMI supplemented with 2% fetal bovine serum (FBS). Cells were counted using a hemocytometer and 50,000 cells were spun at 2500 rcf for 5 mins onto a cytospin slide, fixed with methanol, and stained with eosin and methylene blue (Millipore Harleco Hemacolor Stain Set, Catalog #65044-93). The cells were then assessed for purity under a light microscope (Olympus BX53M).

3.1.2. Diesel exhaust particle preparation

DEPs were obtained from the diesel engine exhaust outlet of the Air Pollution Exposure Laboratory at Vancouver General Hospital. For the *in vitro* assays, DEPs were weighed, suspended in culture media and sonicated for 2 minutes at 60% power (Microson Ultrasonic Cell Disrupter, Heat Systems Ultrasonics Inc.) prior to incubation to improve dispersion of agglomerated particles in the suspension. Dilutions were prepared such that the final concentration of DEPs after adding to cells was 100μg/mL. This concentration was based on the higher range of previous *in vitro* experiments with DEPs, which used between 10-100μg/mL (158, 159).

3.1.3. Neutrophil activation flow cytometry assay

$1x10^6$ isolated neutrophils were stimulated for 30min in Eppendorf tubes with 20nM phorbol myristate acetate (PMA; positive control), media alone (negative control), or 100μg/mL DEPs at a final concentration of $1x10^6$ cells/mL. Cells were centrifuged at 300 rcf and resuspended in media ($1x10^6$ cells/mL) before staining 100,000 cells for flow cytometric targets CD66b, activated CD11b, and viability dye (Table 3.1) for 20 minutes. Cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, Catalog #P5368), pelleted and resuspended in PBS before recording 60,000 events on a flow cytometer (BD FACSCanto II, BD Biosciences). For this assay, and all flow cytometry-based assays within this thesis, compensation beads incubated with the same antibodies as the assay were used to calculate compensation for the experiment (ThermoFisher Scientific OneComp eBeads, Catalog #01-1111-42). One exception is the NETs flow cytometric assay (Section 3.1.5), in which ionomycin-stimulated single-stained cells were used instead of beads, in order to include a compensation control for the DAPI

channel. Compensation in the viability dye channel was calculated with an FVS660-stained sample containing 50% heat-killed cells (1 min at 65°C + 1 min on ice) and 50% fresh cells, to produce a positive and negative peak. Fluorescence minus one (FMO) controls were performed once for each experiment, and used to set the location of the gates. A comparison between a heat-killed sample (which stains positive for viability dye) and normal sample was used to set the location of the gate in the viability channel.

Table 3.1. Antibodies and fluorescent dyes used to assess *in vitro* neutrophil activation

Target	Fluorochrome	Ex/Em	Clone	Isotype	Product Info	Final Concentration
CD66b	PE	496/578	Monoclonal, G10F5	Mouse IgM	BD Biosciences, Cat #561650	1:25
CD11b (Activated)	BV421	407/421	Monoclonal, CBRM1/5	Mouse IgG	BD Biosciences, Cat #566313	1:25
Viability Dye	FVS660	649/660	-	-	BD Biosciences, Cat #564405	1:4000

The CD11b antibody used in this study was a monoclonal antibody recognizing an epitope of the activation-specific form of CD11b, which is expressed only after a signal from a simulus such as a chemoattractant. This activated form makes up approximately 10% of total CD11b.

3.1.4. Neutrophil ROS production assay

The effect of DEPs on neutrophil ROS production was assessed with a widely-used ROS indicator 2'7'-dicholordihydrofluorescein diacetate (H_2DCFDA) (113, 160). This non-fluorescent probe freely permeates cells, and once inside, is cleaved by intracellular esterases to produce the cell-impermeable product, H_2DCF. Upon subsequent oxidation, it is converted to the bright fluorescent compound 2'7'-dicholorofluorescein (DCF) and detectable with 492-495nm excitation and 517-527nm emission. H_2DCFDA is a generalized assay for ROS production, as it does not specifically measure any one compound, but rather, a range of oxidative reactions. Isolated peripheral blood neutrophils were incubated with 10μM H_2DCFDA for 15 minutes in an incubator at 5% CO_2 and 37°C. Under the same conditions, cells were stimulated for 45 min with

4μM of the calcium ionophore ionomycin (positive control), media alone (negative control), or 100μg/mL DEPs, for a final concentration of $1x10^6$ cells/mL. Viability dye was added for the final 20 minutes of incubation. Cells were centrifuged at 300 rcf and 4°C for 5 min, placed on ice, and resuspended in cold PBS before recording 60,000 events on the flow cytometer.

3.1.5. NETs flow cytometry assay

NETs were identified using a previously described flow cytometric assay (161), whereby NET-releasing neutrophils were defined as cells staining triple-positive for three hallmark, extracellular components of NETs: DNA, MPO and citrullinated histones (H3cit) (Table 3.2). $1x10^6$ isolated peripheral blood neutrophils were stimulated for 2h with 4μM ionomycin (positive control), media alone (negative control), 100μg/mL DEPs, or a combination of 4μM ionomycin + 100μg/mL DEPs. Following stimulation, cells were pelleted and resuspended in fresh RPMI media. 50,000 cells from each condition were spun onto a cytospin slide and stained with eosin and methylene blue for qualitative visualization. 400,000 cells from each condition were stained for flow cytometric quantitative analysis of NETs. Without a permeabilization step, anti-MPO, anti-H3cit, and DAPI stain were added for 15 minutes. Cells were washed with PBS, pelleted, and resuspended, before adding the secondary antibody for H3cit for 15 minutes. A final PBS wash was performed prior to collecting 60,000 events on the flow cytometer.

Table 3.2. Antibodies and fluorescent dyes used to assess *in vitro* NETs with flow cytometry.

Target	Fluorochrome	Ex/Em	Clone	Isotype	Product Info	Final Concentration
MPO	FITC	495/519	Monoclonal, 2C7	Mouse IgG	Abcam Cat, #11729	1:25
H3cit	-	-	Polyclonal	Rabbit IgG	Abcam Cat, #5103	1:350
Rabbit IgG	APC	650/660	Polyclonal	Goat IgG	Jackson ImmunoResearch Cat, #111-136-144	1:500
DNA	DAPI	358/461	NA	NA	BD Biosciences, Cat #564405	1:2000

MPO = myeloperoxidase, H3cit = citrullinated histone H3, Rabbit IgG = secondary antibody for H3cit.

3.1.6. NETs immunocytochemistry assay

120,000 isolated peripheral blood neutrophils were placed on a sterile, round, glass coverslip for 1h in an incubator (5% CO_2 and 37°C) to allow adherence onto the coverglass. Adherence was visually assessed using a light microscope, and excess liquid was aspirated off before stimulating with 4μM ionomycin (positive control), media (negative control), or 100μg/mL DEPs for 2h in an incubator (5% CO_2 and 37°C). Slides were transferred to a drop of 4% paraformaldehyde on a parafilm-covered rack for 30 min (Figure 3.1). Cells were washed with Tris-Buffered Saline (TBS, made with Fisher BioReagents NaCl, Catalog #BP358313 and Tris Base, Catalog #BP152-500), permeabilized for 20 min (ThermoFisher Fix & Perm Cell Permeabilization Kit, Catalog #GAS003) and blocked for 30 min (ThermoFisher BlockAid Blocking Solution, Catalog #B10710). Cells were then incubated with primary antibodies for NE and citrullinated histone (H3cit) overnight (Table 3.3). The following day, cells were washed and transferred to secondary antibodies for 1h, and stained with Hoechst 33342 DNA stain, prior to washing and mounting on glass slides with antifade mountant (ProLong Diamond Antifade Mountant, ThermoFisher,

Catalog #P36970). Cells were visualized with confocal microscopy (Zeiss LSM780 laser scanning confocal microscope).

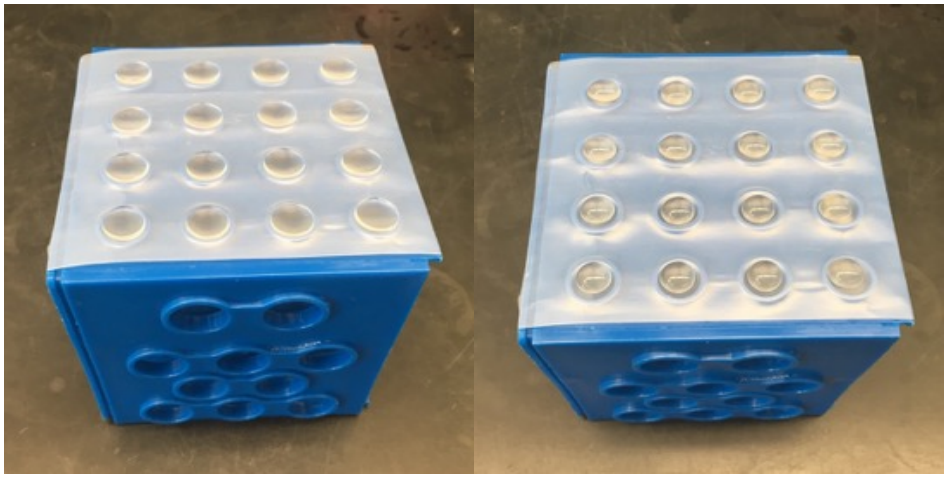

Figure 3.1. Immunocytochemistry staining performed on parafilm-covered rack. 12mm round glass coverslips (left) were transferred onto fresh 200ul drops of reagents (right) during the staining procedure to eliminate aspiration steps potentially resulting in NETs DNA loss.

Table 3.3. Antibodies and fluorescent dyes used to assess *in vitro* NETs with microscopy.

Target	Fluorochrome	Ex/Em	Clone	Isotype	Product Info	Final Concentration
NE	-	-	Monoclonal, NP57	Mouse IgG	Dako, Cat #M0752	1:100
H3cit	-	-	Polyclonal	Rabbit IgG	Abcam, Cat #5103	1:300
Rabbit IgG	Alexa555	555/565	Polyclonal	Goat IgG	Invitrogen, Cat. #A21428	1:2000
Mouse IgG	Alexa488	495/519	Oligoclonal	Goat IgG	Invitrogen, Cat. #A28175	1:500
DNA	Hoechst 33342	350/461	-	-	BD Biosciences, Cat. #561908	1:5000

NE = neutrophil elastase, H3cit = citrullinated histone H3, Rabbit IgG = secondary antibody for H3cit, Mouse IgG = secondary antibody for NE

3.1.7. Statistical analyses

The mean of each group was compared using a one-way repeated measures ANOVA, followed by Tukey's post-hoc test in GraphPad Prism 6 (GraphPad Software Inc.), and p-values <0.05

were considered statistically significant. A summary of assays and endpoints can be found in Table 3.4.

Table 3.4. Overview of *in vitro* experiments.

Assay	Endpoints
Neutrophil activation (flow cytometry)	CD66b and CD11b median fluorescence intensity
Neutrophil ROS production (H_2DCFDA)	DCF median fluorescence intensity
NETs (flow cytometry)	% Neutrophils staining triple positive for NETs markers (DAPI, MPO, H3cit)
NETs (immnocytochemistry)	Qualitative assessment of NETs morphology and co-localization of DNA, NE and H3cit

H2DCFDA = 2', 7'-dicholorodihydrofluorescein diacetate (ROS indicator); DCF = 2'7'-dichlorofluorescein (oxidized ROS indicator); DEPs = diesel exhaust particles, ROS = reactive oxygen species production; NETs = neutrophil extracellular traps; DAPI = 4',6-diamidino-2-phenylindole (DNA stain); MPO = myeloperoxidase, NE = neutrophil elastase; H3cit = H3 citrullinated histone.

3.2. Determining the effects of diesel exhaust exposure on neutrophil release, migration, and function *in vivo*

3.2.1. COPD Originates in Polluted Air (COPA) human exposure study design

This study was approved by the ethics review boards at the University of British Columbia and the Vancouver Coastal Health Research Institute, and the clinical trial was registered at clinicaltrials.gov (Identifier: NCT02236039). Written informed consent was obtained from each subject. In total, eight subjects from the COPA study were included in this thesis and all human exposure study procedures were performed between September, 2017 and April, 2018 at the Air Pollution Exposure Laboratory at Vancouver General Hospital. This facility is led by Dr. Christopher Carlsten, and is equipped to perform acute, controlled human exposures to DE (162). Participants underwent one telephone and one in-person screening. Research participants were exposed to DE for 2 hours on one occasion, at a dose representative of large urban cities or

occupational exposures (300µg/m^3), and FA on another occasion in a randomized crossover design controlled exposure protocol. By definition of the randomized crossover design, this has the advantage of virtually eliminating potential individual confounding factors, and increasing the power of a given sample size relative to a traditional parallel-group design (163). An overview of the study design can be found in Figure 3.2. The order of exposures was randomized and balanced, and exposures were separated by a minimum 4-week washout period to minimize the potential for carryover effects.

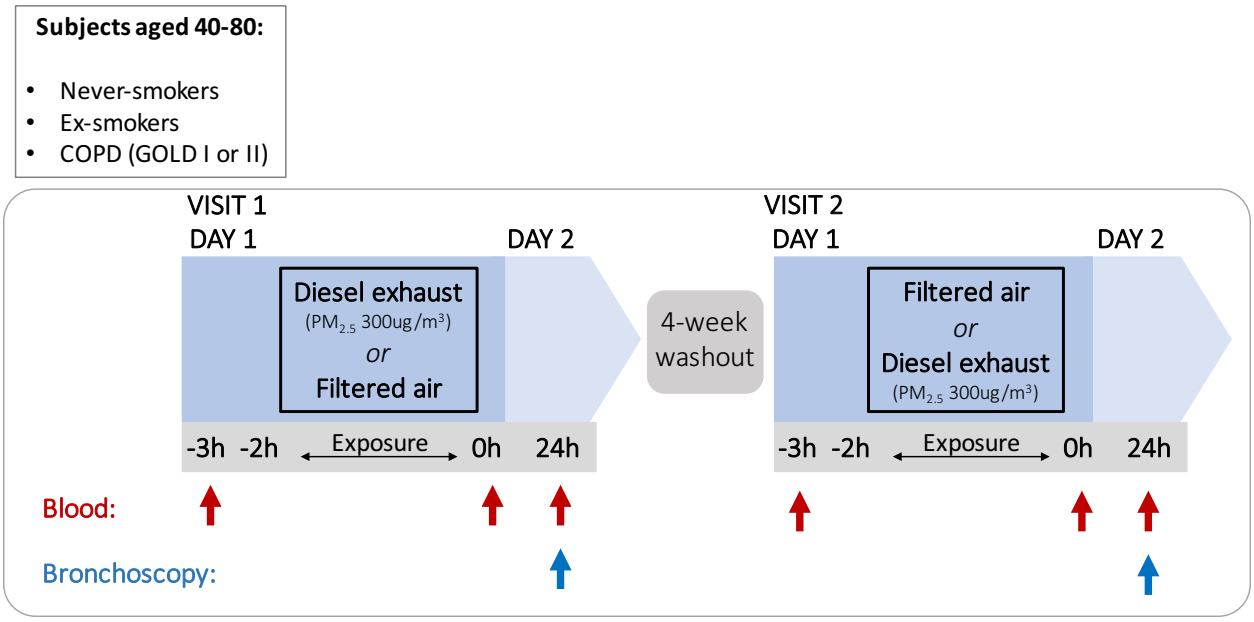

Figure 3.2. COPA study design. The COPA study followed a randomized, double-blinded controlled human exposure crossover design. Participants visited the lab for two 2-day visits, separated by a minimum 4-week washout period. Individuals underwent a 2h exposure to diesel exhaust (DE; 300µg/m^3 $PM_{2.5}$) on one occasion, and filtered air on the other. Blood samples were collected before and after exposures, and bronchoalveolar lavage (BAL) was obtained during a bronchoscopy procedure 24h after exposure. Blood and BAL samples were used to assess the effects of DE exposure on neutrophil transit through the circulation to the lungs, and functional activity in the lung.

Participants visited the lab on two separate occasions for a 2-day study procedure. On the first day, subjects completed the common cold questionnaire prior to beginning study procedures, and visits were rescheduled if there were signs of infection. Baseline blood samples were obtained

before beginning the 2h exposure to DE (300μg/m^3 $PM_{2.5}$) or FA. Twenty minutes into each hour of the exposures, participants performed 15 minutes of light exercise (30% VO_2-max, determined during an exercise test at secondary screening) on a stationary bicycle to increase ventilation. Blood was collected again immediately after exposure. On the second day (24h post-exposure), blood samples were collected prior to a bronchoscopy procedure, performed by the primary investigator Dr. Christopher Carlsten, to obtain BAL.

3.2.2. Subjects

Subjects aged 40-80 were recruited through local flyers, newspaper advertisements and clinical referrals in accordance with the University of British Columbia Research Ethics Board. After a telephone screening, individuals from three groups were included: (1) never-smokers, (2) ex-smokers with >10 pack-years smoking history (quit >6 months prior to enrollment and FEV_1 % predicted >80%), or (3) mild-moderate COPD (quit smoking >6 months prior, FEV_1 % predicted ≥50%). Individuals were excluded based on the following criteria: (1) pregnant/breastfeeding, (2) current smoker, or quit smoking <6 months prior, or smoking history 0-10 pack-years, (3) asthma diagnosis, (4) inhaled corticosteroid use, (5) regular bronchodilator use, (6) significant co-morbidities judged by principal investigator to increase cardiovascular risk.

At a secondary in-person screening visit, study procedures were described in full detail, and participants were given ample opportunity to ask questions prior to providing written informed consent. Lung function testing was performed in accordance with American Thoracic Society (ATS) standards (164) to confirm the presence of airflow limitation in the COPD group, defined as FEV_1/FVC <0.7. Prior to exposures, individuals were asked to abstain from bronchodilator

medications (48h), acetylsalicylic acid (5d), caffeine (12h), and nutritional supplements (i.e. vitamins, due to their antioxidant properties) (48h).

3.2.3. Diesel exhaust and filtered air exposures

DE and FA exposures were 2h in duration and performed at the Air Pollution Exposure Laboratory at Vancouver General Hospital. DE was generated using a previously described exposure system (162). Briefly, an Environmental Protection Agency (EPA) Tier 3-compliant 6.0kW diesel engine was operated under a constant 2.5kW load using standard ultra-low sulfur diesel and a two-stage dilution system. The exhaust was first diluted 9:1 with compressed air, then further diluted 25:1 with high-efficiency particulate air (HEPA)-filtered air. Exhaust was then aged for approximately 4 minutes prior to entering the 4x6x7-foot exposure booth. This concentration was selected to mimic particulate matter concentrations representative of a smoggy day in a polluted urban city such as Beijing, or in a busy urban sub-location such as a bus terminal or high-traffic road (162). This level is also considered representative of occupational DE exposures, such as near highway toll booths, railroads, or heavy machinery such as at construction sites (24). For FA exposures (control condition), facility room air was fanned into the delivery system, passed through a HEPA-filter, then into the exposure chamber. Levels of the gaseous and particulate components in the exposure chamber were monitored in real-time for safety and characterization, and can be found in the results (Section 4.2.2).

3.2.4. Laboratory methods

3.2.4.1. Blood sample collection

Peripheral venous blood was collected into EDTA tubes and gently inverted to prevent coagulation. Tubes were kept cold and blood was processed immediately.

3.2.4.2. Blood smears

Peripheral blood band cells were counted on blood smears as a surrogate marker of bone marrow stimulation. A minimum of two blood smears was prepared for each time point. Blood was allowed to dry for 30 minutes prior to fixing in ethanol and staining with eosin and methylene blue (Millipore Harleco Hemacolor Stain Set, Catalog #65044-93). Slides were air dried overnight and cover slipped with Permount Mounting Medium (Fisher Chemical, Catalog #SP15-100). A minimum of 500 leucocytes were counted for each time point (Baseline, 0h, 24h) by one observer who was blinded to the exposure conditions. Cells were identified as eosinophils, segmented neutrophils, band cells, or other cells. There are multiple criteria for the definition of band cells, but the current study employed the College of American Pathologists definition for consistency with previous air pollution literature (165). Under this definition, band cells are distinguished from segmented neutrophils by the lack of clearly segmented nucleus, such that there must be complete separation of nuclear lobes with a thread-like strand connecting them to classify as a segmented neutrophil (166).

3.2.4.3. Blood neutrophil activation flow cytometry assay

To address whether DE exposure alters blood neutrophil activation in a human model, 50μL of whole blood was stained for neutrophil lineage and activation markers CD45, CD16, CD66b,

activated CD11b, and CD182 as well as viability for 25 minutes at 4°C (Table 3.5). Red blood cells were lysed for 7 minutes at 4°C (BD Facs Lysing Solution, BD Biosciences, Catalog #349202), then cells were pelleted and resuspended in PBS prior to recording 100,000 flow cytometric events. Live neutrophils were gated based on expression of CD45+/CD16+/CD66b+ (167) and exclusion of the viability dye. Surface expression of CD16, CD66b, CD11b and CD182 was quantified in terms of MFI.

Table 3.5. Antibodies and fluorescent dyes used to assess neutrophil activation in blood and BAL from the human exposure study.

Target	Fluorochrome	Ex/Em	Clone	Isotype	Product Info	Final Concentration
CD45	APC-Cy7	650/785	Monoclonal, 2D1	Mouse IgG	BD Biosciences, Cat #557833	1:20
CD16	PE-Cy7	496/785	Monoclonal, CB16	Mouse IgG	ThermoFisher, Cat #25-0168-41	1:20
CD11b (Activated)	BV421	407/421	Monoclonal, CBRM1/5	Mouse IgG	BD Biosciences, Cat #566313	1:25
CD66b	APC	650/660	Monoclonal, G10F5	Mouse IgM	ThermoFisher, Cat #17-0666-41	1:25
CD182	BV510	405/510	Monoclonal, 6C6	Mouse IgG	BD Biosciences, Cat #744196	1:25
Viability Dye	LIVE/DEAD Red Stain	595/615	-	-	ThermoFisher, Cat #L23102	1:25

The CD11b antibody used in this study was a monoclonal antibody recognizing an epitope of the activation-specific form of CD11b, which is expressed only after a signal from a simulus such as a chemoattractant. This activated form makes up approximately 10% of total CD11b.

3.2.4.4. BAL sample collection

BAL was obtained from 2x50mL or 2x60mL saline instillations into the upper lobe, which was performed on opposite lungs, and with consistent volume, in the two bronchoscopies for each subject. BAL was placed on ice, and immediately transported to the lab for further processing. Samples were poured through a 40μm mesh filter to remove debris, then spun at 475 rcf at 4°C

for 15 minutes. Cells were resuspended in phenol red-free RPMI and counted using a hemocytometer.

3.2.4.5. BAL cytospin preparation

75,000 cells were spun onto cytospin slides, allowed to dry for 30 minutes, then fixed in ethanol and stained with eosin and methylene blue (Millipore Harleco Hemacolor Stain Set, Catalog #65044-93). Slides were air dried overnight then cover slipped the following morning with Permount Mounting Medium (Fisher Chemical, Catalog #SP15-100).

3.2.4.6. BAL neutrophil activation flow cytometry assay

200,000 cells were stained for 25 minutes in the dark at 4°C for flow cytometry, with an identical panel as was used in the blood (section 3.2.4.3). After staining, cells were pelleted and resuspended in PBS before recording 100,000 events.

3.2.4.7. BAL neutrophil ROS production assay

200,000 cells were incubated with antibodies against neutrophil surface markers (CD45, CD66b, CD16) for 15 minutes prior to adding H_2DCFDA ROS indicator dye for 20 minutes at 5% CO_2 and 37°C (Table 3.6). Cells were then stimulated with 4μM ionomycin, a positive control known to induce ROS production (from previous literature (168) as well as from own *in vitro* work presented in this thesis), or media (negative control), and incubated for 45 minutes, with viability dye (eBioscience Fixable Viability Dye eFluor450; Catalog# 65-0863-14) being added for the last 20 minutes. Cells were pelleted and resuspended in PBS prior to immediately collecting 60,000 events using flow cytometry.

Table 3.6. Antibodies and fluorescent dyes used to assess neutrophil ROS in BAL from the human exposure study.

Target	Fluorochrome	Ex/Em	Clone	Isotype	Product Info	Final Concentration
CD45	APC-Cy7	650/785	Monoclonal, 2D1	Mouse IgG	BD Biosciences, Cat #557833	1:20
CD16	PE-Cy7	496/785	Monoclonal, CB16	Mouse IgG	ThermoFisher, Cat #25-0168-41	1:20
CD66b	APC	650/660	Monoclonal, G10F5	Mouse IgM	ThermoFisher, Cat #17-0666-41	1:25
Viability Dye	eFluor450	405/450	-	-	ThermoFisher, Cat #65-0842-85	1:500
H_2 DCFDA	DCF	593/522	-	-	ThermoFisher, Cat #D399	10μM

3.2.4.8. BAL neutrophil extracellular traps assay

150,000 cells were seeded onto autoclaved 12mm uncoated round glass coverslips for 1h at 5% CO_2 and 37°C. Cell adherence was visually assessed using light microscopy prior to fixing for 30min with 4% paraformaldehyde. Cells were stained over two days using an identical protocol to the *in vitro* NETs immunocytochemistry assay (Section 3.1.6), with fluorescent stains for H3cit, NE and DNA (described previously in Table 3.3). At least one unstained specimen, and one stained only with secondary antibodies, was prepared whenever there were sufficient BAL returns and cell numbers. Images were acquired using an EVOS FL Auto Imaging system (ThermoFisher Scientific). Between one and three large photos of each specimen, in each of the three channels, was acquired by stitching 16 photos at 60X magnification in a 4x4 grid (approximately 360,000 μm^2).

The percentage of DNA-covered area associated with NETs was quantified in ImageJ on the best quality photo for each subject visit, by a blinded observer. To do so, NETs structures were defined by areas meeting all three of the following criteria (adapted from Grabcanovic-Musija et

al. (125)): 1) clear extracellular fibrous strands; 2) positive DNA staining; 3) positive NE staining. Although we stained for H3cit in the same manner as the *in vitro* flow cytometry-based experiment, it was not used as defining criteria since NETs from the lungs of COPD patients do not always contain citrullinated histones (130). The quantity of NETs in each image was assessed in terms of area, normalized to total nuclear area on the slide, which is drawn from previous work using this method to quantify NETs in COPD sputum (125). First, the area covered by NETs matching the criteria above was traced with an outline in the overlay image (Hoechst 33342, NE, H3cit) and quantified. Then, total nuclear area of the image was quantified in the Hoechst 33342 channel image. To do so, fluorescence images were converted to binary (black and white) using the color threshold tool in ImageJ software, and the nuclear area was quantified automatically using the particle analysis tool. The traced NETs area divided by the total nuclear area was used to determine the percentage of DNA area associated with NETs.

3.2.5. Statistical analyses

The effect of exposure on the endpoints of interest, described below (Table 3.7), was assessed in R Studio software version 1.1.383. To account for the crossover design of the COPA study, linear mixed effects models were employed using the 'nlme' package (version 3.1-131) with the random effect of subject ID, and three possible combinations of fixed effects. In the first model, the sole fixed effect of exposure was used to test the primary hypotheses for the effect of DE on our outcomes. In the second model, the interaction between the fixed effects of patient group (e.g. never-smoker, ex-smoker, COPD) and exposure was used to assess whether the effect of exposure was modified by patient group. In the third model, the interaction between the fixed

effects of exposure and order (i.e. exposure to FA first vs. DE first) was performed to assess the potential for carryover effects. A p-value <0.05 was considered statistically significant.

Table 3.7. Overview of *in vivo* experiments.

Assay	Endpoints
Proportion circulating band cells (Blood smears)	% Band cells in venous blood
Blood neutrophil activation (Flow cytometry)	CD66b, CD11b , CD16 and CD182 Median fluorescence intensity
BAL neutrophil counts (Cytospins)	% Neutrophils in BAL
BAL neutrophil activation (Flow cytometry)	CD66b, CD11b, CD16 and CD182 Median fluorescence intensity
BAL neutrophil ROS production (H_2 DCFDA)	Baseline BAL neutrophil DCF mfi and Ionomycin-induced change in BAL neutrophil DCF mfi
BAL NET formation (Immnocytochemistry)	Assessment of NETs in BAL, defined using categorical criteria to assess DNA, NE and H3cit fluorescence staining

BAL = bronchoalveolar lavage; ROS = reactive oxygen species; H2DCFDA = 2', 7'-dicholorodihydrofluorescein diacetate (ROS indicator); DCF = 2'7'-dichlorofluorescein (oxidized ROS indicator); NETs = neutrophil extracellular traps; NE = neutrophil elastase; H3cit = citrullinated histone H3

3.2.6. Power calculation

Power was calculated based on a previous controlled human exposure crossover study (52), with DE-induced neutrophilia in the bronchial epithelium as the outcome. Based on the mean change and estimated standard deviation in this study, and an alpha cutoff of 0.05, a minimum sample size of 13 would be required to achieve 80% power. However, due to time constraints, only eight subjects were able to complete the study for inclusion in this thesis. Therefore, results from these eight will be presented herein.

3.2.7. Data completion

Due to optimization of lab techniques, data collection for some assays began after the first subject had already completed the study. Furthermore, BAL assays were limited to the number of cells obtained in each bronchoscopy. Therefore, the number of subjects whose data is included for each assay is outlined here: Blood smears for band cell counts (n=7), blood flow cytometry panel (n=8), BAL cytospins for cell differentials (n=7), BAL flow cytometry (n=8), BAL ROS assay (n=5), BAL NETs assay (n=7).

Chapter 4: Results

4.1. Effect of DEPs on neutrophil function *in vitro*

4.1.1. Neutrophil isolation

The first objective of this thesis is to assess the effects of DEPs on neutrophil functions *in vitro.* Neutrophil isolation from the blood, performed using negative magnetic selection, took approximately 40 minutes and yielded neutrophils with 93.8 ± 2.6% purity (assessed by counting 300 cells per cytospin slide, Figure 4.1). 94.5 ± 3.9% of cells remained viable after isolation, as determined by flow cytometry (LIVE/DEAD Fixable Red Stain, ThermoFisher Scientific). Given the fact that neutrophils have a short lifespan (169), and can undergo activities such as ROS production during cell death (170), properly assessing neutrophil viability in this experiment was important. Therefore, the viability dye was tested prior to these experiments on heat-killed cells, and was confirmed to positively stain dead cells.

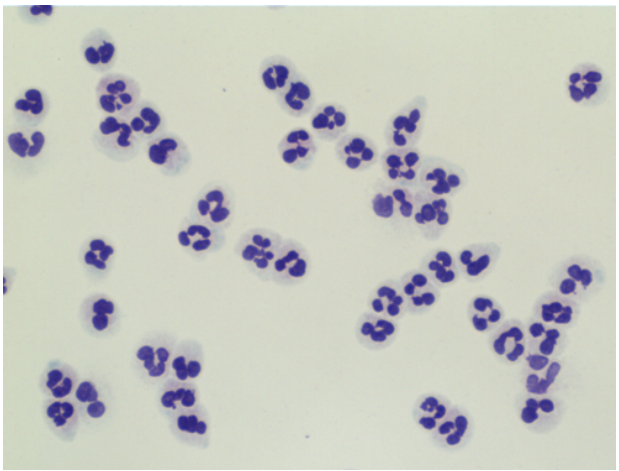

Figure 4.1. Peripheral blood neutrophils after isolation. Cells were spun onto a slide and stained with eosin and methylene blue. Neutrophils were isolated from whole blood using a magnetic negative selection kit obtaining neutrophils with 93.8 ± 2.6% purity and 94.5 ± 3.9% viability (mean ± SD).

4.4.2. DEPs increased CD66b expression with no effect on CD11b

It was hypothesized that incubating DEPs with isolated neutrophils would increase the expression of activation markers CD66b and CD11b. All results herein are expressed as mean change (95%CI) in relation to the unstimulated sample, unless otherwise stated. The expression of CD66b, and an activated form of CD11b (a subunit of Mac-1, or CD11b/CD18 complex), on neutrophils was assessed after excluding contaminating lymphocytes, debris, and doublets, based on light scattering properties, and dead cells based on exclusion of the viability dye in live cells (See Figure A.1 for gating strategy). Figure 4.2 shows that in accordance with our hypothesis, the positive control (PMA, which is known to increase expression of both CD66b and CD11b) (171, 172), and DEPs significantly increased CD66b MFI +93 (43 to 143, $p<0.01$) and +65 (15 to 115, $p<0.05$), respectively. Contrary to our hypothesis, only PMA increased CD11b MFI +61 (6 to 116, $p<0.05$) while DEPs did not +24 (-80 to 31, $p>0.05$).

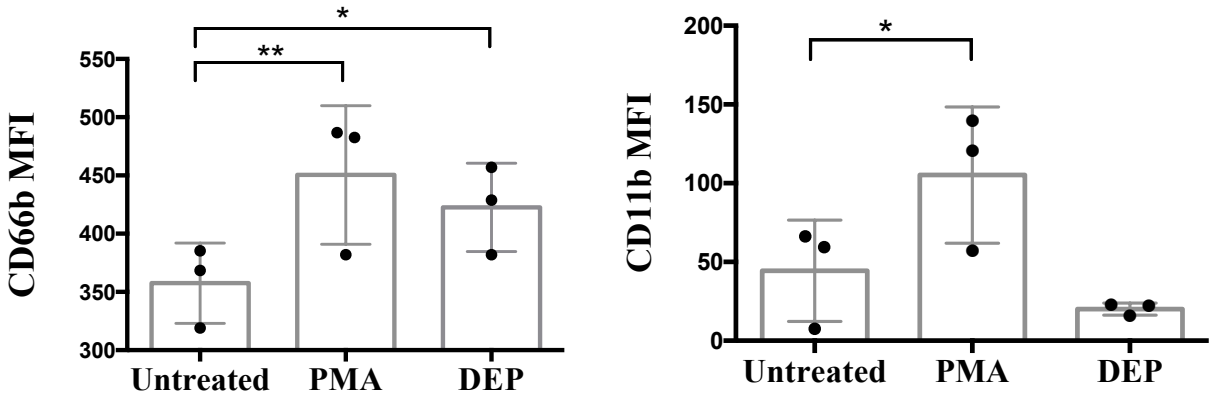

Figure 4.2. Effect of DEPs on neutrophil surface marker expression. Isolated peripheral blood neutrophils were stimulated for 30min with media alone (untreated), 20nM phorbol myristate acetate (PMA; positive control), or 100μg/mL diesel exhaust particles (DEP), then assessed for surface activation marker expression by flow cytometry. Bars and error bars represent mean and SD, respectively. MFI = median fluorescence intensity. Effect assessed using RM-ANOVA with Tukey's post-test. Significance expressed as * = $p<0.05$, ** = $p<0.01$.

4.4.3. DEPs increased neutrophil ROS production

To address the hypothesis that DEPs induce intracellular ROS production, neutrophils were incubated with the colourless H_2DCFDA indicator, which fluoresces upon oxidation by NADPH oxidase and other ROS products, to form DCF. Therefore, increased DCF fluorescence in this experiment (assessed by flow cytometry) indicates increased intracellular ROS. A representative flow cytometric gating strategy, and a fluorescence intensity plot of the DCF indicator, can be found in Figure 4.3. Using the same strategy as described for the activation panel above, live, singlet neutrophils were gated based on light scattering properties and exclusion of dead cells using a viability dye. DCF MFI in the live neutrophil population was assessed. In support of our hypothesis, both ionomycin (the positive control, known to stimulate the NADPH oxidase complex) and DEPs both significantly increased DCF MFI +473 (77 to 869, $p<0.05$) and +670 (274 to 1066, $p<0.01$), respectively (Figure 4.4). No statistical differences were observed between DEP and ionomycin-induced DCF fluorescence. Overall these data are consistent with our hypothesis, indicating the DEPs induce ROS production.

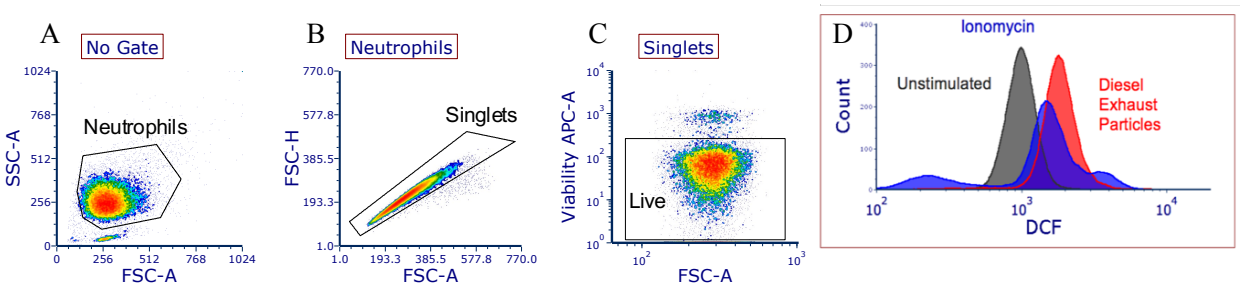

Figure 4.3. Representative gating strategy for *in vitro* assessment of the effects of diesel exhaust particles on neutrophil ROS production. First, neutrophils were gated based on light scattering properties (panel A), then singlets were selected (panel B), and dead cells were excluded (panel C). Median fluorescence intensity of the oxidized probe (DCF) was assessed after stimulation (panel D; gray = unstimulated, blue = 45 min stimulation with 4µM ionomycin, red = 45 min stimulation with 100µg/mL diesel exhaust particles).

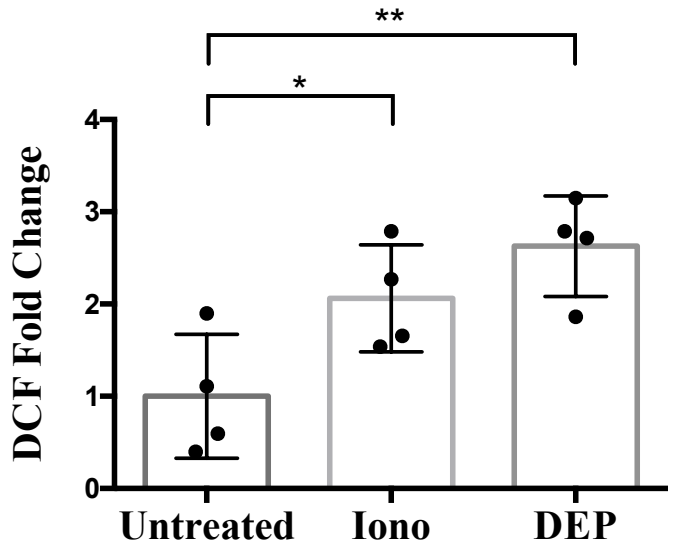

Figure 4.4. Effect of DEPs on neutrophil oxidative burst. 45 min stimulation of isolated peripheral blood neutrophils with media alone (untreated), 4μM ionomycin (Iono) and 100μg/mL diesel exhaust particles (DEP) on reactive oxygen species production, determined by the median fluorescence intensity of intracellular DCF (the fluorescent, oxidized form of the normally colorless ROS indicator H_2DCFDA). Bars and error bars represent mean fold change and SD, respectively. Unstim = unstimulated, DEP = diesel exhaust particles, significance expressed as * = $p<0.05$, ** = $p<0.01$.

4.4.4. DEPs induced NET release and enhanced ionomycin-induced NET release

The potential for DEPs to induce NET release from isolated blood neutrophils was assessed using a previously published flow cytometric assay (161). This assay identifies NETs as events staining triple-positive for DNA, MPO and citrullinated histones (a histone modification proposed to facilitate chromatin decondensation during NETosis). Importantly, there was no permeabilization step performed, so positive staining indicates extracellular presence of these components. Contaminating lymphocytes and debris were excluded based on light scattering properties, and the percentage of neutrophils forming NETs was quantified (See Figure A.2 for gating strategy and Figure A.3 for FMO controls). Consistent with previously published results (129, 161), the percentage of NET-forming neutrophils was significantly increased 25% (1 to 50%, $p<0.05$) after 2h stimulation with 4μM ionomycin. Interestingly, in accordance with our

hypothesis, stimulation of neutrophils with 100μg/mL DEPs also significantly increased NET-forming neutrophils by 36% (12 to 61%, $p<0.01$) relative to unstimulated cells (Figure 4.5). The combination of ionomycin and DEPs together increased NET release by 51% (27 to 75%, $p<0.001$) relative to unstimulated, and by 26% (1.5 to 50%, $p<0.05$) relative to ionomycin alone. Therefore, DEPs not only induced NET formation, but also enhanced the NETs response to the positive control, indicating that DEPs and ionomycin may act through unique pathways.

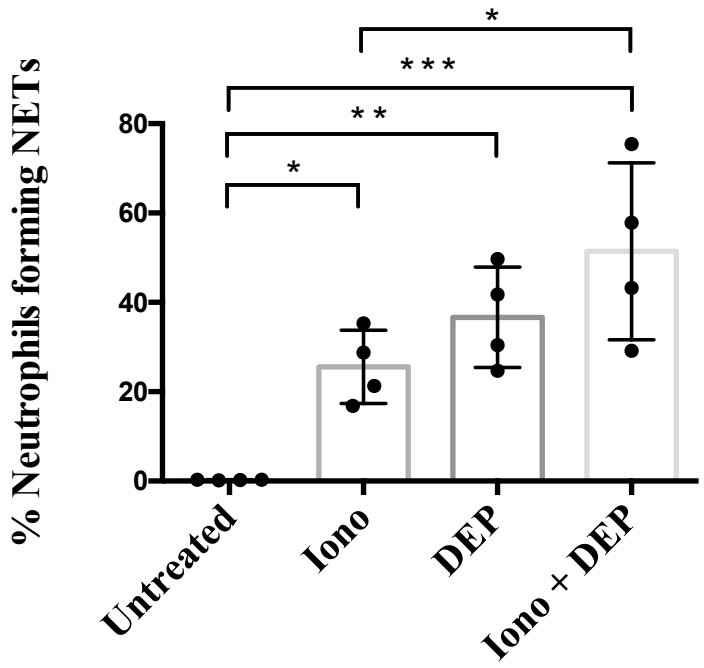

Figure 4.5. Effect of DEPs on NET formation. Isolated neutrophils were stimulated for 2h with media alone (untreated), 4μM ionomycin (Iono), 100μg/mL diesel exhaust particles (DEP), or a combination of ionomycin and DEP, and NET formation was quantified using flow cytometry. Results are expressed as the percentage of neutrophils staining triple positive for DAPI, citrullinated histone, and myeloperoxidase. Bars and error bars represent mean and SD, respectively. Unstim = unstimulated, DEP = diesel exhaust particles, Iono = ionomycin. Differences assessed by repeated measures ANOVA with Tukey's post-test; significance expressed as * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

To further confirm NET formation following stimulation with DEPs, NETs were also qualitatively assessed using fluorescence microscopy in two samples. NETs can be identified by their morphology (protrusion of long web-like DNA strands) and their protein contents (173). Histones may become hyper-citrullinated during NET formation, which removes the positive charge on the histone surfaces, and facilitates chromatin decondensation (173). Therefore, the presence of citrullinated histones, as well as other NET-associated proteins such as MPO or NE, in web-like structures serve as a specific means of identify NETs (174). Qualitative assessment of the effects of DEPs on NET formation can be seen in cytospin slide images acquired using brightfield microscopy (Figure 4.6) and immunocytochemistry images obtained by confocal microscopy (Figure 4.7).

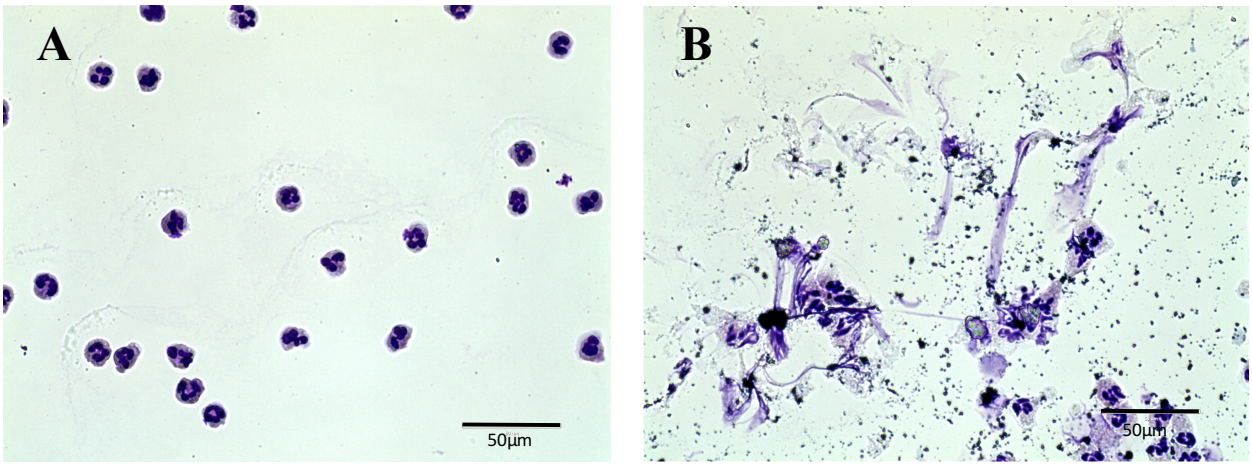

Figure 4.6. Brightfield images of DEP-induced NETs. Effect of 2h incubation of isolated peripheral blood neutrophils with media alone (panel A) or 100µg/mL diesel exhaust particles (panel B) on NET formation, assessed by morphology. 50,000 cells were spun onto slides prior to fixing with ethanol and staining with eosin and methylene blue.

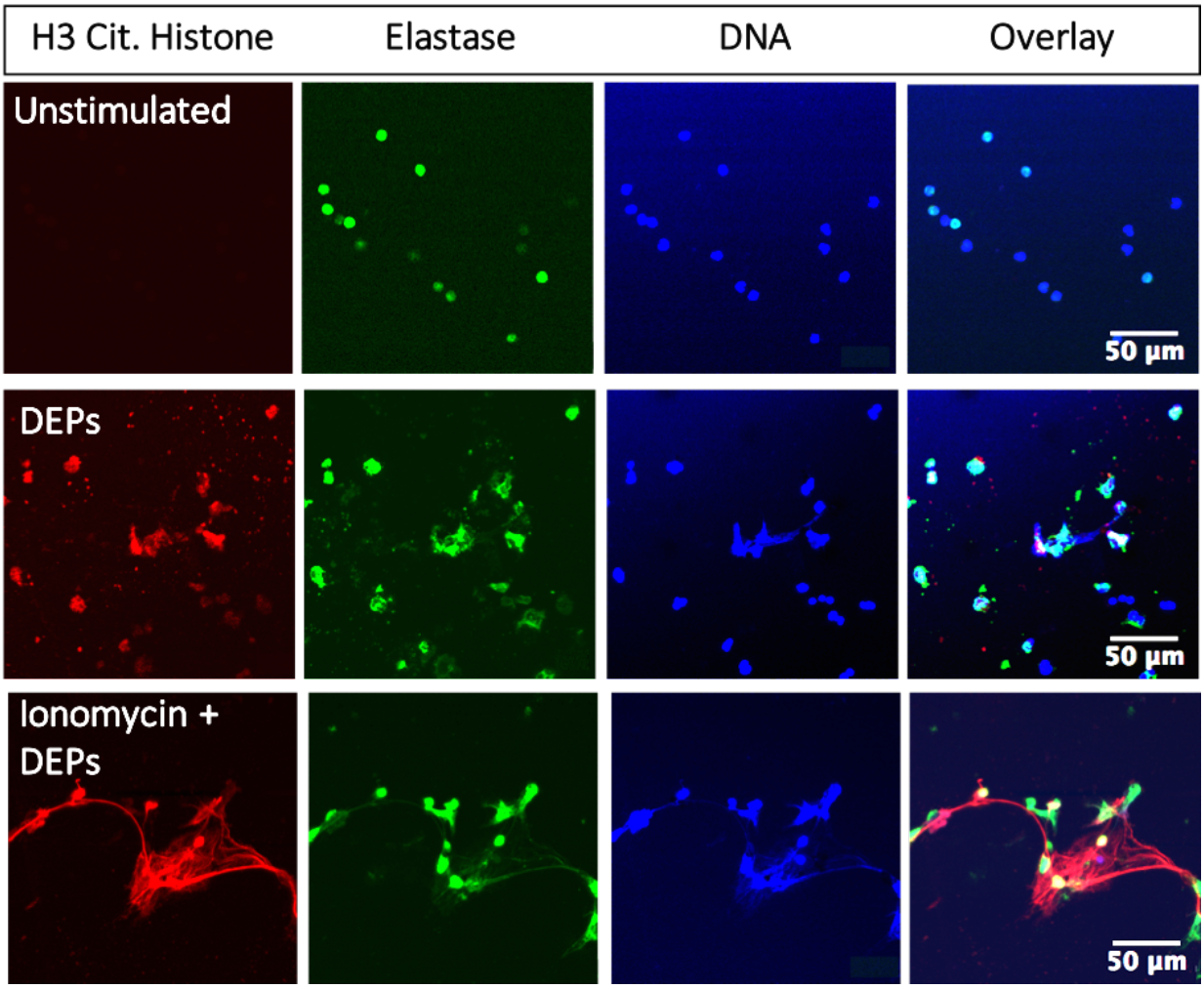

Figure 4.7. Confocal fluorescence images of DEP-induced NETs. Qualitative assessment of neutrophil extracellular traps after 2h stimulation with 100µg/mL DEPs alone, or DEPs in combination with 4µM ionomycin. Colocalization of these three stains in web-like morphologic structure is indicative of NETs. H3 Cit. Histone = H3 citrullinated histone, DEPs = diesel exhaust particles.

It should be noted that, although ultrafine PM is reportedly capable of translocating from the airways to the bloodstream and other organs (175, 176) this concentration of DEPs is likely higher than what would appear in the blood following an inhaled human exposure. However, these experiments served to optimize laboratory methods prior to the *in vivo* study, and to do a preliminary assessment of the direct effects of DEPs on isolated neutrophils in a controlled setting.

4.2. Effect of acute human exposure to DE on neutrophils *in vivo*

Objectives 2 and 3 of this thesis are to assess the potential mechanistic role of neutrophils in the inflammatory response to DE, in terms of their release and migration in the blood, recruitment to the lung, and functional activity. These effects were assessed using a controlled human exposure crossover design, with blood and BAL samples.

4.2.1. Subject characteristics

Eight subjects completed the study (2 never-smokers, 2 ex-smokers, 4 COPD; 6 males; age 68±7; mean±SD). FEV_1/FVC for participants with COPD was below the fixed ratio of 0.7 as well as the lower limits of normal (177). Characteristics are found in Table 4.1.

Table 4.1. Subject characteristics

						post-bronchodilator		
Subject	**Group**	**Sex**	**Age (yrs)**	**Smoking History (Pack-yrs)**	**Time since Quitting (yrs)**	**FEV_1 (L)**	**FEV_1 % Predicted**	**FEV_1/FVC**
1	Never-Smoker	M	63	0	-	3.93	127	0.78
2	Never-Smoker	M	58	0	-	4.46	134	0.75
3	Ex-smoker	M	73	30	42	3.00	109	0.91
4	Ex-smoker	M	70	90	19	2.52	93	0.72
5	COPD	M	80	10	30	2.33	98	0.54
6	COPD	F	66	24	16	1.87	86	0.54
7	COPD	F	70	12	33	1.79	88	0.48
8	COPD	M	67	53	11	2.62	77	0.49

FEV_1 = Forced expiratory volumne in one second. FVC = Forced vital capacity. Pack-yrs = packs/day times number of years.

4.2.2. Exposure characteristics

Levels of pollutants within the exposure booth were monitored during FA and DE exposures for characterization (Table 4.2).

Table 4.2. Exposure characteristics

	$PM_{2.5}$ ($\mu g/m^3$)	CO_2 (ppm)	CO (ppm)	NO_2 (ppb)	NO (ppb)	NO_x (ppb)	TVOC (ppb)
DE	279 ± 19	1169 ± 50	19 ± 1	67 ± 17	2716 ± 424	2782 ± 420	2002 ± 351
FA	3 ± 2	563 ± 49	7 ± 1	9 ± 6	152 ± 78	162 ± 83	124 ± 32

Values are expressed as mean ± SD. DE = diesel exhaust; FA = filtered air; $PM_{2.5}$ = particulate matter sized 2.5 microns or less in diameter; CO_2 = carbon dioxide; CO = carbon monoxide; NO_2 = nitrogen dioxide; NO = nitric oxide; NO_x = nitrogen oxide; TVOC = total volatile organic compounds; ppm = parts per million; ppb = parts per billion.

4.2.3. Assessment of carryover effect

There was no significant effect of order on any outcomes ($p>0.05$ for effect modification by exposure order). For the results described below, the main effect of exposure will be discussed for all outcomes regardless of whether the result was significant, while the interaction of patient group with exposure is only discussed where it is statistically significant. Results are expressed in parentheses as follows: (mean change relative to FA, 95%CI, p-value), unless otherwise stated.

4.2.4. DE immediately reduced the proportion of circulating band cells

Band cells are neutrophil precursors, and can thus be used as a marker of bone marrow stimulation. We investigated the effect of DE on band cell counts in blood smears, due to the fact that band cell sequestration in the lung (i.e. reduced proportions in blood) is connected to

microvascular damage (178, 179), while bone marrow stimulation (i.e. increased proportions in the blood) is observed following exposure to other types of particulate pollutants (165, 180, 181). In this section, immature neutrophils lacking a clearly segmented nucleus are referred to as 'band cells', while neutrophils with a clearly segmented nucleus (polymorphonuclear cells) are referred to as 'PMNs', and the collection of both are referred to as 'neutrophils'. Baseline cell counts were subtracted from the post-exposure counts for each individual (to obtain delta values at 0h and 24h) prior to mixed effects analysis.

Band cells were quantified as a percentage of neutrophils as well as a percentage of total leucocytes, since bone marrow stimulation leads to the release of both mature and immature (band) neutrophils into the bloodstream (179). DE significantly reduced the proportion of circulating band cells at 0h when expressed as a percentage of neutrophils (-5.2%, -3.5 to -6.9%, $p = 0.001$) and as a percentage of total leucocytes (-2.9%, -1.7 to -4.2%, $p = 0.004$) (Figure 4.8), while there was no significant change in the percentage of circulating neutrophils at 0h. When expressed as a percentage of total leukocytes, the effect of DE was significantly modified by patient status, such that COPD patients saw a greater reduction than never-smokers (estimate for COPD + DE vs. never-smoker + DE = -3.9%, $p = 0.03$). There was a similar trend in the ex-smoker group, though it did not reach statistical significance (estimate for ex-smoker + DE vs. never-smoker + DE = -2.8%, $p = 0.11$). There was no effect of exposure on the proportion of band cells at 24h. The percentage of neutrophils (band + PMN) tended to decrease 24h after DE, but this effect was not statistically significant (-6.4%, -13.4 to 0.5%, $p=0.082$). Overall, the immediate effects are consistent with previous literature, which has demonstrated band cell disappearance from the blood in response to an inhaled insult, and is postulated to be the result of

sequestration in the lung microvasculature (178, 179). In the current study, the DE-induced reduction in band cells was exaggerated in COPD patients. Contrary to our hypothesis, there was no indication of bone marrow stimulation at 24h.

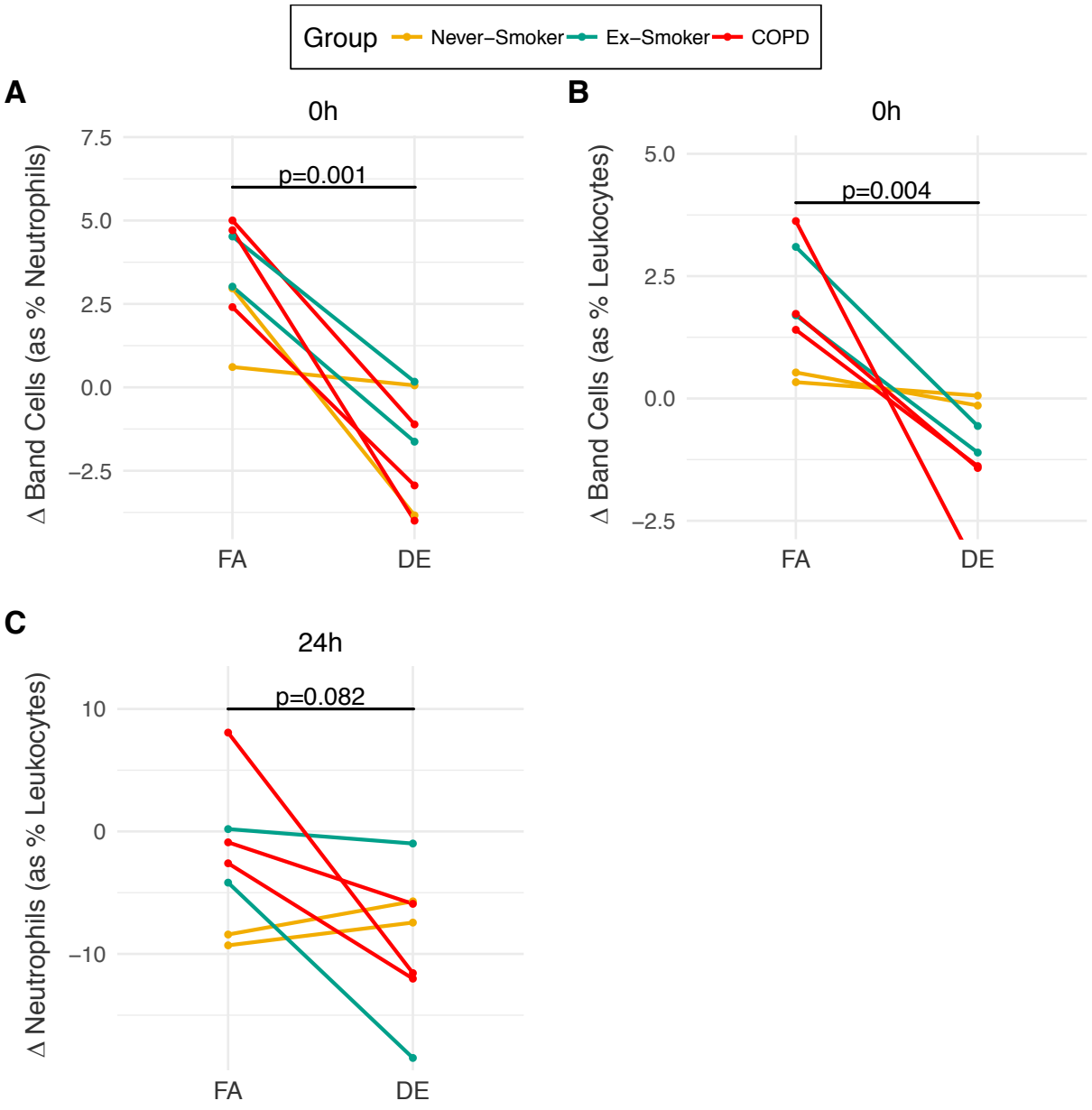

Figure 4.8. Effect of DE exposure on band cells. The proportion of circulating band cells after a 2h exposure to diesel exhaust (DE) was determined from blood smear differential cell counts. Each point represents the change from pre-exposure to post-exposure for one subject. Subjects are connected by a line. FA = filtered air.

4.2.5. DE differentially altered CD182 expression in patient groups

Live neutrophils were gated on based on positive staining for CD45, CD16 and CD66b, and exclusion of the viability dye (See Figure A.4 for viability dye test, Figure A.5 for gating strategy, and Figure A.6 for FMO controls). There was no main effect of exposure on the surface expression of activation markers (Table 4.3). However, 24h after exposure, patient status was an effect modifier for the effect of DE exposure on CD182 expression, such that the DE-induced change in CD182 expression was more positive in individuals with COPD relative to never-smokers (estimate for COPD + DE vs. never-smoker + DE = +80.87, p=0.038) (Figure 4.9). The effect in ex-smokers appeared intermediate, though it was not significantly different from either of the other groups.

Table 4.3. Summary of blood neutrophil activation from human exposure study.

Outcome	Time	FA Mean (MFI)	DE Mean (MFI)	DE Effect Mean (95% CI)	p-value
CD16	0h	-34.9	-41.6	-6.7 (-183.2 to 169.8)	0.936
CD66b	0h	17.4	-16.4	-33.8 (-73.2 to 5.8)	0.101
CD11b	0h	7.4	-3.9	-11.3 (-29.1 to 6.4)	0.252
CD182	0h	-17.60	-9.7	7.9 (-52.7 to 68.6)	0.807
CD16	24h	39.9	90.9	51 (-67.1 to 169.0)	0.371
CD66b	24h	15.9	0.9	-15 (-47.7 to 17.8)	0.345
CD11b	24h	1.9	-0.5	-2.4 (-13.6 to 8.8)	0.646
CD182	24h	-2.70	15	17.7 (-23.5 to 58.8)	0.374

Time post-exposure is indicated. Values displayed are baseline-subtracted (i.e. -3h timepoint). MFI = median fluorescence intensity, FA = filtered air, DE = diesel exhaust.

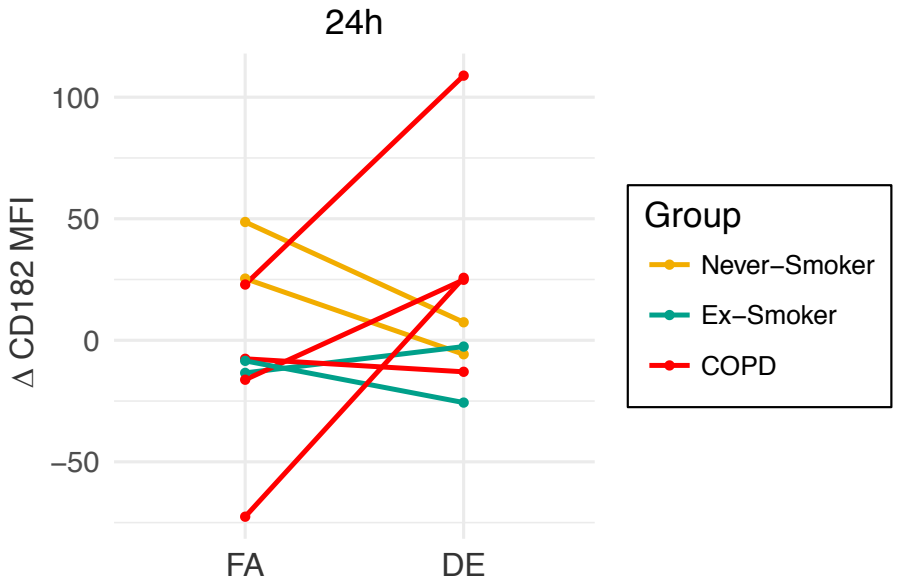

Figure 4.9. Effect of DE exposure on CD182 expression in peripheral blood neutrophils. The effect of diesel exhaust (DE) exposure on peripheral blood neutrophil CD182 expression was significantly modified by subject group, such that COPD subjects saw an increase while never-smokers saw a decrease. Each point represents the change in CD182 expression across the exposure (ie. 24h minus baseline) for one subject. Individual subjects are connected by a line. FA = filtered air; MFI = median fluorescence intensity.

4.2.6. DE exposure led to lymphocytic inflammation in BAL

To assess neutrophil migration to the lung, BAL cytospin slides were prepared. Differential cell counts were performed by two independent observers who were blinded to the exposure conditions. Each observer counted a minimum of 500 cells, and identified them based on their morphological and staining characteristics as macrophages/monocytes, neutrophils, eosinophils, basophils, lymphocytes or epithelial cells. Inter-observer count differences averaged less than 3% for each outcome (Figure 4.10). A summary of the effect of DE on BAL cell counts can be found in Table 4.4. Contrary to the hypothesis, there was no effect of exposure on the percentage of neutrophils in the BAL. However, DE caused an increase in the percentage of lymphocytes

(9.19%, 1.38 to 17.00%, p=0.036), and a decrease in the percentage of monocytes/macrophages (-12.65%, -23.74 to -3.71%, p=0.021) in the BAL.

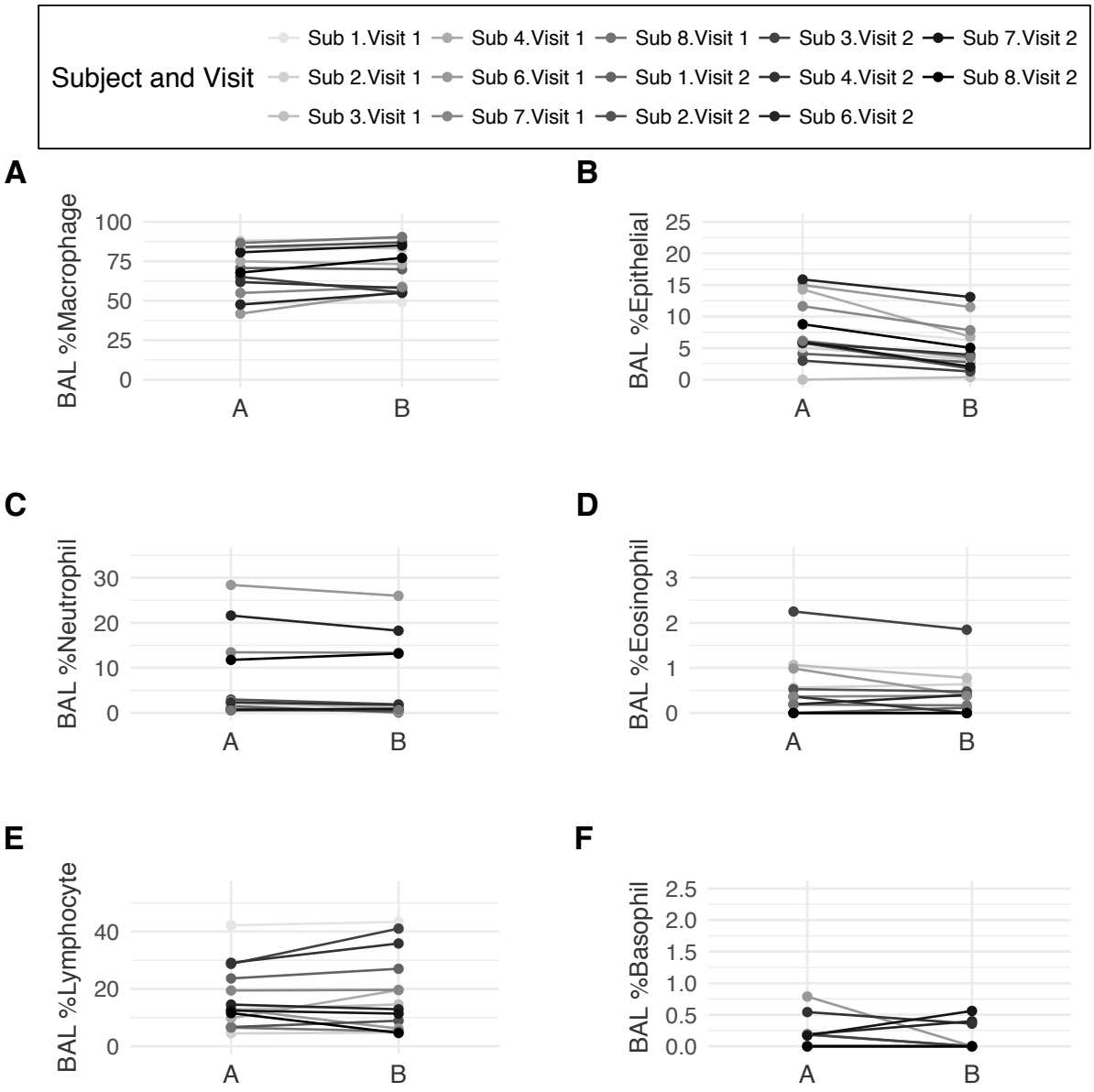

Figure 4.10. Observer agreement for BAL cytospin count differentials. The x-axis shows the two observers, labelled A and B. Lines connect the two observers counts of the same slide. The average disagreement between observers for each cell type was below 3%.

Table 4.4. Summary of BAL cell counts from human exposure study.

Outcome	FA Mean (%)	DE Mean (%)	DE Effect Mean (95% CI)	p-value
Epithelial Cells	7.58	9.25	1.67 (-1.47 to 4.81)	0.27
Macrophage/Monocytes	76.14	63.49	-12.65 (-23.74 to -3.71)	**0.021**
Lymphocytes	11.91	21.1	9.19 (1.38 to 17.00)	**0.036**
Neutrophils	4.38	6.61	2.23 (-4.20 to 9.14)	0.435
Eosinophils	0.33	0.49	0.16 (-0.25 to 0.57)	0.407
Basophils	0.12	0.12	0 (-0.16 to 0.16)	1.000

Results are expressed as a percentage of total cells. BAL = bronchoalveolar lavage, FA = filtered air, DE = diesel exhaust. Significant p-values ($p<0.05$) are in bold.

4.2.7. DE increased NETs in BAL, with no effect on ROS or activation markers

Objective number 3 of this thesis was to assess the effects of DE exposure on neutrophil functional activity. Activation marker expression (flow cytometry), ROS production (H_2DCFDA assay) and NET formation (fluorescence microscopy) were quantified in BAL neutrophils after exposure to DE and FA. BAL flow cytometric analysis used the same gating strategy as in the blood (see Figure A.7 for representative gating strategy in BAL, and Figure A.8 for FMO controls). Although these markers have been used previously to identify blood, BAL and sputum neutrophils (167), the flow cytometry panel was further verified in this study by comparing the percentage of neutrophils in the CD45 gate to the percentage of neutrophils counted by observers on the cytospin slides. Comparison of the two methods revealed that the flow cytometric method was within 3% of cytospin counts for each subject visit (see Figure 4.11). Contrary to the hypothesis, there was no significant effect of DE exposure on the level of activation markers on BAL neutrophils after DE (Table 4.5).

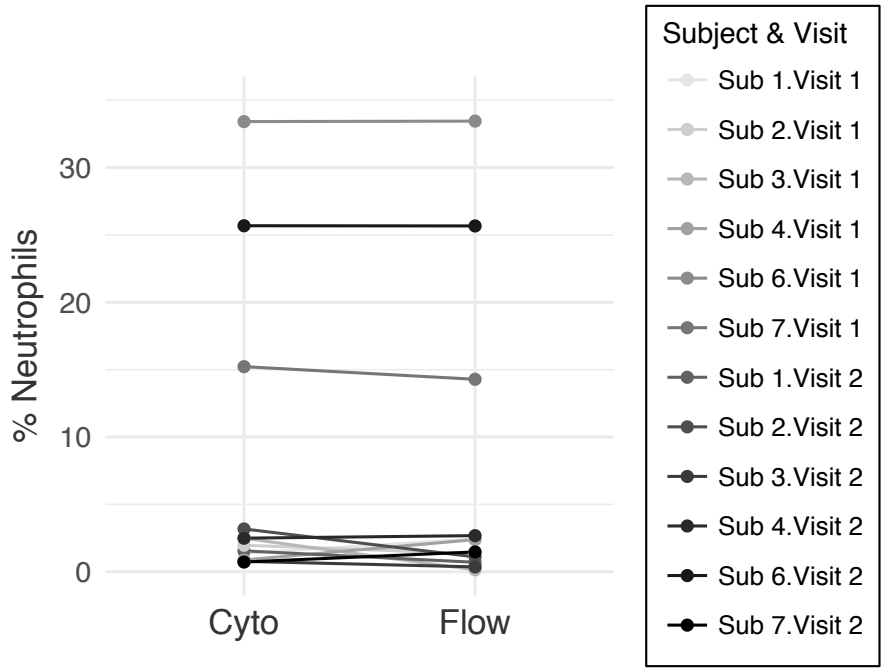

Figure 4.11. Agreement between cytospins and flow cytometry for identifying BAL neutrophils. Agreement in % neutrophils between cytospin counts (expressed as % of leucocytes) and flow cytometry (expressed as % CD45+ events) was compared for each subject at each visit as verification for the flow cytometric panel.

Table 4.5. Summary of BAL neutrophil activation from human exposure study.

Outcome	FA Mean (MFI)	DE Mean (MFI)	DE Effect Mean (95%CI)	p-value
CD16	885.2	353.4	-531.8 (-1706.0 to 642.5)	0.350
CD66b	1190.9	559.7	-631.2 (-1510.6 to 248.1)	0.156
CD11b	204.1	223	18.9 (-103.0 to 140.9)	0.741
CD182	12.20	28.6	16.4 (-21.1 to 54.0)	0.365

BAL = bronchoalveolar lavage, MFI = median fluorescence intensity, FA = filtered air, DE = diesel exhaust

Also contrasting with the hypothesis, there was no significant effect of DE on BAL neutrophil intracellular ROS production (Table 4.6), which was quantified in two ways using the H_2DCFDA assay (described in Figure 4.12). First, the background ROS production was assessed

by the shift in DCF fluorescence after incubation with H_2DCFDA. Second, the ROS response to a stimulus was assessed by the shift in DCF fluorescence after 45min stimulation with 4μM ionomycin.

Table 4.6. Summary of oxidative burst assay in BAL neutrophils from the human exposure study.

Outcome	FA Mean (MFI)	DE Mean (MFI)	DE Effect Mean (95%CI)	p-value
Resting DCF shift	316.4	224.3	-92.1 (-297.2 to 113.0)	0.434
Ionomycin-induced DCF shift	148.3	57.2	-91.1 (-235.3 to 53.2)	0.192

BAL = bronchoalveolar lavage, MFI = median fluorescence intensity, FA = filtered air, DE = diesel exhaust, DCF = dichlorofluorescein (oxidized form of the indicator H_2 DCFDA)

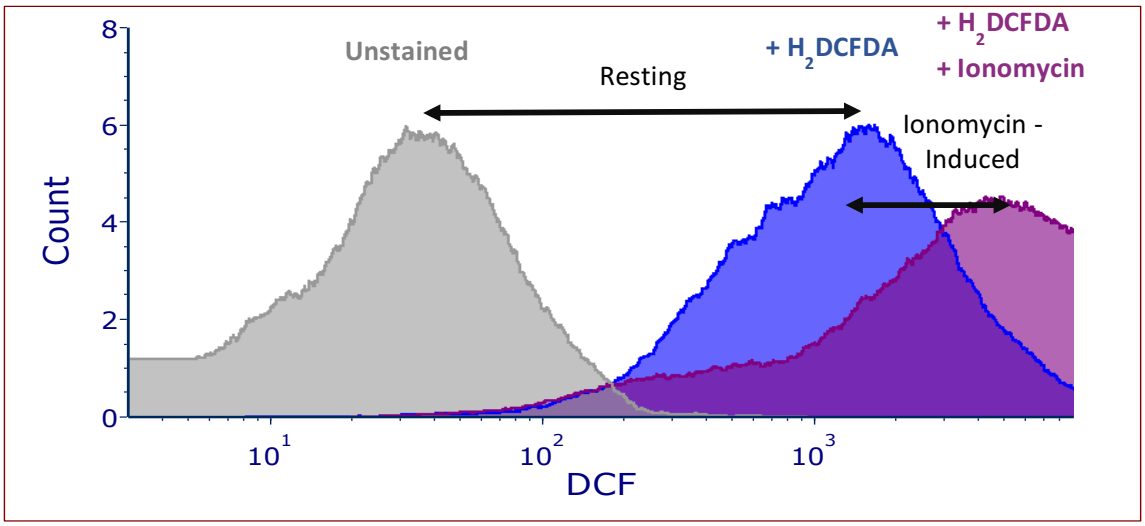

Figure 4.12. Representative flow cytometric analysis of ROS production in BAL neutrophils. Bronchoalveolar lavage (BAL) neutrophils were assessed for their oxidative burst activity 24h after exposure to diesel exhaust or filtered air. Resting and ionomycin-induced intracellular ROS production were assessed by quantifying the shift in median fluorescence intensity of the oxidized indicator. DCF (oxidized form) = 2',7'-dicholorofluorescein; H_2DCFDA = 2',7'-dihydrodicholorofluorescein diacetate.

Lastly, to quantify the effect of DE exposure on NET formation in the lung, the NET-covered area was quantified on one large stitched image (4x4 images acquired at 60X magnification) for each subject in each visit, then normalized to the total DNA-covered area. DE exposure significantly increased the % DNA area associated with NETs (15.9% increase, 3.8 to 30.0%, p=0.024) (Figure 4.13). Representative immunofluorescent images of the photos used for quantification can be found in Figure 4.14. Interestingly, in accordance with previous work investigating NETs in COPD patients (130), some specimens had pronounced NET formation but little or no citrullinated histones contained within (Figure 4.15).

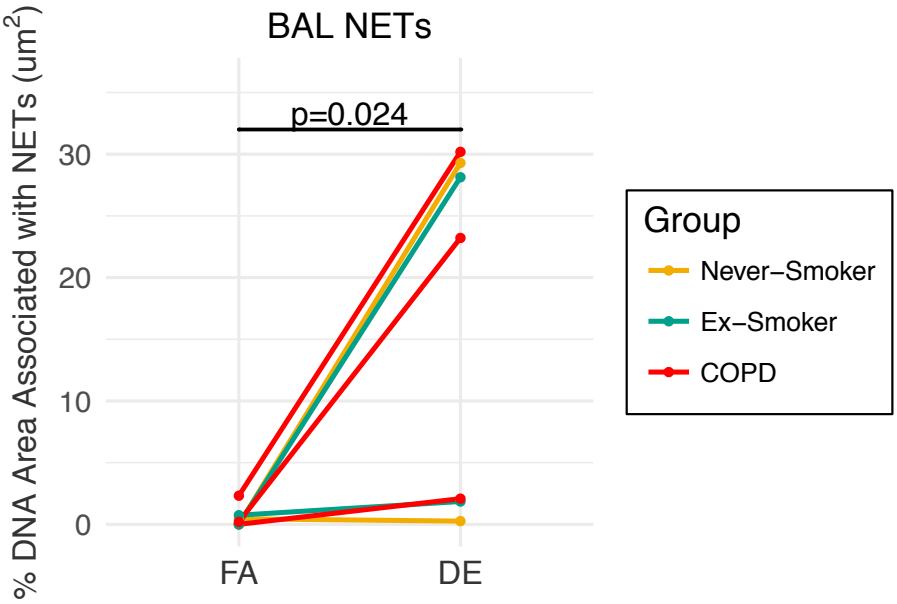

Figure 4.13. Effect of DE on BAL neutrophil extracellular trap formation. Each point represents the percentage of total area covered by NETs in a single large 4x4 stitched image for one subject.

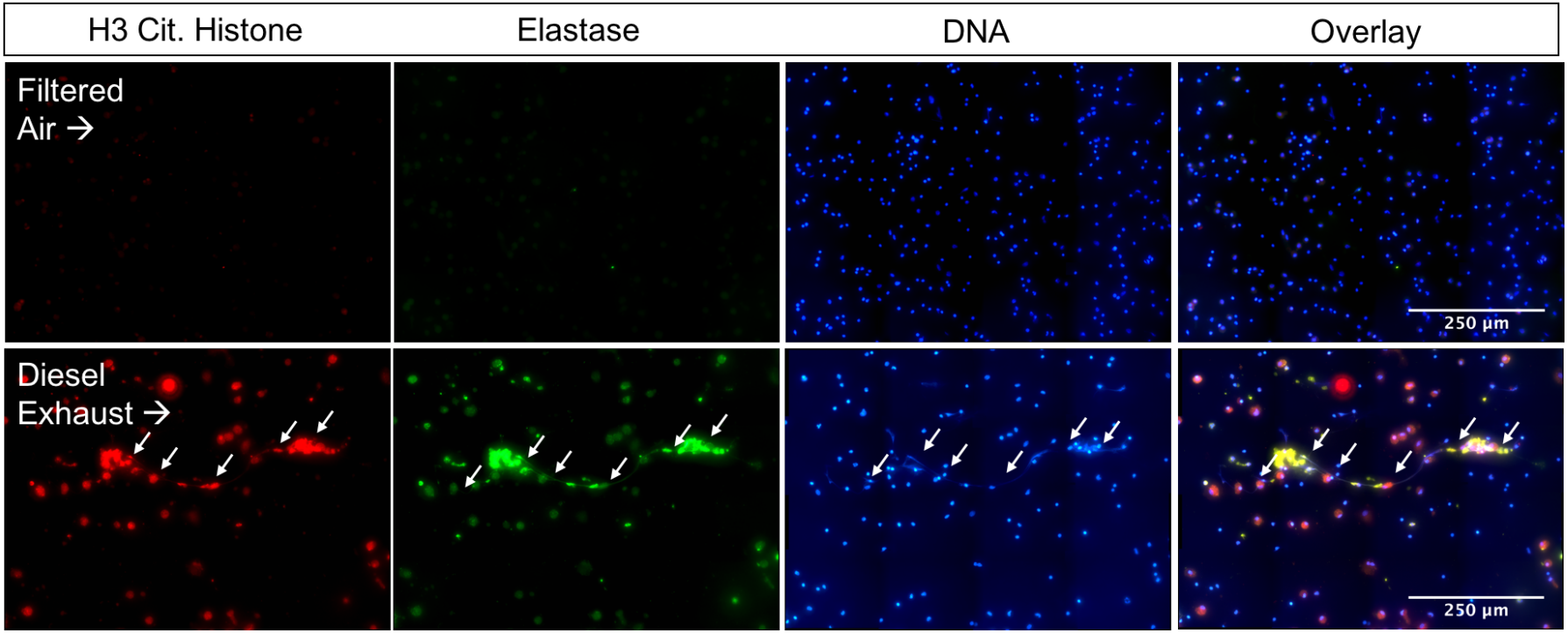

Figure 4.14. Stitched fluorescence images of NETs in the BAL after FA and DE. Representative images (4x4 images at 60X magnification) from immunostained BAL, acquired with fluorescence microscopy, with arrows pointing to NETs structures. BAL was stained for citrullinated histones in red (H3cit, a common component of NETs), neutrophil elastase in green, and DNA (Hoechst 33342) in blue. Long fibrous structures containing H3cit, elastase, and DNA can be observed in the specimens obtained after diesel exhaust exposure.

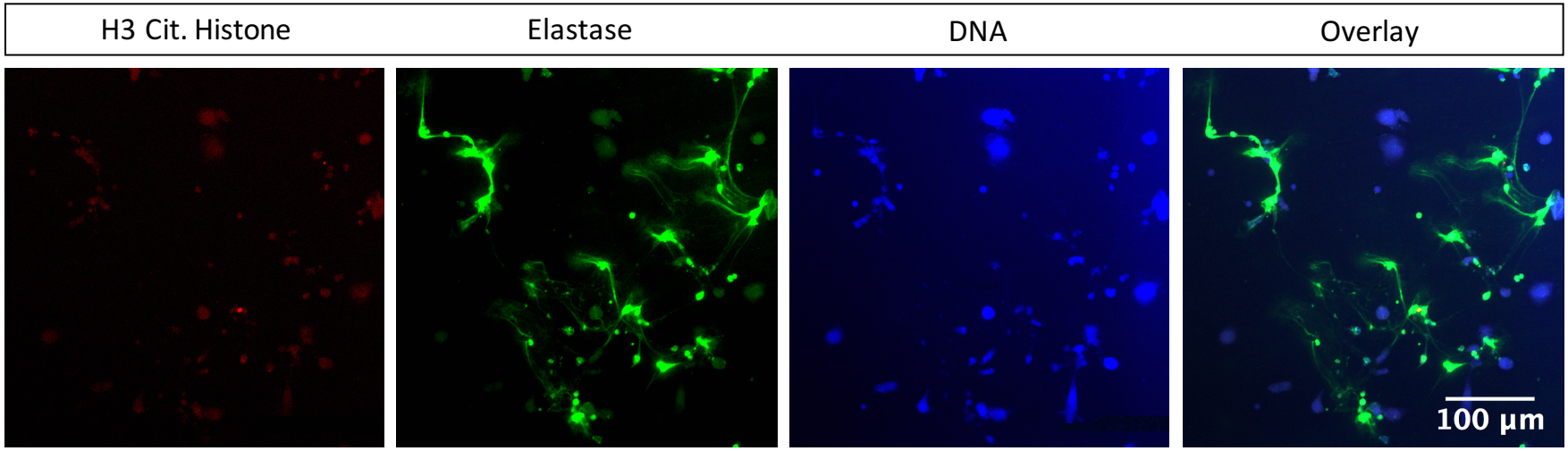

Figure 4.15. Representative confocal fluorescent photomicrographs of NETs lacking H3cit from a COPD patient after a DE. Although citrullinated histones, depicted here as H3cit, are commonly thought of as a hallmark component of NETs, some reports have identified NETs lacking H3cit, including one study in COPD patient sputum.

Chapter 5: Discussion

Neutrophils are consistently implicated in the inflammatory response to DE (or DEPs), as reflected by neutrophil influx to the lung and increased blood neutrophil counts after human (52, 54, 148, 150) and animal exposures (182, 183). However, no research to date has investigated the functional role of neutrophils in response to DE, beyond their mere recruitment. Airway neutrophilia is also central to COPD pathophysiology (96, 139), where neutrophil number and function is inversely related to clinical outcomes (125, 184-186). This is the first reported controlled human DE exposure study focused on participants with COPD. Understanding the effects of DE on neutrophil function, both in healthy and at-risk populations (COPD and ex-smokers) can shed light on the mechanistic events potentially leading to the development or worsening of COPD. In spite of what may seem apparent to observers of developing literature on the topic, there is no expert consensus that air pollution causes COPD. In fact, the American Thoracic Society has supported an international workshop on May 19, 2018, motivated in part by the need to review the evidence on this topic so as to inform a potential consensus therein. Accordingly, this study can provide key experimental evidence to support the emerging epidemiology demonstrating a role for air pollution in COPD.

5.1. Neutrophil response to DEPs *in vitro*

In this thesis, I demonstrated that neutrophils became functionally activated after stimulation with DEPs. Specifically, DEPs increased the expression of CD66b (a surface marker involved in adhesion and migration (187), and a marker of neutrophil degranulation (188)) but not CD11b. In addition, DEPs led neutrophils to produce intracellular ROS, and release NETs. The mechanism

through which DEPs interact with neutrophils to induce such effects, as well as which component(s) of DEPs are responsible, may be an interesting area of future investigation. Previous work has identified that the organic material from DEP surfaces has pro-inflammatory properties itself (159). Additionally, DEPs have been shown to carry large amounts of free radicals on their surfaces, which could potentially induce neutrophil activity (189). Some work has also identified the involvement of the pattern recognition receptor TLR4 in the inflammatory response to fine ambient particulate matter (190) and diesel exhaust particles (191), though this has not been specifically identified in the context of neutrophils.

Although neutrophil activity plays a central role in protection against pathogens, their activation and resulting antimicrobial mechanisms includes the release of highly toxic substances that can perpetuate further inflammation, promote mucus hypersecretion, and contribute to emphysematous tissue damage in the lung (192). Therefore, these data present a potential biological mechanism through which neutrophils could contribute to the tissue-damaging and pro-inflammatory effects of exposure, through their activation by DEPs.

In these experiments, neutrophils were incubated with DEPs *in vitro*. There are three likely scenarios in which neutrophils would theoretically come into contact with DEPs in a real-world human exposure. First, in diseases characterized by neutrophilic pulmonary inflammation such as COPD (80), neutrophils are already present in high numbers in the lungs and may come into direct contact with DEPs immediately upon inhalation. Second, a number of human exposure studies have demonstrated neutrophil recruitment to the lungs in healthy subjects within the time frame of 6h to 18h after DE exposure (52-54, 147, 150). Given that DEPs are highly

biopersistent (36), these newly recruited neutrophils may also interact with the previously deposited DEPs once inside the lung. Lastly, previous work has demonstrated that inhaled DEPs can exit the lung and translocate into the bloodstream (58), thus interacting with peripheral blood neutrophils in this manner. The current study, which employed isolated peripheral blood neutrophils, has potential relevance for the scenario of interaction in the lung as well as in the blood, each with certain limitations in mind. First, neutrophils are known to alter their phenotype and activation status upon extravasation to the tissue (193). Therefore, neutrophils in the lung may not behave in the same manner as the peripheral blood neutrophils, used in these experiments. On the other hand, while the scenario of translocation into the bloodstream is more relevant in terms of neutrophil phenotype, the fine particulates that may translocate are a tiny fraction of the total inhaled particulates (194-196), which was in the upper range of previous *in vitro* work (158, 159). With these caveats, these data demonstrate proof-of-concept that DEPs may directly interact with neutrophils to increase their functional activity. Optimal modeling of real-world conditions is explored in the controlled human exposure section of this thesis.

The current findings, showing increased CD66b but not CD11b, are somewhat consistent with previous work which demonstrated that both CD66b and CD11b are upregulated on isolated blood neutrophils following stimulation with cigarette smoke extract, as well as electronic cigarette (e-cig) smoke extract *in vitro* (197, 198). Neutrophils store CD66b and CD11b in their gelatinase and specific granules, and constitutively express these proteins at low levels on their cell surface (199). Therefore, increased surface expression is reflective of mobilization of these granules, and can be used as a proxy for granule exocytosis (200). To this end, in the context of cigarette smoke extract, granule proteins were directly measured in the supernatants, and the

increased CD66b and CD11b expression was in fact accompanied by increased secretion of granular proteins MMP-8 and MMP-9 (198), both of which contribute to emphysematous tissue damage and are correlated with the severity of emphysema (201). However, secreted proteins were not measured in this thesis, so the effect of DEPs on MMPs cannot be drawn conclusively.

CD66b surface expression is a marker of neutrophil activation and degranulation (202), and also plays a role in adhesion with the endothelium by mediating interactions with E-selectin (187). Therefore, these data are consistent with human exposure studies which show enhanced neutrophil recruitment to the airspaces following DE exposure (52-54, 147, 150). Although CD11b expression did not increase as hypothesized, it important to note that we used a monoclonal antibody that only recognizes an epitope on the activated form of the CD11b protein which contains a conformational change in the α-chain of the integrin. The constitutively expressed CD11b does not take its activated form (clone CBRM1/5) until an activating signal (i.e. a cytokine or chemokine) is transduced, after which CD11b becomes activated and capable of binding its ligands, such as ICAM-1 (203). The activated form of CD11b only makes up a small subset of total CD11b (203), which may help explain the discrepancy between our findings and those in previous studies with cigarette smoke and e-cig extracts. Alternatively, differences in the composition of the particles themselves, or the fact that extracts in the cited studies include all components of smoke, as opposed to particles alone, may also explain this disagreement.

Isolated neutrophils also responded to DEPs by increasing oxidative burst activity. This is in accordance with previous *in vitro* studies, which have shown that neutrophils stimulated by fine ambient PM, road dust, and cigarette smoke extract produce ROS (16, 204, 205). However, other

particulate pollutants such as coal fly ash and SiO_2 fail to induce ROS release, indicating that this response is pollutant-dependent (205). This is the first study to demonstrate these specific effects in the context of DEPs. Induction of oxidative stress is one of the most prominent proposed mechanisms for DE toxicity (36). For example, ultrafine particles have previously been shown to translocate into the blood stream and cause systemic oxidative stress, resulting in pro-thrombotic effects (206-209). Our data is consistent with this notion, and demonstrates the potential for peripheral neutrophils to contribute to systemic oxidative stress by increasing their oxidative burst activity upon stimulation by DEPs that traversed into the blood. However, *in vivo*, the systemic effects of DE are thought to result mainly from inflammatory mediators spilling over from the lung into the blood, rather than the particles translocating into the blood themselves, though both may occur and, the relative contribution of each pathway to systemic oxidative stress is debated (210).

One concern when measuring particulate matter-induced ROS is that ROS can be generated from reactions with the particle itself (particle-derived ROS), as well as from inflammatory reactions (cell-derived ROS) mechanisms (211). In the current study, intracellular ROS was assessed using flow cytometry, which quantifies the median fluorescence intensity of each event (i.e. each cell), and not the media within which it is suspended, as would be measured in a plate-based assay. Furthermore, the oxidized form of the indicator has low cell permeability, meaning that H_2DCFDA oxidized to DCF by particles in the media would theoretically not contribute to fluorescence intensity in the flow cytometric events (212). Therefore, the increase in ROS production detected was likely the result of true intracellular (cell-derived, rather than particle-derived) ROS.

Finally, neutrophils stimulated with DEPs increased the formation of NETs, which were first described in 2004 as a novel form of cell death distinct from necrosis and apoptosis (123). Dysregulated NETosis has been implicated in a number of diseases including sepsis (126), asthma (213, 214), COPD (125, 130, 214), cystic fibrosis (127) and autoimmune diseases (215). Furthermore, NETs are capable of directly inducing epithelial and endothelial damage (124), and occluding vasculature which contributes to thrombosis (216, 217). *In vitro,* NETs can be stimulated by range of non-physiological (i.e. PMA, ionomycin) and physiological (i.e. bacteria, fungi, LPS, IL-8, TNF-α) stimuli (122). However, no previous study has investigated whether DEPs can induce NET formation. In agreement with our hypothesis, DEPs incubated with isolated neutrophils increased NET formation. Although our experiment was not designed to assess the mechanism through which DEPs induced NETosis, these findings are in agreement with the observation that DEPs also increased oxidative burst, as ROS generated by the NADPH oxidase complex are essential in at least one NETosis pathway mechanism (129). These ROS stimulate NE translocation from the granules into the nucleus, where it proteolytically disrupts chromatin packaging and begins the process of nuclear decondensation required for NET formation (218). One limitation of the flow cytometry-based NETs assay is that the presence of citrullinated histones was a requirement for identification of NETs in this assay. Histone citrullination has been termed an essential step in NET release (135, 219), though this is now being challenged as being potentially stimulus-dependent, as other reports have identified NETs lacking this histone modification (130). However, identification of NETs from the positive control ionomycin in this study was likely unaffected, as it is known to produce NETs which contain citrullinated histones (161, 220). If anything, the observed effect that DEPs induce NETs

could have been underestimated in magnitude, if DEPs are capable of inducing NETs lacking citrullinated histones.

Overall, the *in vitro* work in this thesis demonstrated that DEPs increase the functional activity of neutrophils, in such a way that could facilitate migration into the lungs, and microbial killing activities with the potential to also damage lung tissue. Generally, these experiments satisfied the aim of performing an initial plausibility assessment of the effects of DEPs on neutrophil activation, building upon an experimental design consistent with previous work (221, 222).

5.2. Neutrophil release and migration following DE *in vivo*

I determined that acute exposure to DE immediately reduced the proportion of circulating band cells in the blood, and that this effect was more pronounced in COPD patients. A reduction in circulating band cells has similarly been described after cigarette smoke inhalation, and is suggested to be the result of preferential sequestration in the lung microvasculature (178). Due to their limited deformability, band cells may remain in the lung microvessels for a longer duration that mature neutrophils (179). There is some evidence that band cells can contribute to smoke-induced emphysema by spending more time in the vasculature, where they can release tissue-damaging proteolytic enzymes (178, 179). Within the pulmonary circulation, band cells are susceptible to activation by means of inflammatory mediators, or inhaled particulates spilling over from the lung into the circulation (178). Therefore, if the loss of band cells in this study is due to their sequestration in the pulmonary vasculature, they have the potential to contribute to lung tissue damage in this manner. Previous work demonstrating the release of neutrophil chemoattractants following exposure to DE indicates the potential for these signaling molecules

to spill over into the nearby vasculature (147). Therefore, this data identifies a potential mechanism through which repeated DE exposure could plausibly contribute to emphysema. However, confirmation that band cells indeed migrated to the lung cannot be confirmed with this data, as it would require a blood sample from the pulmonary vasculature.

As noted above, the DE-induced reduction in band cells was modified by patient status, such that COPD patients saw a greater reduction, while there was a similar trend for ex-smokers. These data are consistent with epidemiological evidence identifying these individuals as potentially more susceptible to the effects of environmental exposures (65, 223, 224). The reason for this differential effect cannot be determined from the current data, but could potentially be related to differences in pulmonary endothelial function (225). Specifically, the pulmonary vasculature in COPD patients exhibits impaired ability to maximally relax, while tobacco consumption in general also leads to thickening of the pulmonary arterial intima (225). Alternatively, ex-smokers and COPD patients have more pronounced inflammation in the lung, which may have increased the chemotactic signaling and resulted in this difference between groups (97). Finally, COPD patients may have elevated particle deposition in the lung, resulting in an increased particulate matter dose to the lung despite equal exposures (23). In the current study, we sampled blood immediately after exposure, and 24h after. Future studies would benefit from increased sampling frequency to better capture the dynamic process of neutrophil release from the bone marrow, transit through the blood, and migration to the lungs. Although the patient status was a significant effect modifier, these results are limited by a low sample size (2-3 subjects per group), and the stability of this interpretation across a broader population should be considered with caution.

Contrary to the hypothesis and previous literature, there was no indication of bone marrow stimulation upon exposure at 24h, as measured by the proportion of band cells in circulation. Therefore, the DE-induced neutrophil recruitment observed in multiple previous human exposure studies (52-54, 147, 150) may reflect recruitment to the lung from the existing circulating pool, rather than the release of new neutrophils from the bone marrow. The hypothesis was drawn from observational studies, which showed a strong positive association between high levels of particulate air pollution and circulating band cell levels (165, 181). The strength of this relationship is supported by evidence of the reverse, such that in one study, when individuals relocated from Japan to Antarctica (where particulate matter levels are very low), the proportion of band cells in the blood decreased; upon returning to Japan, band cell levels increased again (180). These changes were hypothesized to be the result of pro-inflammatory signaling induced by air pollution exposure, as serum IL-6 and G-CSF were reduced upon arrival in Antarctica, and elevated again upon returning to Japan. These studies imply that particulate air pollution stimulates the bone marrow to increase the rate of release of leukocytes and their precursors into circulation. However, circulating band cells have never been the endpoint of a TRAP study, nor have they been investigated with an acute, controlled human exposure study design. The fact that band cell count did not increase in our study may be explained by some important differences from our study design and previous observational studies (165, 180, 181). Firstly, blood sampling in the cited studies took place after a minimum of one full day of exposure (165, 180, 181). Our acute 2h exposure may not have been a sufficiently long duration to signal the bone marrow, and thus increase the appearance of band cells in the blood. The observation that total neutrophils also did not increase (i.e. band + mature) further suggests that the bone marrow was

not stimulated in the current study. A second major difference in our study was the type of exposure. The current study employed DE, while the observational studies noted above were in the context of forest fire smoke or ambient air pollution. Differences in the various components of these exposures, or in the types of particles themselves, could be responsible for this discrepancy. Lastly, we sampled blood 0h and 24h after exposure. If the bone marrow response occurred between or after these time points, we may not have detected it. However, PM_{10} levels are most closely associated with band cells after a 1-d lag, suggesting that our 24h time point was theoretically an appropriate time to detect a difference if there was one (165). Of final note, blood smears were used to quantify the percentage of band cells in relation to the percentage of neutrophils or total leukocytes. However, future work would benefit from the addition of total band cell counts, to differentiate between absolute numbers and relative proportions.

In conclusion, acute neutrophil recruitment after DE exposure may originate from the existing circulating pool rather than new production from the bone marrow. It is unclear whether these effects would be modified by longer-term exposure to DE. Immediate loss of band cells from circulation after DE in this study illuminates a potential mechanism for alveolar damage through their sequestration in the microvasculature and consequent release of destructive mediators, in the context of TRAP.

It was hypothesized that peripheral blood neutrophils would increase their expression of activation markers in response to DE, lending mechanistic support to their increased recruitment to the lung. Although increased CD66b was observed in the *in vitro* study, and altered expression of activation markers including CD16, CD11b and CD182 occurs in the blood following

exposure to biomass smoke (226, 227) and cigarette smoke (228), we observed no significant effect of DE on surface marker expression in the blood in this *in vivo* study. These data indicate that the neutrophil recruitment observed in previous studies may be due to enhanced chemoattractant signaling rather than alterations in expression of surface markers. Alternatively, it is possible that neutrophils expressing higher levels of these receptors, which assist in adhesion and migration, may have been recruited to the lung, removing them from circulation and resulting in no detectable change in the venous blood.

Interestingly, subject group significantly modified the effect of DE on CD182 (also known as CXCR2) expression, such that COPD patients had an average increase in expression while never-smokers had an average decrease after DE exposure. CD182 is a chemotactic receptor which binds neutrophil chemoattractants including IL-8 and GRO-α, and mediates neutrophil migration to sites of inflammation (229). Increased CD182 expression is associated with elevated chemotactic responses to IL-8 (230), suggesting that neutrophils from COPD patients may be more sensitive to migratory stimuli following DE exposure relative to never-smokers. A previous human exposure study demonstrated that DE exposure increases IL-8 and GRO-α mRNA and protein expression in the bronchial epithelium (147). Therefore, previous work and the current study together lead to the hypothesis that neutrophil chemoattractants are increased by DE exposure and that neutrophils from COPD patients may be particularly responsive to these stimuli. It is unclear what led to these changes, but CD182 expression can be modulated by a number of signals, including downregulation by TNF-α (231) and IL-8 (230), and upregulation by LPS (232). In our exposure system, LPS is essentially absent (162), but previous acute human exposure studies have demonstrated increases in IL-8 in the lung (53, 147), and TNF-α in the

blood (57) following acute exposure to DE. This apparent increase in sensitivity to these chemoattractants in the COPD population was not reflected in the BAL (i.e. there was no modulating effect of patient status on the percentage of neutrophils in BAL), but may have occurred in other regions of the lung, such as the central airway lumen or the airway wall, which tend to be the main site of neutrophilic inflammation following DE (52-54, 147).

The percentage of neutrophils in the BAL was used to quantify neutrophil migration to the lung following DE; however, there was no change induced by the exposure. Although increased appearance of neutrophils in the airways is a clear and consistent feature of acute exposure to DE, this phenomenon has only been described in the BW, airway wall, and sputum, but not BAL, which is consistent with our results. I used BAL to address the objectives of the current project for two main reasons. First, the primary aim of this study was to assess neutrophil function, since increased neutrophil number in the lung following DE has already been demonstrated. Although the mentioned samples from previous work, which are generally more reflective of the central airways, would have been preferred, practical limitations (namely very low cellular yield in BW, limited ability to perform functional assays in tissue specimens, and inconsistency in being able to obtain sputum samples) made them problematic for the current objective. Furthermore, this is the first controlled human exposure study to include the at-risk ex-smoker and COPD populations, who are known to have elevated neutrophils in the BAL, suggesting that this population could be more prone to neutrophil recruitment to the BAL following DE (100, 139, 140). Ultimately, evidence that DE produces an inflammatory environment in the BAL, with characteristics such as increased IL-8, GRO-α, and IL-1β, suggested that neutrophil functions could plausibly be altered in the BAL despite no change in

their numbers (233, 234), particularly in subjects who may already have neutrophilia in the BAL, such as those included in the current study. Therefore, given that BAL is consistently obtainable with adequate cell yield, it was chosen for the assessment of neutrophil functions in response to DE. The current study is thus consistent with previous literature in the BAL from healthy subjects, adding that BAL neutrophilia does not occur after exposure to DE, even in ex-smokers and COPD patients. However, these null results should be interpreted with the caveat that low power in the current study poses increased risk of type II error.

Also consistent with previous work, DE exposure did lead to lymphocytosis in the BAL (52, 54), which was met with a concurrent decrease in the percentage of macrophages. It is unclear whether the observed change in proportion was the result of lymphocytic infiltration with no change in macrophages, or the opposite, or combination of both. However, the combination seems likely as there was no change in absolute cell counts for either cell type (defined as cells/mL in BAL). Lymphocytes and macrophages encompass a broad range of cells that can be further subdivided into lymphocyte subsets (B cells, CD4+ T cells, CD8+ T cells, natural killer T cells) and macrophage subsets (resident alveolar macrophages, recruited monocyte/macrophages, M1 type/M2 type etc.). The complexity of their functional roles is beyond the scope of the current project, and therefore discussion of these results will only be discussed if applicable in the context of modulating or relating to neutrophil functions, presented in the next section. Nonetheless, it is worth noting that similar findings (increased lymphocyte and decreased macrophage proportions) were described in the lung following acute road tunnel exposure (235), which lends support to our use of DE as a model for TRAP. In addition, previous work in an animal model demonstrated that blocking CD4+ and CD8+ T lymphocytes using monoclonal

antibodies abrogated the inflammatory response to DE (measured as the number of inflammatory cells in BAL), suggesting that these cells play a role in orchestrating the response to DE (236). Overall, these findings are consistent with previous work which shows that lymphocytes are prominent in the inflammatory response to TRAP (52, 235, 236) and adds new information that these findings are applicable to the current study population, which is the first to include ex-smokers without COPD and also COPD patients.

5.3. Neutrophil activity in the lung after DE *in vivo*

It was hypothesized that DE would increase neutrophil functional activity in the lung. This was based on *in vitro* experiments described above, and the fact that human exposure studies increase the amounts of MPO in the lung (53, 237), which is suggestive of neutrophil activation. However, MPO is also found in lower amounts in monocytes and macrophages, and further insight into neutrophil functions is required (151, 238).

Contrary to our *in vitro* results, there was no overall effect of DE exposure on the expression of activation markers in BAL neutrophils, or on oxidative burst in BAL neutrophils. There are a few possible reasons for this discrepancy. Although the *in vitro* experiments used diesel particulates from the same source of DE as our human exposures, the mode of stimulation was different. In the human exposure study, blood neutrophils likely came into contact with far lower concentrations of particulates, and this exposure may have been insufficient to induce similar alterations. In the BAL, neutrophils are quite functionally distinct from blood neutrophils, as the process of extravasation from the blood stream leads to alterations in surface marker expression

(193), which may have also contributed to discrepancies between the *in vitro* results (which employed isolated blood neutrophils) and *in vivo* results (employing BAL neutrophils).

This study is, however, the first to demonstrate that an acute exposure to DE increases the amount of NETs in the BAL. NETs have an important role in protecting against pathogens, but high levels of NET formation is considered pathogenic, as they may contribute collateral tissue damage, and have been associated with a number of disease states (125, 126, 128, 130, 213, 214). In COPD, increased amounts of NETs in sputum are related to clinical outcomes, such that the amount of sputum NETs increases during exacerbation, and is inversely correlated to airflow limitation (125). The DE-induced increase in NETs in the current study suggests a potential mechanistic link between DE exposure and COPD pathogenesis. Firstly, NETs expose proteases that are normally contained safely within the neutrophilic granule (121), making them capable of damaging endothelial and epithelial tissue (124). Second, NETs expose histones to the extracellular environment, which also act as DAMPs and can promote inflammation through TLR and inflammasome activating pathways (239). Therefore, in the context of chronic or repeated exposures, NETs could potentially contribute to pulmonary inflammation and emphysematous tissue loss. In the current study, high levels of NE were identified within BAL NETs, which itself can also stimulate goblet cell degranulation, and potentially contribute to airflow obstruction through peripheral airway mucus plugging (145). On the other hand, the ability of NETs to trap and kill bacteria raises the question of whether NETs may be protective in the response to DE (123, 240). Further work is required to elucidate the effects of DE-induced NETs in the lung.

It is interesting that DE increased (non-significantly) the percentage of neutrophils by a slight 2% on average, while the amount of DNA-area associated with NETs significantly increased on average by 16%. These data highlight the importance of investigating functional effects of inhaled exposures, which can occur even in the absence of recruitment. Further insight into the effects of DE on neutrophil functions in the proximal airways, where neutrophilic inflammation is much more prominent both at rest and after DE exposure (52, 53, 241), would be beneficial if practical limitations can be overcome.

In addition, the effect of DE on NETs varied widely between individuals. The DE-induced change in NETs-associated DNA was in the range of -0.9% to 2.1% for three subjects, and 23.0% to 29.3% for four subjects. These differences were not explained by patient status, nor were they explained by mere changes in neutrophil abundance. Therefore, the potential for individual differences such as genetics to contribute to the effects of DE on BAL NETs may be an area of future interest.

It is unclear what mechanism led to the increase in NETs in the BAL. DE may have induced the release of pro-inflammatory signaling molecules known to stimulate NETs (242), and elevated by DE (147). Alternatively, the particles themselves may have interacted with neutrophils leading this to occur. Lastly, the increase in NETs may have resulted from impaired clearance by macrophages (243) rather than increased formation, which is consistent with the observed reduction in the proportion of BAL macrophages in this study. Nonetheless, it is interesting that histone citrullination was not always present in the NETs observed in the current study. This was rather intriguing, as the literature on NETs lacking citrullinated histones is sparse, but one of the only studies where it has been described is in the context of COPD-derived sputum (130). In that

report, the issue was raised that the lack of citrullination may have consequences on the effectiveness of bacterial killing (130). Although the ways in which histones are bactericidal are not known, it has been suggested that their positive charge strongly binds anionic bacterial cell wall components, compromising the bacterial wall barrier integrity (244). Therefore, histone citrullination (which removes the positive charge) is hypothesized to assist in NET release by facilitating nuclear decondensation, while potentially hindering NET-mediated microbial killing (130, 135).

There are important limitations to the findings of this controlled human exposure study. Limited sample size is a challenge with all controlled human exposure studies, and particularly in the current thesis, which had low statistical power due to time constraints. Although acute human exposures can lend insight to the biological mechanisms contributing to disease in chronic exposure settings, we cannot conclude causative relationship between exposure and disease using this model. The dose of exposure (300 $\mu g/m^3$ $PM_{2.5}$) is in the upper range of what humans are likely to encounter in the real world. However, this high level is required to elicit a physiological response that can be studied as a model for potential effects of lower-level, longer-term exposures in the real world. Additionally, as discussed previously, the major site of neutrophilic inflammation in the lung is in the central airways rather than the alveolar compartment, but samples from the central airways were not used for the current study due to practical challenges. Finally, the overall human exposure study was not designed solely for the purposes of neutrophil investigations, and the sample time points (0h and 24h) may not have optimally captured the neutrophil response to DE. Only one previous human exposure study assessed a time-course of the pulmonary response to DE, by testing both a 6h and 24h time point, and found a pronounced neutrophil response in the sputum at 6h, while there was no significant effect at 24h (148). The

latest DE-induced neutrophilic response in previous acute exposure studies occurred 18h post-exposure (53), suggesting that our 24h time point was likely later than the peak response.

Nonetheless, this study has a number of strengths. Controlled human exposure crossover studies facilitate the investigation of air pollution effects in a controlled experimental setting, eliminating the potential for most confounders present in epidemiological studies (156). The crossover design has the added benefit of increasing statistical power by eliminating between-subject variability from the overall error (163). Although human exposure studies cannot absolutely prove causation, they add crucial supporting information, by elucidating mechanisms and lending biological plausibility to observational data which has already identified a strong role for air pollution in morbidity and mortality relating to the global burden of respiratory disease (3). This study addresses the recent call-to-action in the field to delineate the effects of air pollution in susceptible populations (156), and to strengthen our knowledge of how air pollution may be linked to COPD, which has been referred to previously as a likely, but suggestive relationship requiring further study (79).

5.4. Conclusions

To summarize the findings of this thesis, I demonstrated that select neutrophil functions are altered by DEPs *in vitro* (CD66b expression, oxidative burst, and NET release). Furthermore, I showed that acute human exposure to DE results in a reduction of peripheral blood band cells and increase in NET formation in the lung. These results are consistent with the notion that neutrophils play an active functional role in the response to DE, and may contribute to the harmful effects of exposure. These data strengthen the biological plausibility implicating these

cells in the response to TRAP exposure. Furthermore, I demonstrated that the band cell response to DE, as well as the peripheral neutrophil CD182 expression was modified in the COPD population, while these effects were moderate, though not statistically significant, in ex-smokers. This is the first study to assess biological pathways potentially contributing to differences in sensitivity to TRAP within these particular susceptible populations, using a controlled human exposure crossover study design.

5.5. Future Directions

The finding that DE increased the amount of NETs in the lung is novel and intriguing, given the potential for NETs to induce epithelial and endothelial tissue damage (124), both of which are characteristic of emphysema (84). A growing body of evidence has identified NETs as key players in inflammatory diseases (245) and correlated them with clinical outcomes such as COPD exacerbation and lung function decline (125). Future investigation into the potential role of DE-induced NETs in protection against infection, contribution to lung tissue damage, and/or clinical effects such as lung function or mucus production would clarify whether they play a harmful or beneficial role in response to DE. To this end, the effect of DE on reducing the proportion of band cells in circulation was suggestive, but not confirmative, of band cell sequestration in the lung. Additional investigation would be required to confirm the ultimate destination of band cell migration explaining their decreased numbers in circulation. Delineating the role of band cells in potential disease processes relating to DE would be an interesting future direction, as band cells have already been implicated in the physiological response to longer-term exposures (165, 180, 181), as well as emphysematous tissue damage in animal models (178, 179, 246), prior to this acute exposure study. Overall, the field of controlled human exposure studies

has already begun to address the effects of DE in healthy subjects. Continued efforts should work toward a better understanding of air pollution exposures in susceptible individuals, given the apparent discordance between their prominent contribution to the global burden of air pollution (3) and the degree to which they are the focus of controlled human exposure studies. Further efforts should also be made to connect experimental and epidemiological approaches to understand the relationship between air pollution and airways disease – these disciplines remain too separate, though exceptions are illuminating (247). This information is crucial to our understanding of air pollution as a potential risk factor for COPD, and is paramount for influencing the policy-driven regulation of such ubiquitous exposures as TRAP. Given that COPD has no cure, elucidating risk factors and investing in prevention strategies may be one of the most effective ways to reduce the massive global burden of this disease.

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Appendix A

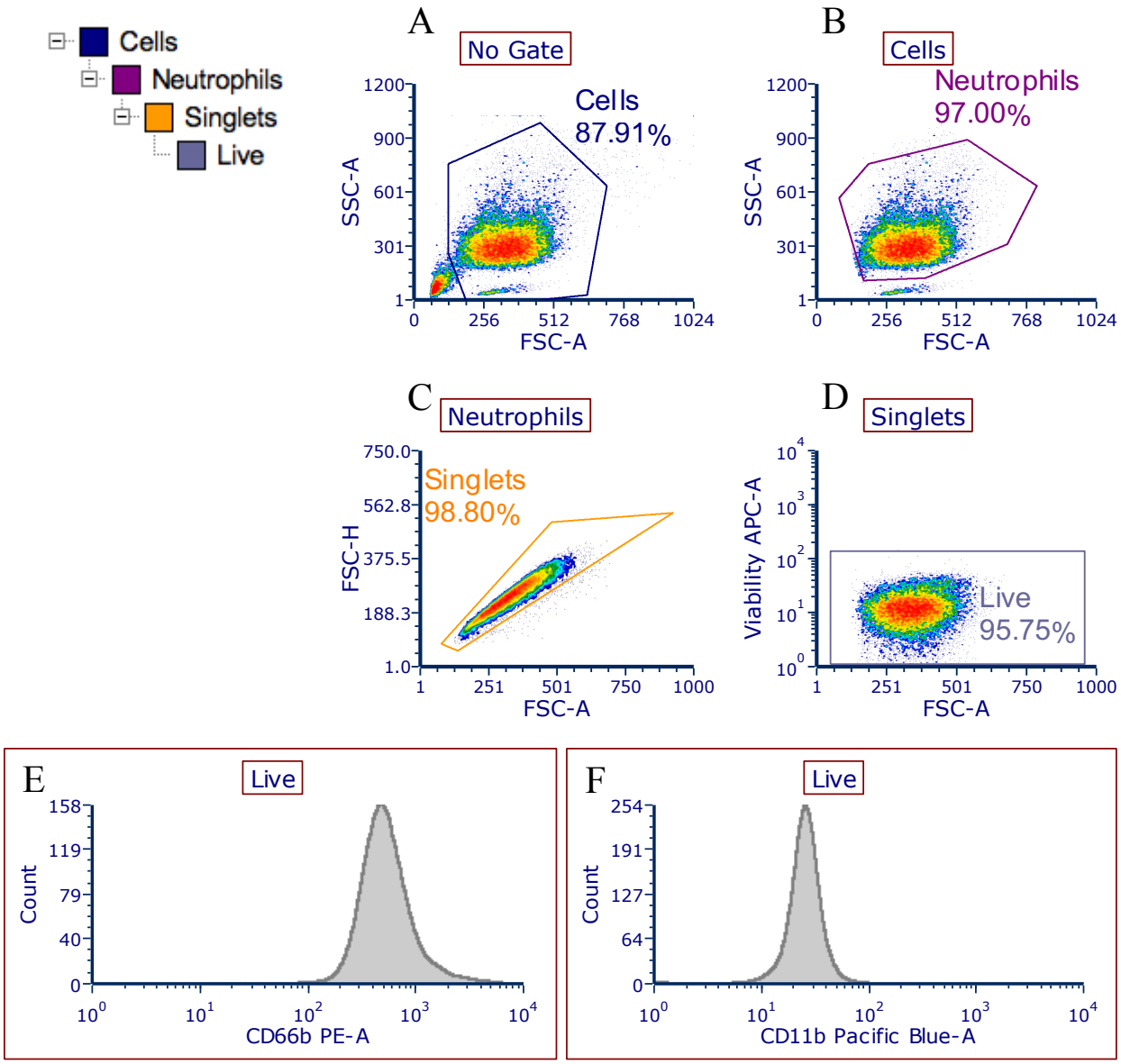

Figure A.1. Representative gating strategy for *in vitro* neutrophil activation panel. Example is shown in a DEP-stimulated sample. Debris, contaminating lymphocytes, and doublets were excluded based on light-scattering properties (panels A, B, and C respectively). Dead cells were excluded by gating negatively in the viability channel (panel D), and median fluorescence intensity of the two activation markers was quantified (panels E and F).

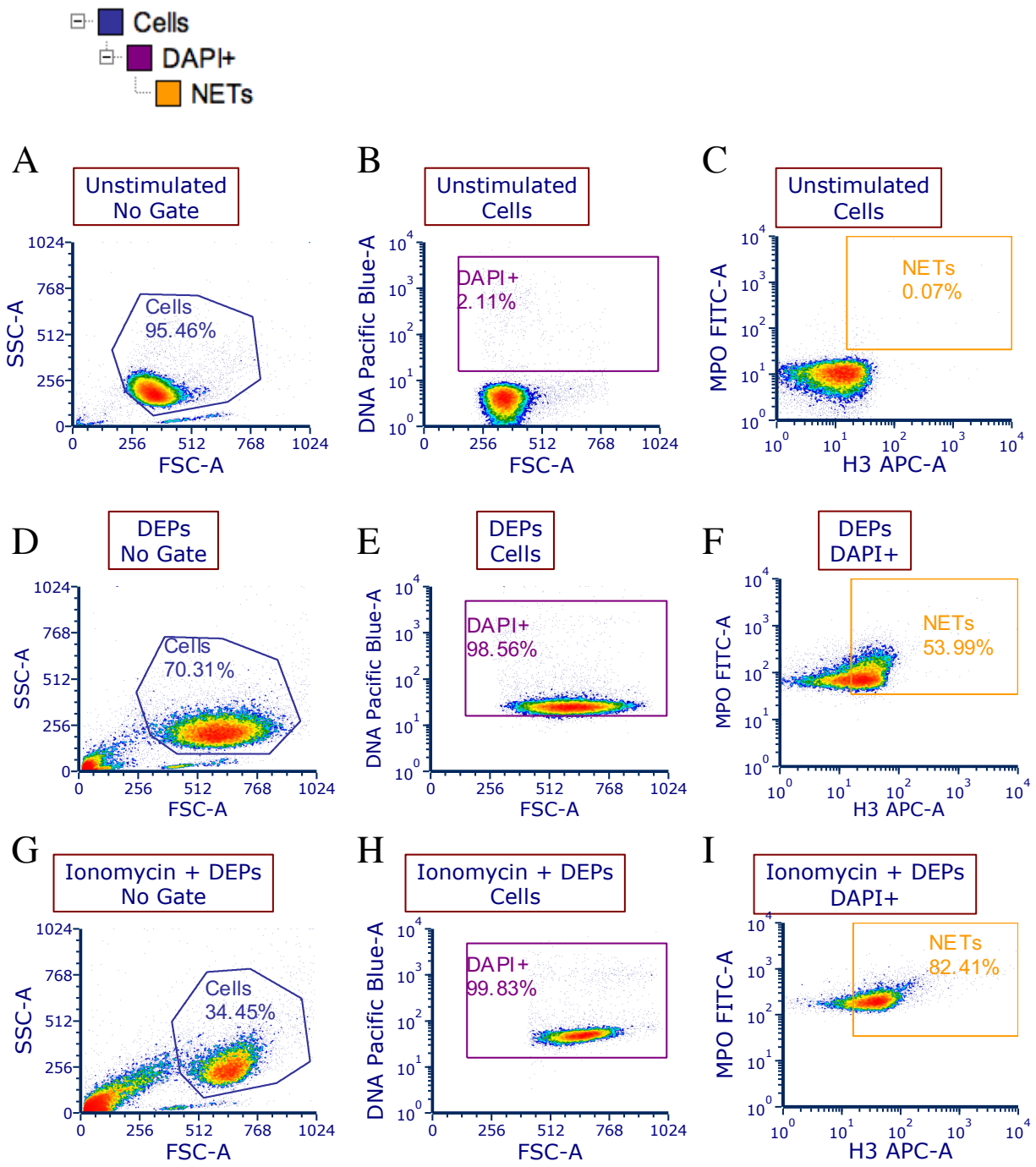

Figure A.2. Representative gating strategy for *in vitro* NETs flow cytometry assay. Peripheral blood neutrophils were isolated using magnetic negative selection, and incubated *in vitro* with media (negative control, panels A-C) ionomycin (positive control, not shown), diesel exhaust particles (DEPs; panels D-F), or a combination of ionomycin + DEPs (panels G-I). Gates were set based on FMO controls shown in Figure A.3. Neutrophil extracellular traps (NETs) were identified with a previously described strategy, where NETs are defined as events staining triple-positive for DAPI, myeloperoxidase (MPO) and H3 citrullinated histone (H3) staining.

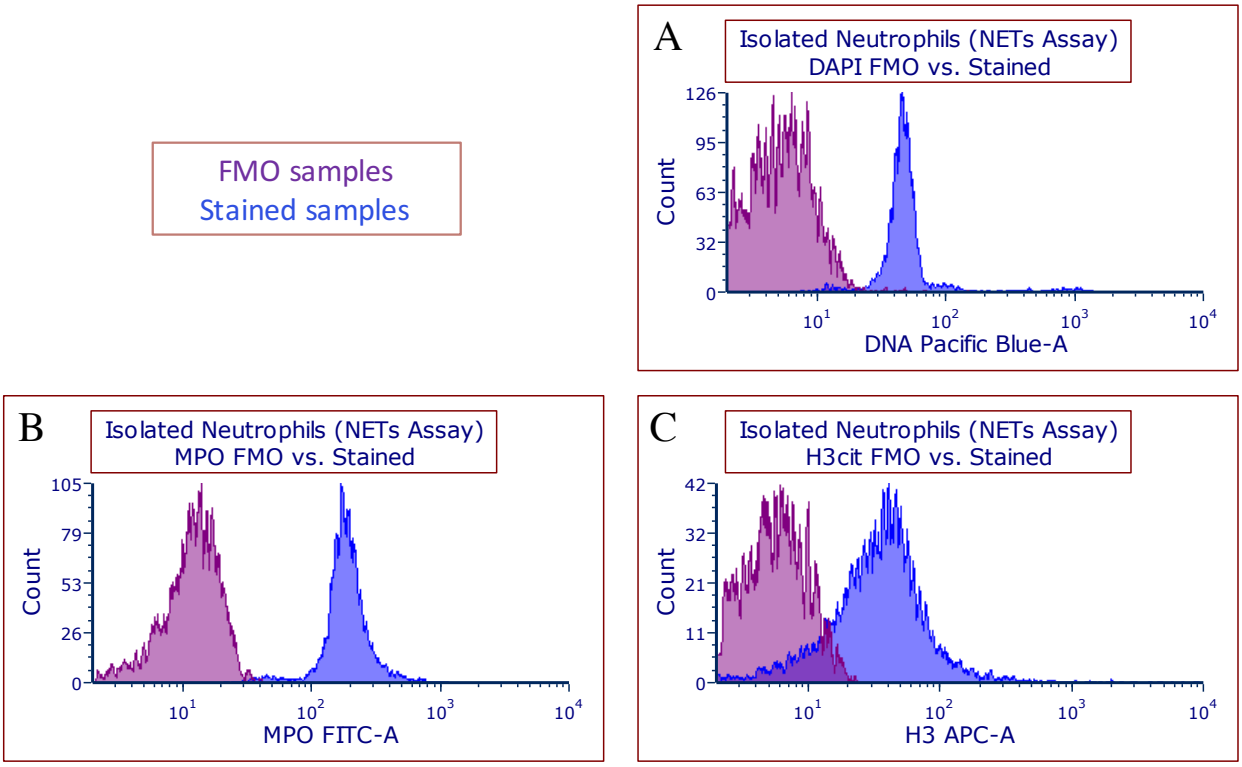

Figure A.3. FMO controls *in vitro* NETs flow cytometry assay. Ionomycin + diesel exhaust particles were used to stimulate isolated neutrophils are they were shown to release high amounts of NETs (and thus display a strong positive fluorescence signal) in the *in vitro* assays. Fluorescence minus one (FMO) controls for the three dyes used in this assay (Panel A = DAPI, Panel B = myeloperoxidase; MPO, Panel C = Citrullinated histone; H3cit).

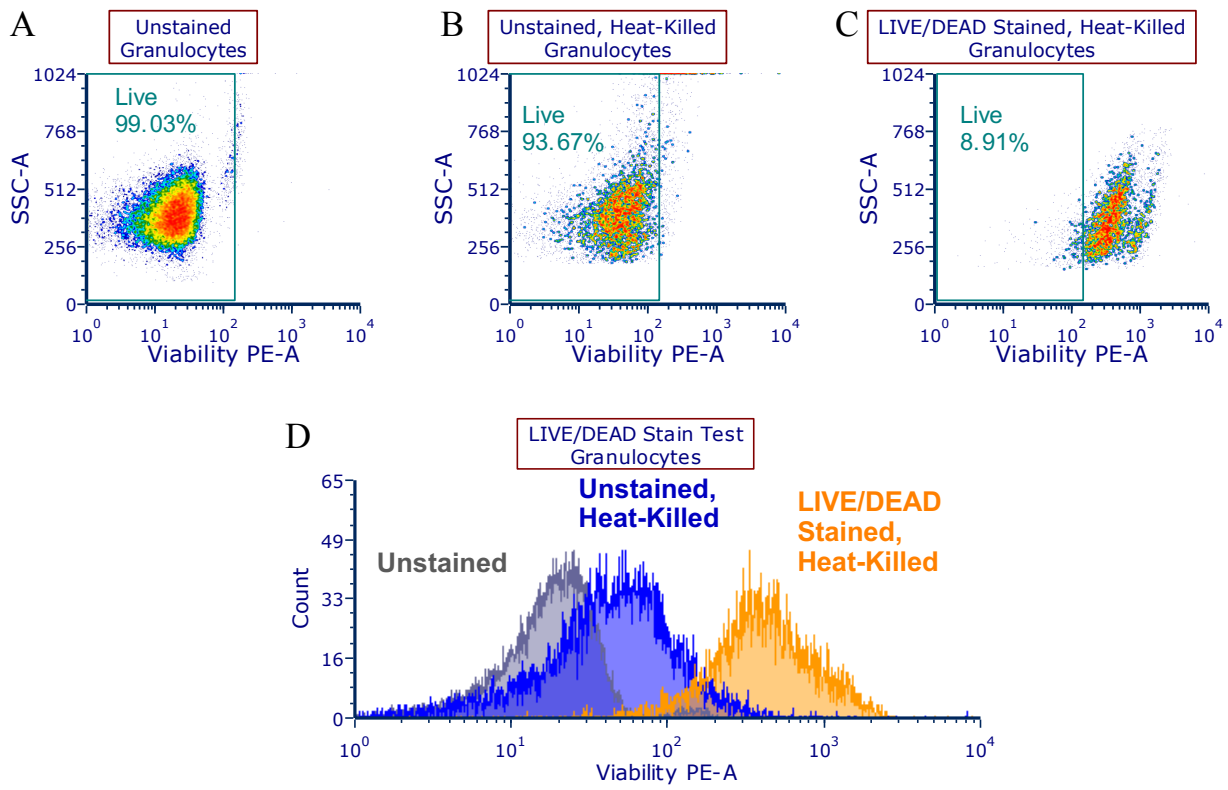

Figure A.4. Viability dye tested against heat-killed cells. The ensure that the viability dye used for the flow cytometry panels in the human exposure study was able to effectively stain dead cells, and to determine the correct position of the flow cytometric gate, cells were heat-killed by heating at 65°C for one minute, then placing on ice for one minute. Then, one unstained normal sample (panel A), one unstained heat-killed sample (panel B), and one viability dye-stained heat-killed sample stained (panel C) were prepared. Panel D shows the fluorescence intensity of the viability dye for these samples. The LIVE/DEAD stain positively identified heat-killed cells.

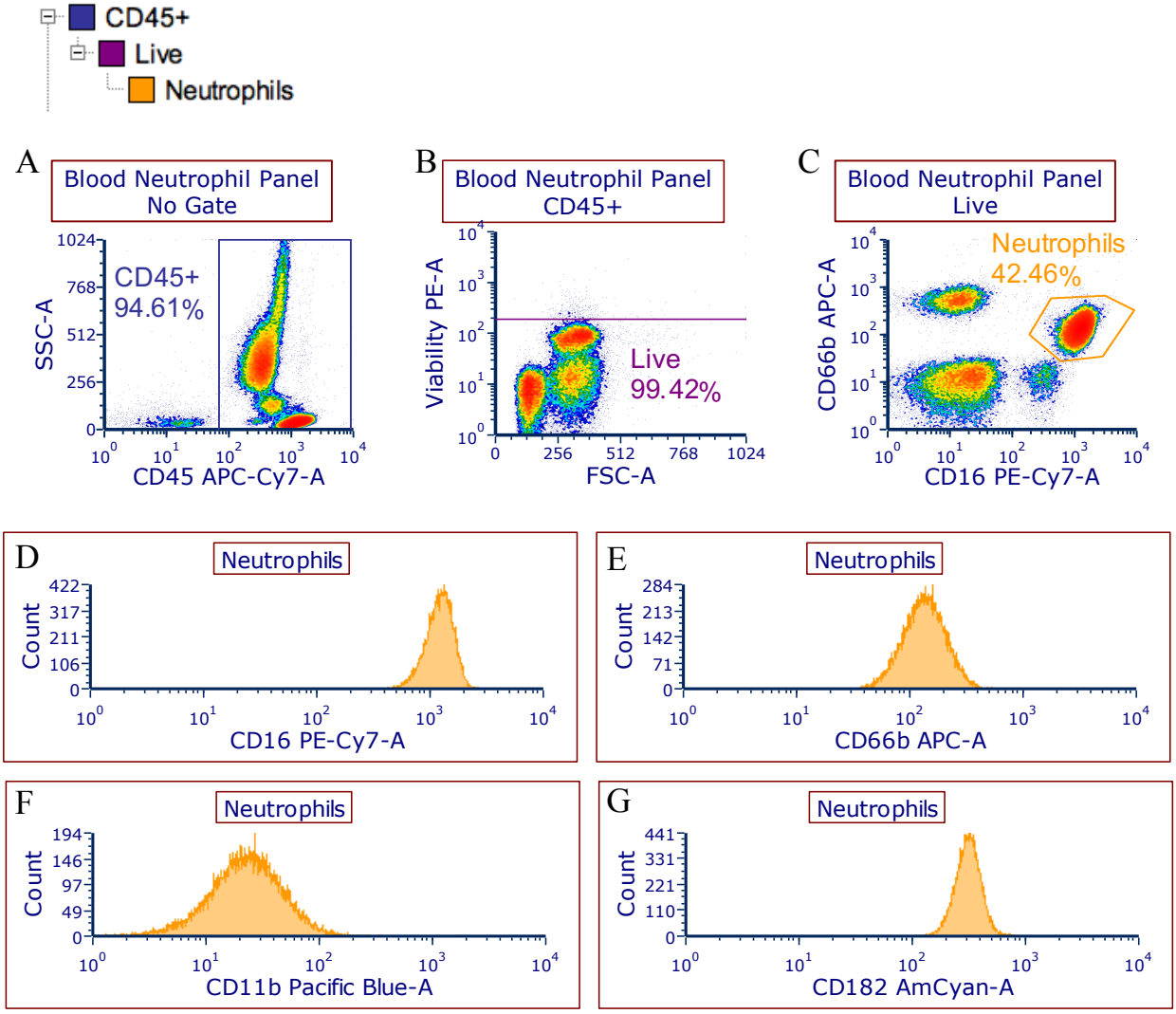

Figure A.5. Representative gating strategy for peripheral blood neutrophil activation panel. Blood samples obtained from the COPA human exposure study (before exposure, and 0h and 24h post-exposure) were stained with surface markers to assess neutrophil activation. Neutrophils were gated on based on positive staining for CD45 (panel A), negative staining for the viability dye (panel B), and positive staining for CD16 and CD66b (panel C). Gates were set based on FMO controls shown in Figure A.6. Median fluorescence intensity of the activation markers CD66b, CD16, CD11b and CD182 was quantified (panel D-G) to characterize the effects of acute diesel exhaust exposure.

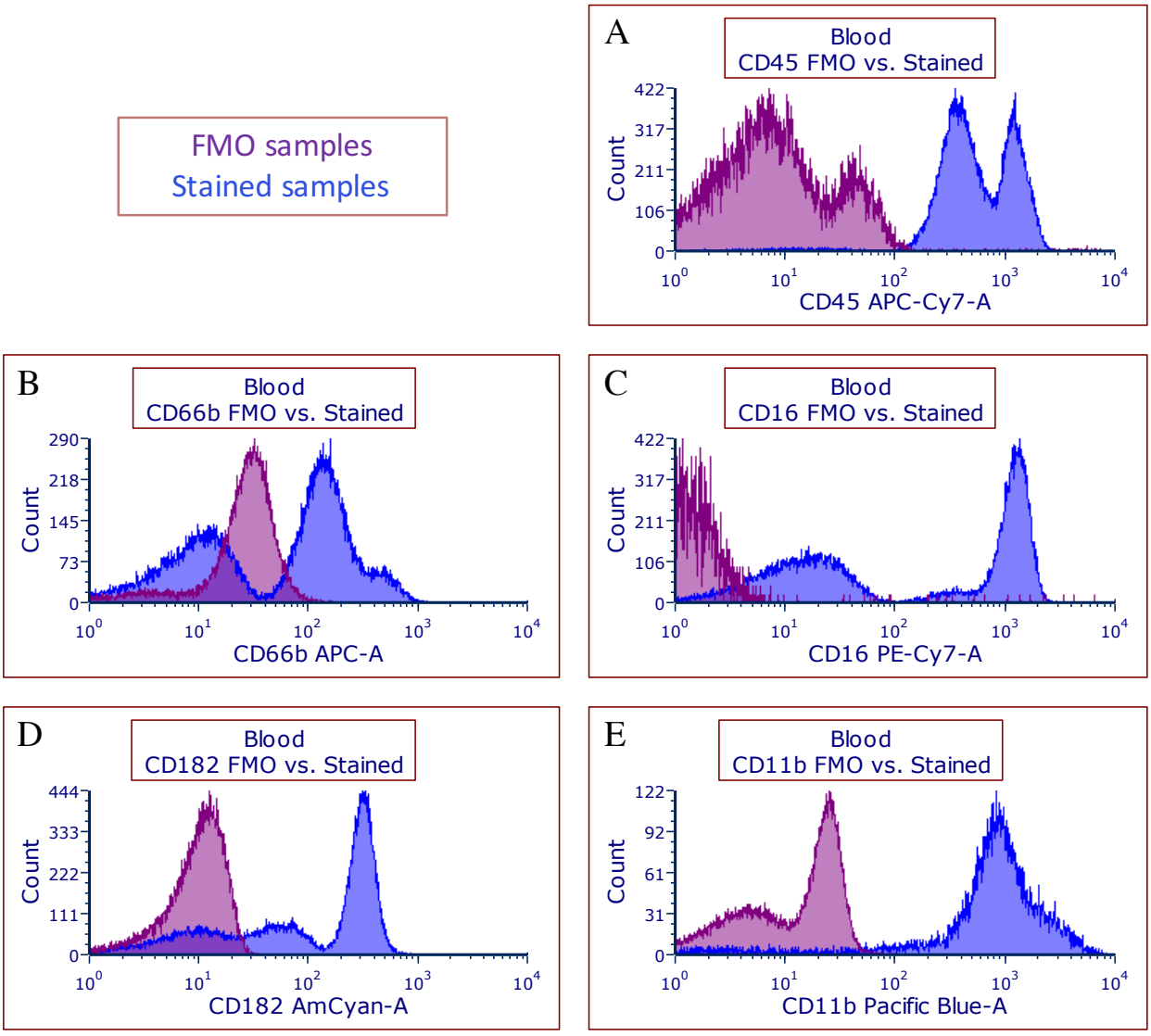

Figure A.6. FMO controls for blood neutrophil activation panel. Peripheral blood was stained with fluorescence minus one controls (FMO; Panels A-E) to assess positive and negative fluorescence for each marker used to set the gates.

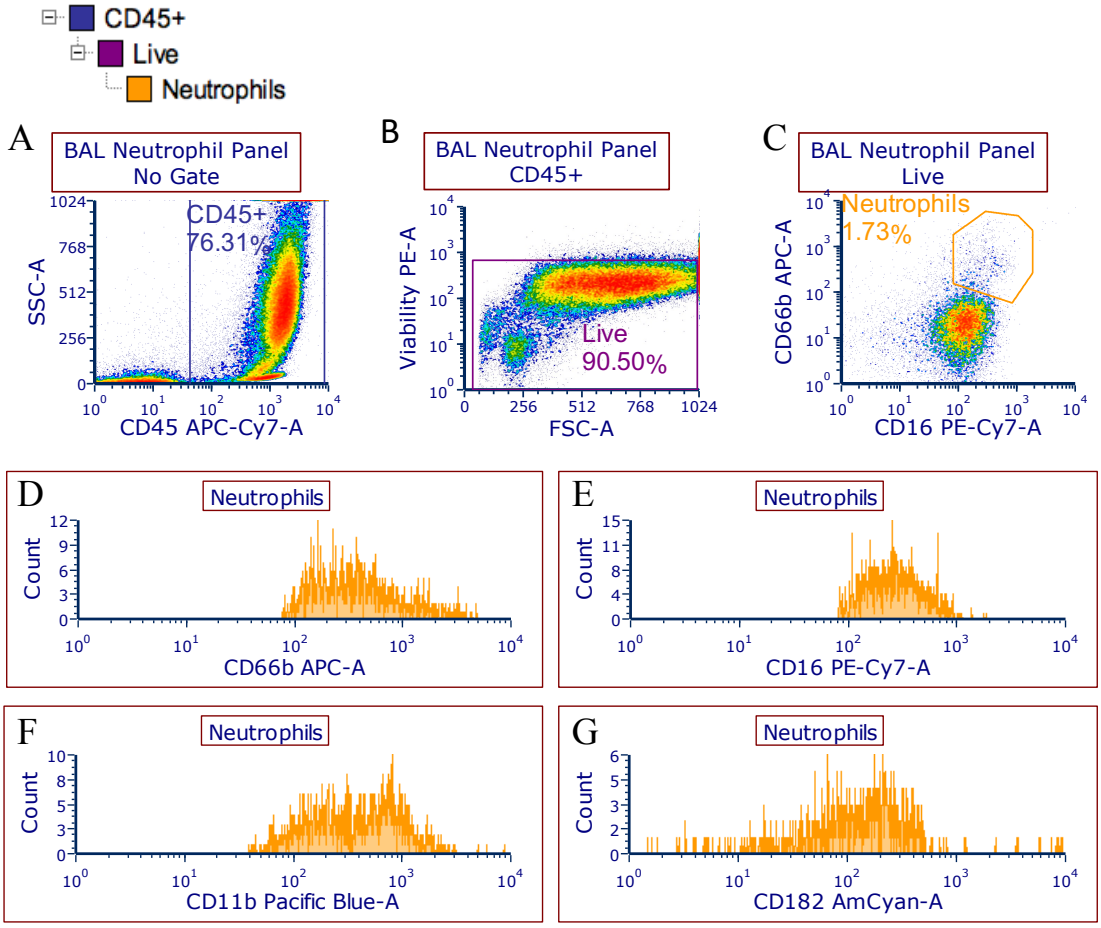

Figure A.7. Representative gating strategy for BAL neutrophil activation panel. Bronchoalveolar lavage (BAL) obtained 24h after exposure was stained for surface markers indicative of neutrophil activation. Neutrophils were gated on based on positive staining for CD45 (panel A), negative staining for the viability dye (panel B), and positive staining for CD16 and CD66b (panel C). Gates were set based on FMO controls from Figure A.8. Median fluorescence intensity of the activation markers CD66b, CD16, CD11b and CD182 was quantified to characterize the effects of acute diesel exhaust exposure on neutrophil activation 24 post-exposure.

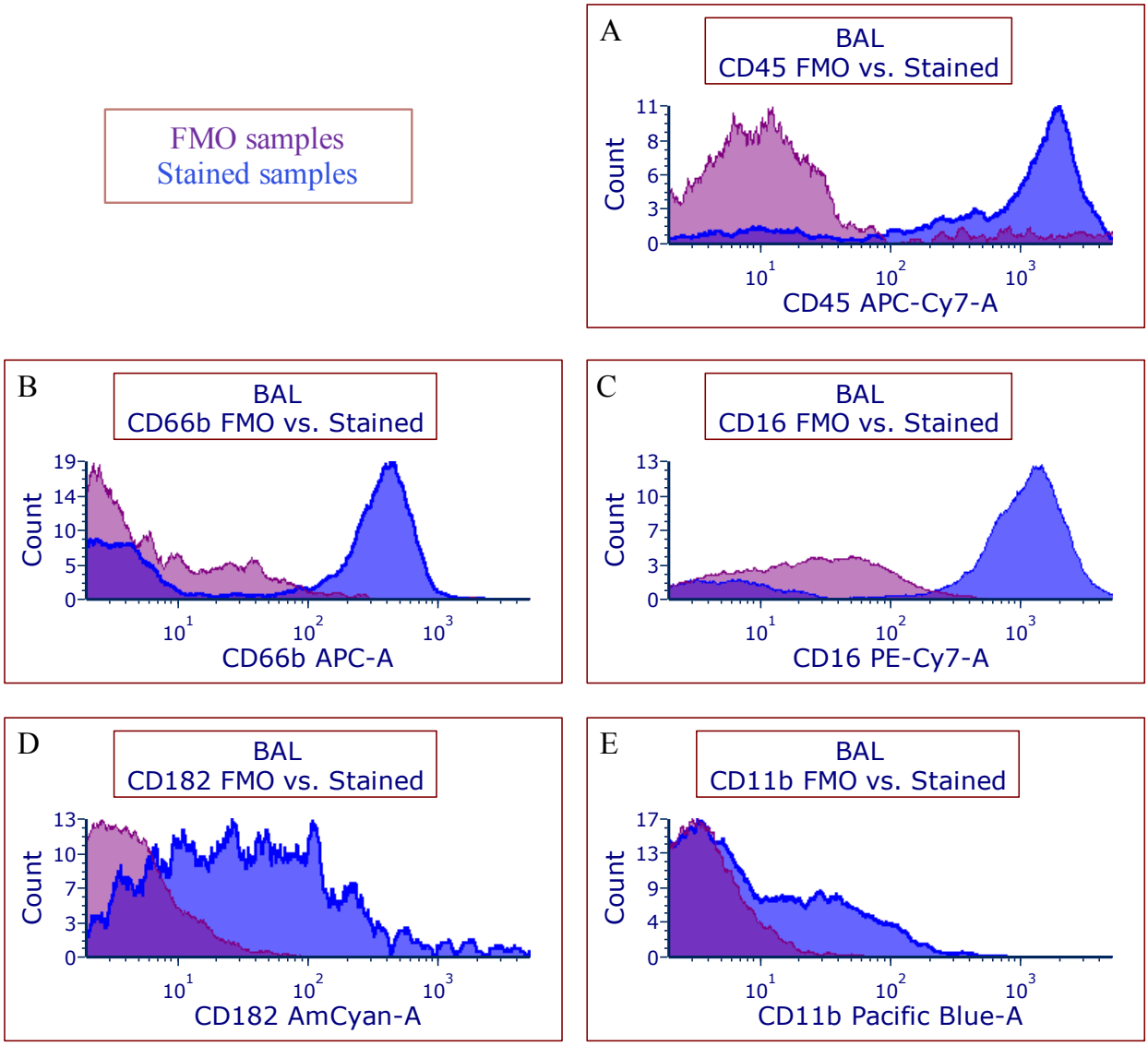

Figure A.8. FMO controls for BAL neutrophil activation panel. Bronchoalveolar lavage (BAL) was stained with fluorescence minus one controls (FMO; Panels A-E) to assess positive and negative fluorescence for each marker used to set the gates.