THE INFLAMMATORY RESPONSE TO CIGARETTE SMOKE AND BACTERIA

MECHANISMS OF CIGARETTE SMOKE-INDUCED INFLAMMATION AND THE EXACERBATED RESPONSE TO BACTERIA IN MICE

By
JAKE (JAMES KENNETH) NIKOTA, BHSc.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Doctor of Philosophy
McMaster University

Descriptive Note

McMaster University DOCTOR OF PHILOSOPHY (2014) Hamilton, Ontario (Medical Sciences)

TITLE: Mechanisms of Cigarette Smoke-Induced Inflammation and the Exacerbated Response to Bacteria in Mice

AUTHOR: Jake (James Kenneth) Nikota, BHSc. (McMaster University)
SUPERVISOR: Dr. Martin R Stämpfli
NUMBER OF PAGES: xiv, 182

Abstract

Chronic obstructive pulmonary disease (COPD) is a leading cause of global morbidity and mortality, with the potential to afflict as many as half of the 1.1 billion smokers in the world. The inflammatory response to cigarette smoke is believed to mediate the progressive and irreversible loss of lung function that characterizes COPD. The greatest burden of the disease arises from episodes of worsened symptoms and inflammation, usually triggered by microbial infection. Currently, the mechanisms that drive cigarette smoke-induced inflammation are being elucidated but ambiguity remains about this response and the inflammatory response engaged in a smoke-exposed lung experiencing a microbial infection. This thesis sought to investigate inflammatory mediators induced by cigarette smoke and those induced by bacteria, the most common cause of infectious exacerbations of COPD, in the context of smoke exposure. In chapter two we investigated the role of Breast Regression Protein-39 (BRP-39), a gene commonly observed to be increased under inflammatory conditions, in the inflammatory response to cigarette smoke. In order to determine the mechanisms of BRP-39 induction, its expression and inflammation was assessed in IL-13, IL-18, and IL-1R1 deficient mice. BRP-39 was found to be redundant in cigarette smoke-induced inflammation, but these data confirmed that IL-1R1 was a crucial mediator of this response. After examining the inflammatory response elicited by smoke alone, we investigated the importance of IL-1 signaling in a model of bacterial exacerbation of cigarette smoke-induced inflammation. We found that the exacerbated neutrophilia that typifies the response of a smoke-exposed lung to bacteria was dependent on IL-1α-mediated production of the CXCR2 ligand CXCL5. This study identified the unique phenomenon that cigarette smoke primes alveolar macrophages to produce excessive amounts of IL-1α in response to bacterial stimuli. The purpose of the final study of the thesis was to more

comprehensively characterize the extent to which cigarette smoke changes the phenotype of macrophages. Examining total gene expression by microarray found that smoke-exposed alveolar macrophages were in a proliferative state expressing a unique profile of inflammatory mediators. Further analysis revealed that this was likely the result of a pulmonary environment rich in growth factors. Taken together, these data provide detail to the understanding of the biological process of inflammation that drives the pathogenesis of COPD. These studies identify a phenomenon that predisposes smokers to experience more severe responses to bacteria and reinforces the targeting of IL-1 signaling in the treatment of COPD.

Acknowledgements

I was fortunate to have completed my graduate studies in the lab of Dr. Martin Stämpfli. Martin has been a fantastic supervisor who was patient when he needed to be, tough when I needed it, and always instilled a true love of the scientific method. He was always accessible to his students and conversations that started over a broken smoke exposure machine could easily end an hour later on the topic of post-World War II political philosophies, or the puck handling strategies of Team Canada's defense, or Swiss population demographics, or… but it was always a lot of fun.

I was also fortunate to have a diverse group of individuals who made up my supervisory committee. I would like to thank Dr. Manel Jordana for always challenging me to have air-tight rationale for each experiment and to think about 'the big picture', Dr. Tony Coyle for repeatedly taking time from his busy schedule to travel to McMaster and give a unique insight and opinion on our research, and Dr. Renée Labiris for friendly advice and a different perspective on lung pathology.

My graduate education was incredibly enriching because of my broad exposure to the field of research through the Stämpfli lab's multiple collaborative efforts. The collaboration with Dr. Sabina Halappanavar at Health Canada made the study pursued in chapter four possible, and our collaborators at Medimmune LLC, particularly Drs. Alison Humbles and Jennifer Kearly, provided valuable resources and a great insight into industrial research.

It has been my pleasure to have supervised the undergraduate thesis work of two extremely talented students, Jagbir Khinda and Kimberly Fernandes. Kim in particular was such a natural at the bench that she earned her nickname as "The Lab Elf". I thank Kim and Jagbir for their long hours and hope the experience affords them the success that they deserve in

medical school and beyond. Additionally, I'd like to thank the many undergraduate volunteers and summer students who passed through the lab, especially Ashling Kelly and Kristen Lambert.

Research is not an individual effort, and over the years I've been part of a constantly changing though consistently resourceful team. I began grad school with very little lab experience, and Nancy Trimble and Dr. Carla Bauer were instrumental in my early training. Dr. Fernando Botelho and I worked extensively together during the first few years, spending many late nights at a flow cytometer, and it led to a publication record that I can be proud of. Drs. Caleb Zavitz and Gordon Gaschler taught me that long hours in the lab could still be fun, and a good conversation with Dr. Mathieu Morissette was one of the best ways to refine an experimental approach. Working with Dr. Abraham Roos never felt too much like work, and Dr. Gilles Vanderstocken's lab treats could make any day better. Pamela Shen was the other half of our 'Dream Team', and I'd like to thank her for the constant assistance and two-person group therapy as we trudged through the grad school process.

Good science starts with good technicians and I thank Joanna Kasinska and Sussan Kianpour for always being ready to step in and help a struggling grad student. Members of other labs in our center were also quick to help, and I'd like to thank members of the Jordana, Xing, Bowdish, and Mossman labs. Additionally, without the administrative support of Marie Bailey, I would have drowned in paperwork.

Ultimately, all of my friends, who know who they are, helped keep me relaxed during the stressful times, and they deserve special thanks for the maintenance of my sanity.

Finally, I would like to thank my family for their support, including all of my aunts, uncles, and cousins. My little sister who continues to find her way and my older brother who would be the only person I insist call me Doctor. My father has been my safety net, helping me

out when life tries to work against grad studies. And though my mother didn't make it to see this end, she was always a staunch supporter of my academic pursuits, and her impact in my life will always give my work an artistic flare.

If the family that raised me is my support, then my new family is my inspiration. My wife Corrine has been my patient rock, helping me with every step of this journey. My newborn son Xavier gives me a reason to try to understand the world, so I can leave it a better place for him.

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List of Abbreviations

AAT	Alpha-1 antitrypsin
AM	Alveolar macrophage
AMCase	Acidic mammalian chitinase
ASC	apoptosis-associated speck-like protein containing a caspase-recruitment domain
ATP	Adenosine triphosphate
BAL	Broncho-alveolar lavage
BRP-39	Breast Regression Protein-39
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EOS	Eosinophils
FEV1	Forced expiratory volume over the first second of exhalation
FVC	Forced vital capacity
GCR	Glucocorticoid receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology

GOLD	Global initiative for chronic Obstructive Lung Disease
HDAC	Histone deacetylase
HDM	House dust mite
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IP	Interferon gamma-induced protein
KO	Knock out (gene deficient)
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MNC	Mononuclear cells
MyD88	Myeloid differentiation primary response gene 88
NE	Neutrophil elastase
NEU	Neutrophils
NRF2	Nuclear factor (erythroid-derived 2)-like 2
NTHi	nontypeable *Haemophilus influenza*
OVA	Ovalbumin
$P2X_7$	P2X purinoceptor 7
Pam3CSK4	Synthetic triacylated lipoprotein
PCLS	Precision-cut lung slices
R	Receptor

RAG	Recombination activating gene
RAGE	Receptors for advanced glycation end-products
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
SCID	Severe combined immunodeficiency
TCN	Total cell number
TGF	Transforming growth factor
Th	Helper T
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPM	Total particulate matter
Treg	Regulatory T
VEGF	Vascular endothelial growth factor
WT	Wild type
YKL-40	Chitinase 3-like-1 (human homologue)

Preface

This thesis contains three studies that have been prepared for publication in peer reviewed journals. Chapter two is already published, while chapter three and four are in the process of submission. While other authors contributed to each study, I was responsible for the majority of the experimental work and manuscript preparation. The introduction of each chapter outlines the specific contributions of each author.

The study presented in Chapter Two represents the work I did while enrolled as a Masters student. Upon transferring to the PhD program, I began the experiments that would comprise the results of Chapter Three. Upon analysis of the findings of Chapter Three, I accessed an older data set generated by a previous graduate student to initiate the analysis and experiments presented in Chapter Four. This entire process took a six and a half year time period.

There were also several avenues of research that were initiated over the course of my graduate studies but based on the experimental results generated, they were not pursued further.

This thesis represents a collection of work that is primarily my own, and my position as first author of each of the included studies has been earned. It is for this reason that I feel as though I am justified in including these documents in the main body of this thesis.

CHAPTER ONE

Introduction

The adverse health effects of cigarette smoke are well known and this addictive habit represents one of the greatest challenges to healthcare systems. Cigarette smoking is a globally ubiquitous practice with varying degrees of usage based on region and socioeconomic status, but collectively one sixth of the global population is an active tobacco product user; translating to over one billion smokers worldwide (1). The high global prevalence of cigarette smoke persists despite a decades-long health education campaign which has led to a decrease in smoking rates in western nations (2). Even at decreased rates, in Canada, 16% of individuals aged 15 and over identify themselves as smokers (3). Cigarette smoke related deaths were tallied at 100 million in the 20th century and smoking is projected to be responsible for the death of 1 billion smokers in the 21st century (4). To combat the smoking epidemic, health education strategies must be supplemented with therapeutics based on an understanding of the biology of smoke-induced disease.

Cigarette smoke exposure is the primary risk factor for the development of Chronic Obstructive Pulmonary Disease (COPD). COPD is a leading cause of chronic morbidity and mortality and is projected to become the fifth leading cause of global disease burden by 2020 (5). Current estimates are that over 200 million people worldwide have moderate to severe stages of COPD (6). Although COPD is characterized as an inflammatory disease, the effectiveness of currently available anti-inflammatory therapies has been the source of controversy and are only recommended in specific situations (5, 7). Additionally, COPD is punctuated by periods of exacerbated symptoms and inflammation in response to pathogenic agents, with bacterial and viral infection being the cause of the majority of these exacerbations (5, 8). These episodes can

be life threateningly severe and represent the primary burden of COPD on healthcare systems (9). Though bacterial infections are a major cause of disease exacerbations, the mechanisms that drive the increased disease severity during these episodes are poorly understood. The purpose of this thesis was to investigate the mechanisms of cigarette smoke-induced inflammation and the inflammatory mechanisms engaged during an experimental model of exacerbation. The underlying hypothesis is that the inflamed environment of the smoke exposed lung is predisposed for an exaggerated response to bacteria.

This chapter is divided into 5 sections to provide an introduction to the concepts relevant to this thesis:

1. COPD
2. Experimental models of COPD
3. Cigarette smoke-induced inflammation
4. Cigarette smoke and bacterial host defense
5. Experimental studies completed for this thesis

COPD

Chronic Obstructive Pulmonary Disease is characterized by persistent airflow limitation that is usually progressive and irreversible in nature (5). This loss of lung function is associated with an inflammatory response in the airways and lungs to noxious particles or gases and, although it is well established that genetic and environmental factors contribute to the development of COPD (10), the disease is found predominately in smokers (11). Other environmental risk factors for COPD include a range of activities involving exposure to inhalable particulate matter. These include occupational risk factors such as the inhalation of organic and inorganic dusts and

chemical fumes (12–14), as well as domestic exposures such as the burning of biomass fuel in poorly ventilated dwellings (15, 16). These other contributing factors should not be completely discounted as they may contribute a larger portion of disease burden in the future as population demographics change; however, cigarette smoke is currently the widely accepted preeminent risk factor for the development of COPD.

The clinical presentation of COPD is defined symptomatically, and the diagnosis of COPD should be considered in any individual experiencing dyspnea (difficulty or labored breathing), chronic cough, and/or sputum production coupled with a history of exposure to disease risk factors (5). Spirometry, a measure of a patient's ability to forcibly exhale, is required for a clinical diagnosis of COPD. This measures the maximum volume of air that can be forcibly expired in 1 second (FEV_1) which is presented as a ratio of FEV_1 to the maximum lung volume that can be forcibly expired without a time constraint, or forced vital capacity (FVC). A patient who has an FEV_1/FVC ratio that is less than 0.70 is considered to have COPD. This measurement is taken after the administration of a bronchodilator which opens the airways and increases airflow. The Global Initiative for Obstructive Lung Disease (GOLD) subtyped disease severity into 4 stages based on the percentage of the predicted FEV_1 value: less than 80% of predicted FEV_1 indicates mild disease (GOLD 1), values between 50% and 80% of predicted FEV_1 indicates moderate disease (GOLD 2), values between 30% and 50% of predicted FEV_1 indicates severe disease (GOLD 3), and a FEV_1 of less than 30% of the predicted normal value indicates very severe disease (GOLD 4) (17). Upon spirometric diagnosis of COPD, physicians assess comorbidies that occur frequently with the disease such as cardiovascular disease, skeletal muscle dysfunction, and lung cancer, as well as assessing the risk of future exacerbations in order to guide therapy.

The term COPD was first used in a series of conferences in 1959 and 1962 to describe the functional consequence of a group of distinct pathologies (18). Previous to that, COPD was discussed as the three separate disease states: chronic bronchitis, bronchiolitis, and emphysema (19–26). Ultimately, each of these pathologies contribute in varying degrees to the loss of lung function that characterizes COPD.

Chronic bronchitis is defined as a persistent inflammation of the central airways and is diagnosed by "a persistent cough that produces sputum and mucus, for at least three months in two consecutive years" (27). In COPD, large airways (with an internal diameter greater than 4mm) are infiltrated by inflammatory cells and this infiltration is associated with enhanced mucus production through an increase in the number of goblet cells and an enlargement of mucous glands (28, 29). Mediators from inflammatory cells drive the exaggerated mucus production and these 'mucous plugs' contribute to limited air flow, by blocking large and small airways, and leads to subsequent loss of lung function (30, 31). Chronic bronchitis is also associated with collagen deposition that leads to a thickening of the central airways (32). This airway remodeling and mucous production are hallmarks of the presentation of COPD.

Emphysema is a term derived from the ancient Greek word for inflation as it describes increased airspace in the lung and is defined as an "increase beyond the normal in the size of airspaces distal to the terminal bronchiole either from dilatation or from destruction of their walls" though the definition is typically used only to describe airspace enlargement that arises from the destruction of the lung architecture (27). This pathology results in the reduction of the total surface area available for gas exchange and poor oxygenation of the blood is a consequence. Additionally, the formation of emphysematous lesions create pockets of poor ventilation where air becomes trapped (21). Specifically, air trapping is contributed to by the destruction of elastin,

a key component of the extracellular matrix of the lung that gives the tissue its elastic recoil properties (33). Air trapping results in hyperinflation of the lungs that reduces the functional volume of the lung available for gas exchange, the consequence of which is a decreased inspiratory capacity in addition to the loss of functional lung volume.

The pathogenesis of emphysematous lesions has been attributed to the chronic presence of inflammatory cells capable of producing proteases which break down the extracellular matrix (ECM) of the lung (33). These observations led to the *protease-antiprotease hypothesis* that postulated that emphysema was the result of an imbalance in proteolytic enzymes and their respective inhibitors (34). The classic example of this imbalance is neutrophil elastase (NE), an enzyme released by neutrophils during inflammation, and its inhibitor, alpha-1 antitrypsin (AAT). Intratracheal administration of NE in animal models led to emphysematous lesion formation, and deficiency in the gene for NE protected smoke-exposed mice from ECM destruction (35, 36). The strongest evidence for the *protease-antiprotease hypothesis* comes from the observation that human smokers with a deficiency in the gene for AAT rapidly develop emphysema, even without a smoking history (37). These data demonstrate the destructive potential of inflammatory cell types and highlights the role of cigarette smoke-induced inflammation in driving COPD tissue pathology.

Chronic bronchiolitis refers to the inflammatory processes that affect small airways (generally, with less than 2 mm of internal diameter). Inflammation within the small airways results in similar physiological changes as the large airways but with greater functional consequence, as was first described by Hogg *et al* (23). In addition to mucus and inflammatory exudates impeding airflow, the thickening of the airway walls reduces the volume of the airway lumen (38). This is the result of repeated damage and repair of the small airways which leads to

smooth muscle thickening and collagen deposition (20, 39). These changes are associated with increasing disease severity as small airway disease correlates with GOLD stage (40). The loss of alveolar architecture in emphysematous lesions also contributes to small airway disease as the destruction of alveolar septa detaches the basement membrane of small airways from the lung parenchyma (41). Under normal homeostatic conditions, alveolar septa tether small airways open; allowing them to resist the pressure exerted on them by the surrounding lung tissue when it is inflated. Without this tethering, the pressure from the surrounding airspace collapses the small airways (41). These data highlight the overlap between the three pathologies that constitute COPD and the necessity to view them as a single disease state.

In a COPD patient, a combination of large and small airways disease and emphysema are usually present with varying degrees, and though there is some controversy as to which pathology is the primary source of lung dysfunction, the unifying mechanism underlying all of these pathologies is inflammation. Despite the importance of inflammation in driving the pathogenesis of COPD, the biological pathways engaged during an inflammatory response to the main etiological factor for the disease, cigarette smoke, are poorly understood.

The clinical course of COPD periodically worsens in episodes described as acute exacerbations. The GOLD definition of an acute exacerbation of COPD is "an event in the natural course of the disease characterized by a change in the patient's baseline dyspnea, cough, and/or sputum beyond day-to-day variations, that is acute in onset and may warrant a change in regular medication in a patient with underlying COPD" (5). Increased inflammation is a hallmark of acute exacerbations and is believed to drive the exacerbated symptoms (42–44), though there is no agreed upon biological definition of an exacerbation. Typically, a COPD patient experiences one to two exacerbations annually and the frequency of exacerbations increases with

disease severity (45). More frequent exacerbations are also associated with a greater amount of inflammatory cells present in the lungs and an accelerated FEV_1 decline (46, 47), implying a cyclical relationship between disease exacerbations and disease pathology. This is further reinforced by observations in COPD patients defined as having a 'frequent exacerbator' phenotype as these individuals have an accelerated decline in FEV_1 (48). The repercussions of disease exacerbations are quite severe and more frequent exacerbations are associated with increased mortality (49). This is why the potential risk of a patient for exacerbation is an important part of the diagnosis of COPD and effects the treatment strategy employed (5).

Most exacerbations of COPD are caused by infectious agents and the majority of these are bacterial in nature (50). Other environmental agents such as air pollutants and airborne allergens are also causative factors in triggering exacerbations, but to a lesser extent (51, 52). Bacterial infection also increases the risk of COPD comorbidities, as the frequency of bacterial infection contributes to a greater risk of developing pneumonia in COPD patients (53, 54). The bacteria most commonly isolated from patients experiencing an exacerbation are nontypeable *Haemophilus influenza* (NTHi), *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* in that respective order (55). However, the lower airways which are traditionally thought of as sterile, are frequently colonized by bacteria in COPD patients (56–58). NTHi, in particular, is frequently found amongst the bacteria in the lower airways of COPD afflicted lungs (59). The presence of bacteria is not sufficient to trigger an exacerbation, it is rather the acquisition of a novel strain of bacteria that is associated with these periods of worsened symptoms (60). Specifically, the acquisition of a new strain of NTHi is the most common cause of a bacterial exacerbation (60). The inflammatory response is also significantly greater in bacterial exacerbations when compared to exacerbations where a new strain of bacteria

could not be detected (55). Inflammation drives the pathology of COPD and its increase during bacterial exacerbations potentially accelerates disease progression. Thus, identifying the mediators of inflammation engaged during a bacterial infection in the context of an inflamed lung will provide meaningful insight into the pathogenesis of COPD.

Experimental models of COPD

Experimental models are important tools for elucidating the underlying biological mechanisms involved in disease states. They are crucial for the identification of therapeutic targets, preclinical screening of therapeutic agents, and provide the ability to examine specific aspects of a disease in greater detail; allowing for a more comprehensive understanding. Developing experimental models of COPD are important as very few therapeutic options are available for the disease and there is a lack of therapeutics that specifically target the pathogenic factors that drive the disease (61). Though some currently available corticosteroid treatments have been shown to slow the decline in lung function (62), a better understanding of the biology of COPD is the key to developing more effective therapeutics, and disease models will be invaluable in this effort.

Despite the well-known identification of the main etiological factor for COPD development, there are many challenges in modeling this disease. As a stimulus, cigarette smoke is very complex, comprising of over 4700 different compounds (63). The habit of cigarette smoking is prone to great variability amongst smokers with potentially wildly divergent levels of exposure, making it difficult to establish a clinically relevant 'dose' of cigarette smoke to utilize. Additionally, the relative importance of other factors, such as those that contribute to comorbidities and exacerbations, in the pathogenesis of COPD are unknown as they may synergize with the damaging effects of cigarette smoke and be necessary to fully recreate a COPD-like pathology. The most restrictive limitation to the development of a comprehensive

model of COPD is time, as the first symptoms of COPD in humans often do not emerge until at least 20 years of smoking 20 cigarettes a day (64). This indicates that a human lung develops pathology after decades of exposure to cigarette smoke. This time frame is considerably longer than the life span of most model organisms and longer than the tenure of most graduate students. Despite these limitations, many models of COPD have been developed and are furthering the understanding of the disease.

The simplest and most economical means of examining a disease is through the use of cell culture systems, and experimental models have exposed various lung cell types to cigarette smoke in culture. These *in vitro* models have utilized various methods of simulating the conditions of a smoke-exposed lung: Studies have exposed cells to the individual components of cigarette smoke (65, 66), exposed cells to cigarette smoke condensate (67–69) , or cultured cells in smoke conditioned media (70). This approach can address specific questions, typically about the effects of cigarette smoke on a specific cell type. Cell culture systems can also be utilized to great effect with cells that are isolated from smoke-exposed lungs. These *ex vivo* experiments can examine how the environment of a cigarette smoke-exposed lung affects the phenotype of individual cells and how they respond to stimuli in isolation. However, due to the narrow scope of *in vitro* and *ex vivo* studies they often have to be accompanied by *in vivo* models to validate the findings in a setting that better reproduces that complexity of biological responses.

To establish COPD models with greater biological relevance, a number of experimental approaches have been pursued in laboratory animals. These *in vivo* models have attempted to reproduce the pathology of COPD in several different ways: Repeated instillation of an inflammatory stimuli such as endotoxin can lead to chronic inflammation and air flow obstruction (71). Instillation of proteolytic enzymes, similar to those released by inflammatory

cells, breakdown the ECM of the lung and form emphysematous lesions in experimental animals (72, 73). Models of induced apoptosis targeted to the lung have also demonstrated that cell death can contribute to emphysema (74, 75). Even models of malnutrition and starvation can lead to decreased lung function and airspace enlargement (76, 77). The limitation of these various methods is that they focus on the resultant pathology observed in COPD-afflicted lungs and do not attempt to produce the initial conditions that may give rise to COPD. As a result they provide data that only indirectly implicates biological phenomena that may drive disease pathogenesis.

The explosion of tools available to manipulate the genes of mice has led to numerous transgenic mouse models that develop COPD-like pathology without exposure to cigarette smoke or treatment with any other substance. Lung specific overexpression of the inflammatory genes tumor necrosis factor (TNF)-α, interleukin (IL)-18, and interferon (IFN)-ɣ lead to alveolar destruction as the experimental mice age that is significantly greater than wild type controls (78–80). Interestingly, the overexpression of a cytokine primarily associated with allergic responses, IL-13, was also capable of inducing airspace enlargement (81). Transforming growth factor (TGF)-β, which is also implicated in allergic responses, has also been shown to lead to spontaneous emphysematous lesion formation as demonstrated in mice deficient in αvβ6 which have increased TGF-β activation (82). More recently, spontaneous lung pathology was observed with the over expression of receptors for advanced glycation end-products (RAGE), which initiate inflammatory responses upon binding ligands released by cell damage (83). The commonality of these transgenic models of COPD is that they involve an enhanced and chronic inflammatory response in the lung. Though these models may not try to reproduce the disease from its environmental etiology, they do provide the proof-of-principle that prolonged and

unregulated inflammation can generate lung pathology similar to COPD. These studies demonstrate that there are many inflammatory mediators capable of eliciting COPD-like pathology; however, they fail to identify the specific inflammatory mechanisms that are engaged by cigarette smoke. An inflammatory gene capable of inducing lung pathology is largely irrelevant to the pathogenesis of COPD if the gene is not induced by cigarette smoke.

Intuitively, animal models that utilize cigarette smoke to generate disease are preferable, and the choice of model species is an important consideration as cigarette smoke exposure has been shown to have species specific effects. There are also practical implications and logistical limitations for various model species. Primates make powerful model species due to their similarity to humans, and though smoking-related disease has been modeled in primates (84), small animals are preferable due to the expense and ethical considerations associated with primate research. Guinea pigs are an excellent model species for COPD research as they develop many of the characteristic disease pathologies in response to cigarette smoke. Increased mucus production, emphysematous lesion formation, and small airway remodeling have been observed in guinea pigs with as little as 3 month of smoke exposure (85–87). Unfortunately, guinea pigs are expensive and are limited to small molecule therapeutics as there are a restrictive amount of antibodies and transgenic reagents available (88). Rats have also been observed to elaborate airspace enlargement, mucus production, and small airway remodeling (89), but rats share similar disadvantages to guinea pigs in terms of expense and the limited availability of reagents (88).

Most *in vivo* models of smoke exposure utilize mice. There are disadvantages to this approach, as measurements of lung mechanics are technically more difficult in mice due to their size. Increased mucus production is also difficult to observe in mice and cigarette smoke even

seems capable of suppressing mucus production in other murine experimental systems such as models of allergic disease (90). Additionally, mice seem incapable of reproducing severe disease (GOLD stage 3 and 4), as cigarette smoke-induced pathological changes indicative of advanced COPD have never been reported in mice. However, the advantages of using mice as a model species outweigh these limitations. There is a vast amount of reagents available for mice in comparison to the other model species and, specifically, there are many reagents for investigating inflammatory phenomena. Despite not being able to observe severe disease in mice, cigarette smoke is capable of producing measurable changes in small airway remodeling, lung function decline, and emphysematous lesion formation (91). Additionally, mice mount an inflammatory response to cigarette smoke that has a cellular profile similar to human smokers (91). For the specific questions that need to be addressed in this thesis relating to the identification of novel inflammatory mechanisms, mice are the best model animal to utilize.

A final caveat to consider in any model utilizing cigarette smoke exposure is the method by which mice are smoke exposed. There is a wide variety of smoke exposure systems available. Laboratories have even used a method of intranasal administration of a cigarette smoke solution similar to culture systems (62), but for obvious reasons exposure to cigarette smoke in a gaseous phase is preferable. There is currently no accepted standard methodology by which mice should be smoke exposed. This has led to a considerable amount of inter-laboratory variation in the effects of cigarette smoke exposure, with some labs reporting almost double the amount of detectable pathology and inflammation with similar smoking regimens (92, 93). One of the only measurements to establish a dose of cigarette smoke is the measure of the total particulate matter (TPM) that flows through the exposure chamber. Though there is no agreed upon TPM for establishing chronic inflammatory or cigarette smoke-induced lung pathology, it has been

established that there is a dose response to increasing TPM in terms of inflammation and pathology (94, 95). Cigarette smoke can also be administered as mainstream smoke, which mimics the smoke inhaled directly from a cigarette, or sidestream smoke, which mimics secondhand smoke exposure. Mainstream cigarette smoke has been shown to have a greater inflammatory effect than sidestream smoke administered at equal TPM concentrations (96). The inflammatory profile and pathological conditions of the lung change over the period of time which smoke exposure is administered (97, 98), thus the chronicity of the smoke exposure should also be considered. All of these conditions coupled with additional variables such as the availability of technical staff to smoke expose mice over weekends and the length of each smoke exposure period, create a large amount of permutations and combinations of different factors that results in most labs having a unique method of smoke exposure, and generates the variability observed between labs in the smoke exposure field.

With so many options for constructing a model of COPD, the specific research questions must be utilized to guide the design of the experimental setup. Ultimately, this thesis is concerned with the biological process of inflammation; both the inflammation induced by cigarette smoke exposure and the inflammatory response elicited by bacteria in the context of a smoke-exposed lung. A whole-body mainstream cigarette smoke exposure system was utilized for these studies because it was capable of inducing an inflammatory response in the lungs with a similar inflammatory cell profile to that of human smokers (99). Of additional benefit, this system has been shown to induce measurable lung pathology with chronic smoke exposure (100). The experiments completed for this thesis utilized the power of an experimental model to isolate specific biological processes and examine them in greater detail, the findings of which will provide greater insight into COPD as a whole.

Cigarette smoke-induced inflammation

The word inflammation derives from the Latin 'to set on fire' as a description of the characteristic symptoms of inflammation: pain, redness, swelling, and heat (101). Initially defined symptomatically, inflammation has come to describe the complex response of any vascularized tissue to infection and damage. This process is reviewed in (102), but briefly: The inflammatory response mobilizes innate immune mechanisms which in turn activate adaptive immune responses that together eliminate the injurious stimuli while initiating repair mechanisms. The ideal outcome of this process is the return of the afflicted tissue to homeostatic conditions. Inflammation is initiated by the sensing of pathogenic stimuli which leads to the production of mediators that increase the permeability of vascular endothelium, allowing the transport of plasma proteins, clotting factors, and complement proteins into the affected tissue. The activated endothelium also expresses addressins that facilitate the adhesion and migration of leukocytes into the tissue. Leukocytes are the essential cell types that propagate immune responses within vertebrate organisms. Once in the tissue, leukocytes follow a concentration gradient of chemokines to migrate to the specific area where immune responses need to be directed. Inflammatory responses must be regulated since this response is capable of damaging the host tissue along with any invading pathogens; as demonstrated by the tissue damage observed in cases of chronic inflammation (103). Inflammation is the precursor of almost every protective response in the human body and initiates powerful host defense mechanisms, but this biological process represents a double-edged sword capable of being the main factor driving the pathogenesis of diseases like COPD.

The inflammatory mechanisms engaged by cigarette smoke drive the pathogenesis of COPD (5, 21), and the accumulation of a specific cellular profile in the lungs has been

implicated in this process. The cell types that are frequently identified in the lungs of COPD patients are primarily macrophages, neutrophils, and lymphocytes (104–110). The mediators released by these cells play a critical role in airflow obstruction by inducing mucus hypersecretion, bronchial constriction, small airway collapse, and alveolar destruction. For example, the destruction of lung architecture that forms emphysematous lesions results from the release of neutrophil- and macrophage-derived elastases that catalyze the breakdown of the pulmonary ECM (111–114). CD8 T cells are the main lymphocyte found in the lungs of COPD patients and this cell's ability to trigger cytotoxicity in infected host cells may contribute to lung destruction, but the importance of CD8 T cells in the pathology of COPD has not been conclusively established (115). For these reasons, considerable emphasis has been placed on understanding how macrophages, and particularly neutrophils, are recruited to the lungs in response to cigarette smoke exposure (116). These mechanisms are not fully understood, but animal models have been used to elucidate some of the inflammatory mediators induced by cigarette smoke that lead to the accumulation of macrophages and neutrophils.

While the exact means by which cigarette smoke-induced inflammation is initiated are unknown, the inflammatory response is likely initiated in part by the sensing of damage to the pulmonary environment. Cigarette smoke is a complex mixture of carcinogens, toxins, reactive solids, and oxidants that can have direct cytotoxic effects and place the pulmonary environment under considerable stress (117). This has been demonstrated in animal models of cigarette smoke exposure where a panel of damage-associated molecular patterns (DAMPs) was measured in smoke-exposed mice, finding many to be increased (118). Extracellular adenosine triphosphate (ATP) is a DAMP that has been observed to be significantly increased after cigarette smoke exposure, and $P2X_7$, a receptor for extracellular ATP, has been implicated in

cigarette smoke-induced inflammation and the resulting pathology (119). Toll-like receptor (TLR) 4 is traditionally thought of as a sensor of bacterial ligands and deficiency in this receptor significantly attenuates cigarette smoke-induced inflammation, but this has been proposed to be a result of TLR4's ability to sense ligands released by cell damage such as heat shock protein (HSP) 70 (120, 121). RAGE has also been proposed to be a sensor of cigarette smoke-induced lung damage and RAGE deficient mice have been shown to have reduced inflammatory cells and cytokines (122, 123), but in a murine model of cigarette smoke extract administration and not exposure to actual cigarette smoke. Though there are many potentially toxic substances in cigarette smoke, the damage is at least partially the result of reactive oxygen species, as deficiency in NRF2, a crucial mediator of the antioxidant response, enhances emphysematous lesion formation (124). These results are promising but there are potentially many DAMPs and their associated receptors that could contribute to the inflammatory response mounted against cigarette smoke and additional work is needed to fully characterize this phenomenon.

Inflammation in response to tissue damage is propagated by cellular and molecular mechanisms associated with innate immunity, implying that cigarette smoke-induced inflammation is largely driven by innate immune mechanisms. This hypothesis has been tested in experimental models of cigarette smoke exposure (99, 125). These studies employed recombination activating gene (RAG)-deficient or Severe Combined Immunodeficiency (SCID) mice, both of which lack cells of the adaptive immune system, to show a similar inflammatory response in wild type and RAG/SCID-deficient mice. These data suggests that adaptive immune mechanisms are redundant to cigarette smoke-induced inflammation, but these findings fail to account for evidence that suggests that autoimmune processes contribute to pulmonary inflammation in COPD patients (126). A likely explanation is that innate immune mechanisms

initially drive cigarette smoke-induced inflammation, but as the exposure becomes more chronic, adaptive immunity can become engaged and further propagate the inflammatory response.

Identifying the importance of innate immunity in initiating cigarette smoke-induced inflammation also aids in the identification of the relevant cell types that mediate this response. Alveolar macrophages (AMs) are an innate tissue-resident cell population in the lung lumen that plays a central role in initiating pulmonary host defense mechanisms. This widely accepted knowledge has led to the hypothesis that AMs are the orchestrators of cigarette smoke-induced inflammation (127). Some ambiguity remains about the extent to which macrophages direct the inflammatory response to cigarette smoke, as this is experimentally challenging to pursue. Depleting macrophages is difficult to accomplish without fundamentally changing the pulmonary environment. Depletion of macrophages typically results in an influx of neutrophils (128), making attempts to measure a potential decrease in cigarette smoke-mediated neutrophil recruitment in macrophage-depleted lungs uninterpretable. Though it is difficult to assess cellular inflammation in this context, depletion of macrophages in a model of cigarette smoke-induced emphysema had a protective effect, lessening tissue destruction and loss of lung function (129). Epithelial cells are another cell type innately present in the lungs that likely plays a role in propagating the inflammatory response to cigarette smoke. Though there are few studies that directly examine the role of the epithelium in this process *in vivo*, epithelial cell cultures stimulated with cigarette smoke condensate identify the epithelium as a potential source of chemokines like the neutrophil attracting chemokine IL-8 (130).

Adaptive immune cells may also propagate cigarette smoke-induced inflammation in the context of long term exposure. As mentioned, CD8 T cells are present in the lungs of patients with advanced COPD (105, 110), and smoke-exposed CD8 T cell deficient mice elaborate a

blunted cellular inflammatory response and emphysema formation (131). Additionally, Th17 cells are a subset of CD4 T cells implicated in chronic neutrophil recruitment, and experimental models have shown them to be significant contributors to chronic inflammation and the formation of emphysematous lesions (132, 133). There are also CD4 regulatory T cells (Tregs) that negatively regulate the inflammatory response, and therapies that enhance Treg responses have been shown to reduce neutrophilic inflammation in a rat model of cigarette smoke exposure (134). Chronic exposure to cigarette smoke is associated with an imbalance between Th17 cells and Tregs (135), and this imbalance may play a role in the continuing inflammatory response. Much ambiguity remains about the antigen specificities of T cells activated by cigarette smoke and whether they are directed against self-antigens. Autoimmune reactivity may play a crucial role in COPD (116), but these adaptive responses are ultimately initiated by innate immune cell types and the primacy of innate immunity in this biological process has made cell types like the alveolar macrophage a main focus in understanding the mechanisms of cigarette smoke-induced inflammation.

Immune cells communicate through the production of cytokines to coordinate an inflammatory response. A wide range of inflammatory mediators have been assessed in COPD patients (reviewed in (136)), and more than 50 cytokines and chemokines of interest have been identified. The list of pro-inflammatory factors measured to be increased in COPD patients include TNF-α,IL-1β, IL-6, and more recently IL-18 (43, 137–143). The list also includes mediators with primarily chemoattractant properties, such as monocyte chemotactic protein (MCP)-1, and the neutrophil recruiting CXCL1 and IL-8 (43, 137, 144, 145). Despite the complex network of molecular signals measured in clinical samples, only a few have been the direct target of interventions in human patients, thus experimental models have been employed to

determine the relevance of these mediators. The classic inflammatory cytokine TNF-α was examined with smoke-exposed gene-deficient mice and found to be a crucial mediator of inflammation and lung pathology (92, 146). Inhibiting the cytokine and growth factor GM-CSF also attenuated neutrophilia (147, 148). Additionally, several chemokines have been examined and experiments targeting CXCR2, CXCR3, CCR-1, and CCR5 identified a role for all of these chemokines in the cigarette smoke-induced inflammatory response (149–152). It is also through animal models that proteolytic enzymes have been directly linked to the destruction of the lung's ECM as mice deficient in either MMP-12 or NE are protected from cigarette smoke-induced emphysema (36, 153). Despite these initial results, many potential inflammatory mediators have not been tested experimentally, continued experimentation is necessary to fully elucidate the biological pathways engaged by cigarette smoke.

The IL-1 family of cytokines has been implicated in both the initiation and persistence of inflammation in a wide range of diseases (154, 155). As such, IL-1 family members are a continuing focus of inflammatory research with cigarette smoke exposure models, with particular interest focused on the ligands for IL-1R1, IL-1α and IL-1β. Initially, Doz *et al* identified the critical role of IL-1R1 in cigarette smoke-induced neutrophil recruitment (154, 156). These findings were supported by a parallel study demonstrating that IL-1R1 KO mice had 60% less emphysema formation following 6 months of cigarette smoke exposure (157). IL-18, another member of the IL-1 family (158), was also shown to play a critical role in cigarette smoke-induced inflammation and emphysema formation (159). This gene deletion data has been further strengthened by studies that over-express IL-1β and IL-18 in the lungs of mice to induce pulmonary inflammation and emphysema without cigarette smoke (160, 161). More recent studies have shown that the crucial IL-1 ligand is IL-1α, and that the primary source of IL-1α is

alveolar macrophages (162, 163). IL-1 production is possibly the result of the sensing of DAMPs released in response to the cell damage and death induced by cigarette smoke as dying cells elicit inflammation through IL-1 pathways (164). Additionally, the $P2X_7$ receptor for the DAMP extracellular ATP has been shown to be required for cigarette smoke-induced IL-1 production (119, 165). These data would suggest that IL-1α production in response to cigarette smoke-induced pulmonary stress is a crucial mediator of the inflammatory response to this insult.

In addition to IL-1 family members, other inflammatory mediators are increased by cigarette smoke exposure, and the functional relevance of these biomarkers of inflammation must be investigated to fully understand the process of cigarette smoke-induced inflammation. Several studies have demonstrated that family-18 glycohydrolases such as the chitinases-like molecule Breast Regression Protein-39 (BRP-39 in mice or YKL-40 in humans), are upregulated in a variety of inflammatory conditions (166–168). Two members of this family of enzymatically active and inactive chitinases, acidic mammalian chitinase (AMCase) and BRP-39 have been shown to be crucial in cellular recruitment in murine models of allergic inflammation (169, 170). Additionally, YKL-40 was found to be significantly elevated in smokers without COPD and further elevated in smokers with diagnosed COPD (171). Furthermore, this study demonstrated that human macrophages stimulated with YKL-40 produced an array of neutrophil associated chemokines such as IL-8. BRP-39 is a promising potential mediator of cigarette smoke-induced inflammatory response, warranting additional experimentation to determine its functional relevance and role with respect to other mediators of cigarette smoke-induced inflammation.

Much progress has been made in understanding the mechanisms of cigarette smoke-induced inflammation utilizing a combined approach of clinical observation and experimental

model systems. However, continuing to elucidate this biological process is necessary as experimental findings have been difficult to translate to human disease. For example, studies by Churg *et al* provided evidence that TNFα plays a critical role in cigarette smoke-induced inflammation and lung destruction (146). These studies showed that TNFα KO mice were 60-80% protected against emphysema after chronic exposure to cigarette smoke (92, 146). In clinical studies, blocking TNFα with the monoclonal antibody infliximab, however, failed to have a beneficial effect for COPD patients; as assessed by symptoms, lung function, and exercise performance (172, 173). These studies highlight the need to better understand the inflammatory process engaged by cigarette smoke so that the differences between animal models and human disease can eventually be elucidated; which is a crucial step in developing novel therapeutics that improve the health of COPD patients.

Cigarette smoke and bacterial host defense

The lung consists of approximately 70^2 m of surface area that is continuously exposed to viruses, bacteria, fungi, and parasites yet symptomatic infections are relatively rare. This is because homeostasis of the lung is maintained by a powerful defense system that protects the host against these harmful agents (reviewed in (174)). This is accomplished through a combination of physical barriers and innate and adaptive immune mechanisms (Figure 1). Physical barriers provide the most fundamental defense mechanisms of the lungs as they separate the internal structure of the lung from the external environment while still allowing for the lung's primary function of gas exchange. This is accomplished through an epithelial layer held together with tight junction proteins covered in a layer of mucus and antimicrobial defensins that continuously elevator any debris out of the lung via the beating action of cilia. Other innate immune mechanisms include alveolar macrophages that intake and destroy invading pathogens by

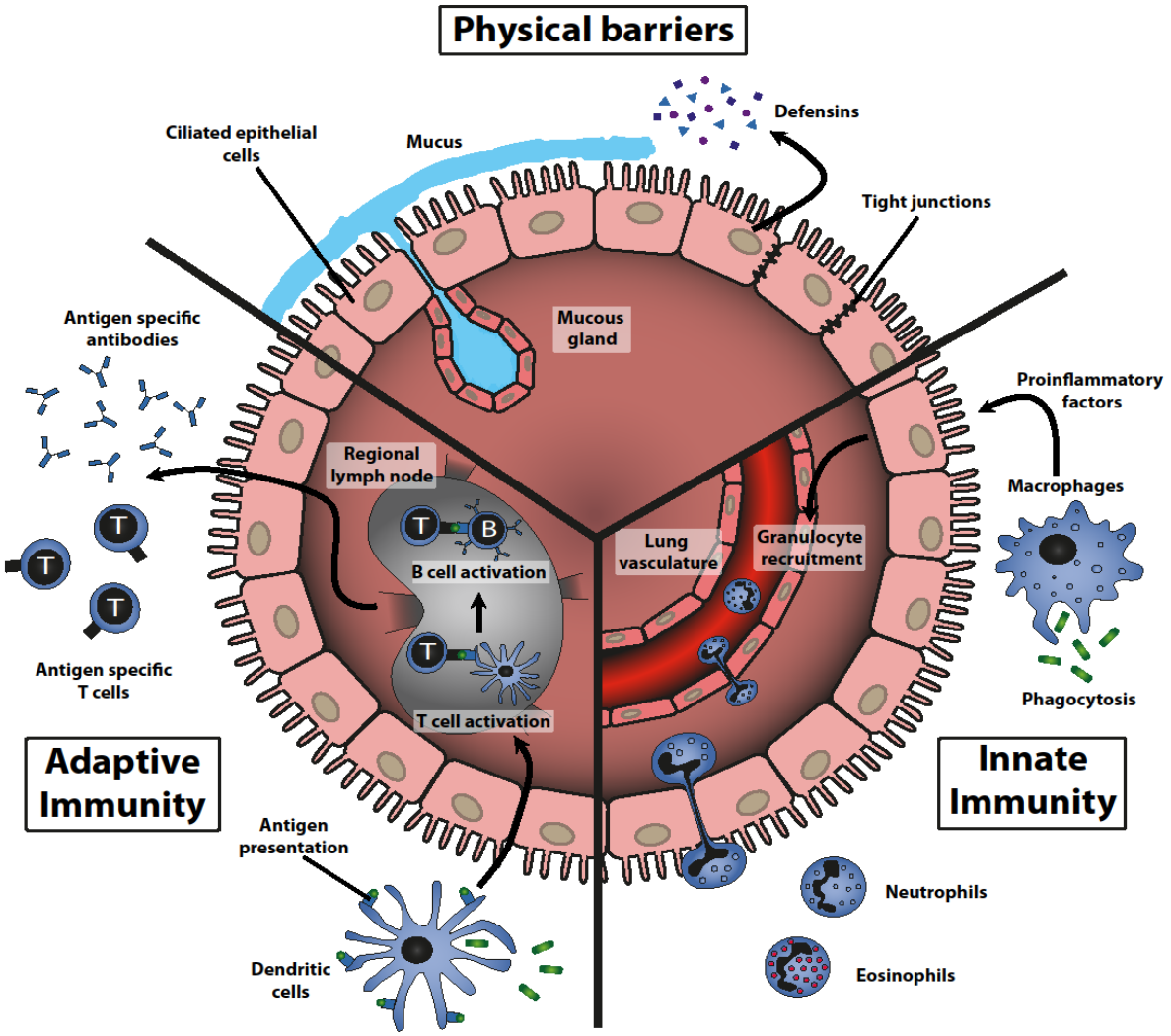

Figure 1 *Summary of host defense mechanisms of the lung*. Illustration created by Jake Nikota as a contribution to the 2014 Surgeon General's report: The Health Consequences of Smoking—50 Years of Progress, available at: www.cdc.gov/tobacco

phagocytosis in addition to initiating inflammatory reactions through the production of proinflammatory factors that recruit monocytes and granulocytes like neutrophils and eosinophils. Subsequent to the innate response, adaptive immunity is engaged by antigen presentation to T cells in regional lymph nodes which ultimately leads to the generation of antigen-specific effector T cells and antibody production. Accumulating evidence suggests that immune homeostasis in the lungs is distorted by cigarette smoke and that altered respiratory host defense contributes to the pathogenesis of COPD (175, 176). This seems to be the consequence of cigarette smoke's ability to both activate and suppress different aspects of the pulmonary host defense system.

The consequences of cigarette smoking for host defense are largely negative, as this addictive behaviour increases the risk and severity of respiratory infections. This is evident from epidemiological studies that show a significantly greater incidence of upper and lower respiratory tract infections in smokers (177, 178), and this greater incidence is independent of whether the smoker has COPD. Additionally, smoking is the strongest independent risk factor for invasive pneumococcal disease among immunocompetent adults (179). The colonization of the bronchial tree of COPD patients with microorganisms is regularly observed, even outside the context of an acute exacerbation (46, 59, 60). Though it is unclear why microbial agents colonize the lower respiratory tract, this phenomenon is likely the result of a suppressed antimicrobial response. There is an interplay between the predisposition of smokers to infection and the clinical course of COPD, as microbial infections are the major cause of COPD exacerbations (5, 180, 181). The increased disease severity observed during such an exacerbation is believed to be the result of an exaggerated inflammatory response to bacterial or viral insult (8, 182–184). Cumulatively, these data suggest that cigarette smoking impairs

antimicrobial host defense of the lung making the pulmonary environment more prone to microbial infections that ultimately cause damaging spikes in inflammation. Cigarette smoke impacts a wide array of immune functions (176), but for the purposes of this thesis, this section will focus on immune mechanisms relevant to bacterial host defense.

The disruptive effects of cigarette smoke on immune function are evident at the most fundamental level of host defense; the physical barriers of the lung. Cigarette smoke has been shown to increase the permeability of respiratory epithelium and reduce mucociliary clearance (185, 186). The specific mechanisms of impaired mucociliary clearance were recently identified by Lam *et al*, who found that impaired ciliary beat frequency and the decrease in the amount of cilia on lung epithelial cells was the result of cigarette smoke induced-autophagy, which triggered the breakdown of cilia components in respiratory epithelial cells (187). Cigarette smoke further disrupts barrier function by inducing apoptosis of the cell types that comprise the physical barrier of the lungs. *In vitro* administration of cigarette smoke extract has been shown to have a pro-apoptotic effect on epithelial cells (188, 189), and *in vivo* models have measured cigarette smoke-induced apoptosis of both lung epithelial and endothelial cells (188). Markers of apoptosis, fragmented DNA and caspase-3, were also observed in the epithelium and endothelium of human smokers (190). These findings have led to the hypothesis that, in addition to compromising host defense, cigarette smoke-induced apoptosis contributes to the pathogenesis of COPD, with the mechanism of this structural cell loss being the cigarette smoke-induced decrease of maintenance factors like vascular endothelial growth factor (VEGF) (191). Structural cells are also a source of cytokines, and cigarette smoke has been shown to attenuate *in vitro* production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-8 by epithelial cells following stimulation with lipopolysaccharide (LPS) (192). Attenuated cytokine

production coupled with the loss of barrier cells, and decreased ciliary action and mucus clearance, contribute to a state of increased risk of microbial invasion.

In addition to being a key mediator of the inflammatory response to cigarette smoke (127), AMs play a central role in sensing and eliminating bacterial agents early in the course of an infection. Cigarette smoke increases the number of macrophages in the lung environment, though a large body of evidence indicates that cigarette smoking functionally impairs these cells (193–198). AMs isolated from human smokers have a reduced ability to produce proinflammatory mediators following stimulation with LPS (199). Specifically, TNFα, IL-6, and Regulated on activation, normal T cell expressed and secreted (RANTES) production in response to the bacterial ligand stimulation was attenuated in smoke-exposed macrophages. Data generated from AMs isolated from murine models show a similar reduction in the production of TNFα, IL-6, and RANTES when stimulated with live bacteria such as NTHi (200, 201). These findings suggest that the role of AMs in sensing bacterial agents and initiating the inflammatory signaling cascade is compromised by cigarette smoke. This theory was challenged by a subsequent study by Gaschler *et al* that suggested that AMs were not suppressed, but rather express a unique profile of inflammatory mediators (202). While expression of the classic inflammatory factors TNFα, IL-6, and RANTES were decreased, AMs from smoke-exposed mice produced significantly greater amounts of chemokines, such as MCP-1, MCP-3, interferon gamma-induced protein (IP)-10, and macrophage inflammatory protein (MIP)-1γ. The cigarette smoke-altered phenotype of AMs extends beyond the process of cytokine production as long-standing evidence suggests that phagocytic activity is decreased in smoke-exposed AMs, which may result in a decreased ability to clear bacteria from the lungs (203–205). Improving NRF2-mediated antioxidant production improves phagocytic ability, implicating oxidative stress in the

cigarette smoke-mediated decrease in phagocytosis (205). The skewing of inflammatory mediators and suppressed phagocytic ability of cigarette smoke-exposed AMs indicates that this cell type is fundamentally altered by cigarette smoke. The full extent of the altered AM phenotype is currently unknown and comprehensive characterization of how cigarette smoke alters AM responses to bacteria could have implications for the management of bacterial infections in any individual regularly exposed to cigarette smoke.

Despite the altered phenotype of AMs, a cigarette smoke-exposed lung does not mount a suppressed inflammatory response to a bacterial infection and instead elaborates an exaggerated response. This exacerbated cellular inflammation has been observed with multiple bacteria species and, in all studies, was primarily neutrophilic in nature (200, 202, 204, 205). Multiplex analysis of the inflammatory mediators present in these lungs revealed a similar altered inflammatory mediator profile as was observed in experiments with AMs isolated from smoke-exposed lungs (202). These data imply a central role for the AM in the cigarette smoke-altered lung environment, and likely a central role in the exacerbated neutrophilic response to bacteria. It is unclear at present which inflammatory mediators are directly involved in neutrophil recruitment as many of the usually suspected inflammatory mediators are suppressed. A logical starting point would be to assess the importance of the neutrophil-recruiting mediators already identified in cigarette smoke-induced inflammation. Currently no study has attempted to identify the neutrophil recruiting mechanisms engaged during a bacterial exacerbation of cigarette smoke-induced inflammation. The study presented in chapter three of this thesis is the first investigation into this phenomenon.

In addition to its effects on innate immunity and physical barriers, there is also evidence that cigarette smoke alters the lung's adaptive immune response to pathogenic agents. Adaptive

immunity is initiated by antigen presentation, and the predominant antigen presenting cell type, dendritic cells (DCs), are recruited to the lungs by cigarette smoke in an IL-1 dependent manner (206). These cells possess an activated phenotype, but currently it is unknown whether there is any alteration in their ability to present antigens. The main effectors of adaptive immunity, T and B cells, have been demonstrated to have signaling defects in the context of cigarette smoke (207, 208), however, these data are controversial and were not able to be reproduced in mice and human peripheral blood cell cultures in a subsequent study (209). This ambiguity may result from variations in the dose of cigarette smoke between labs, as Thatcher *et al* have demonstrated that a high dose of cigarette smoke was sufficient to inhibit T cell function in a murine model of allergy while a low dose was not (210). Recently, Lugade *et al* showed that cigarette smoke attenuated adaptive immune responses to NTHi, and specifically reduced antibody production in response to a potential NTHi vaccine (211). This represents the first study where adaptive immunity was assessed in a model of cigarette smoke exposure and bacterial infection, highlighting the lack of experimental studies that examine the exacerbated phenotype. Though these data would indicate a suppressive effect, with so few relevant experimental studies, much ambiguity still surrounds the effect of cigarette smoke on adaptive immunity.

Due to the frequently observed bacterial colonization of the lower respiratory tract in COPD patients (46, 59, 60), one would expect a negative consequence of cigarette smoke-altered immunity on the ability to clear bacteria from the lungs, yet experimentally results are conflicting. Several *in vivo* models of bacterial infection of cigarette smoke-exposed mice have shown increased bacterial burden in smoke-exposed mice compared to control mice (200, 204, 205). These changes were interpreted to be a result of decreased AM phagocytic ability and impaired mucocilliary clearance. In contrast to these findings, NTHi was cleared more

effectively in smoke-exposed mice in studies by Gaschler *et al* (202, 212). This increased clearance was attributed to a cigarette smoke-induced increase in NTHi-specific IgA (212). The pathogen-specificity of this mechanism of increased bacterial clearance implies that the consequences of altered immunity on bacterial clearance is likely dependent on the specific bacteria utilized in the study and this should be considered when interpreting results from these experimental model systems.

Ultimately, interest in the inflammatory mechanisms engaged by cigarette smoke is due to the perceived role of this biological process in eliciting the pathology associated with COPD. Cigarette smoke alters the response to bacteria which results in an exaggerated inflammatory response. The mechanisms engaged during this increased inflammatory response are likely similar to those engaged during a bacteria-induced acute exacerbation of COPD. Because exacerbations of COPD are associated with an increased rate of lung function decline (46), addressing whether bacteria-exacerbated inflammation can significantly accelerate the pathology induced by cigarette smoke would provide valuable insight into the pathogenesis of COPD. Experimental models have addressed this question indirectly by measuring hydroxyproline, a breakdown product of the extracellular matrix protein collagen, and found it to be significantly increased by the combined stimulus of cigarette smoke and bacteria when compared to smoke-only controls (202). More recently, Ganesan *et al* measured airspace enlargement in a murine model of cigarette smoke-exposure and repeated doses of heat-killed NTHi, and found that NTHi administration could significantly increase cigarette smoke-induced pathology (213). These results indicate that cigarette smoke alters the response to bacteria inducing an inflammatory response that has the potential to contribute to the pathology associated with COPD. The underlying mechanisms that drive this exacerbated inflammation are currently not understood.

Addressing this experimentally will identify cellular and molecular mediators of inflammation that may prove to be valuable therapeutic targets while further elucidating the biological processes the drive the development and progression of COPD.

Studies completed for this thesis

The purpose of this thesis was to investigate the inflammatory pathways engaged in a cigarette smoke-exposed lung and those engaged by bacteria in a smoke-exposed lung. The over-arching hypothesis for the studies completed in this thesis was that cigarette smoke alters the response of the lungs to bacteria and this altered response leads to exacerbated inflammation. This work began by identifying mediators of the inflammatory response elicited by cigarette smoke alone, and subsequently investigated these mediators in a model of bacteria-exacerbated cigarette smoke-induced inflammation. Individual hypotheses were generated for each study and each was completed sequentially with the subsequent hypothesis generated from questions raised by the previous study.

In chapter two we tested the importance of BRP-39 in the inflammatory response to cigarette smoke, based on previous evidence that BRP-39 expression is increased by cigarette smoke and that its expression is a crucial inflammatory mediator in experimental models of allergy. This work identified BRP-39 as a biomarker of the inflammatory response that was not crucial to cigarette smoke-mediated cell recruitment. This work contrasted the inflammatory response between cigarette smoke and allergic airways inflammation. These experiments were performed in tandem with several other studies identifying and characterizing the IL-1 signaling pathway as a crucial mediator of cigarette smoke-induced inflammation. This study presents several key observations that repeat seminal findings demonstrating the importance of IL-1 in the neutrophilic response to cigarette smoke.

Building on the IL-1 data generated in chapter two, we sought to investigate the role of IL-1 signaling in the context of cigarette smoke exposure and bacterial infection. This work identified the phenomenon that cigarette smoke primes AMs to produce exacerbated amounts of IL-1α in response to bacterial stimulation, and that IL-1α is a crucial mediator of NTHi-exacerbated neutrophil recruitment. These data, reported in chapter three, represents the first reported mechanism of neutrophil recruitment in a model of bacterial exacerbation of cigarette smoke-induced inflammation.

The data presented in chapter three added to the findings that demonstrate the altered phenotype of a smoke-exposed AM. To gain a comprehensive perspective on the extent of a smoke-exposed AM's altered response to bacteria, a microarray was performed to analyze the whole genome of AMs isolated from smoke-exposed mice that were stimulated with NTHi. The results of this research are reported in chapter four of this thesis.

The study presented in chapter two has been published, while chapter three has been conditionally accepted for publication. The study in chapter four has been submitted for publication.

CHAPTER TWO

Differential expression and function of breast regression protein 39 (BRP-39) in murine models of subacute cigarette smoke exposure and allergic airway inflammation

This article appeared in *Respiratory Research*, 2011 Apr 7; 12:39.

Chitinase 3-Like-1 has been observed to be increased in a variety of inflammatory conditions (166–168), including upregulated expression in the lungs of COPD patients (171). The importance of BRP-39, the mouse homologue of chitinase 3-Like-1, in the inflammatory response has been established in models of allergy (169), but it had not been investigated in cigarette smoke-induced inflammation. Using a murine model of smoke exposure, we addressed this question, and because of the importance of BRP-39 in allergic disease, we took the opportunity to contrast the inflammatory response elicited by cigarette smoke with the common allergen, house dust mite.

This work was completed as part of a series of studies conducted with Dr. Fernando Botelho investigating the role of IL-1 in cigarette smoke-induced inflammation. For this reason, we examined the role of BRP-39 specifically in the context of IL-1-mediated inflammation. This series of IL-1-focused experiments yielded my coauthorship on the following papers:

IL-1α/IL-1R1 expression in chronic obstructive pulmonary disease and mechanistic relevance to smoke-induced neutrophilia in mice
Published in PLoS One, 2011; 6(12):e28457

A mouse GM-CSF receptor antibody attenuates neutrophilia in mice exposed to cigarette smoke
Published in the European Respiratory Journal, 2011 Aug; 38(2):285-94

Cigarette smoke-induced accumulation of lung dendritic cells is interleukin-1α-dependent in mice
Published in Respiratory Research, 2012 Sep 19; 13:81

Dr. Martin Stämpfli and I were responsible for the experimental design and the analysis of the data. I was responsible for conducting the experiments, and for writing the article. Dr Fernando Botelho assisted technically, and provided mentorship for the development of experimental skills and techniques. Dr. Carla Bauer assisted technically with experiments and the editing process. Drs. Manel Jordana, Anthony Coyle, and Alison Humbles provided advice on the experimental protocols and design, and provided essential experimental reagents. The final manuscript was edited by Dr. Martin Stämpfli.

RESPIRATORY RESEARCH

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Differential expression and function of breast regression protein 39 (BRP-39) in murine models of subacute cigarette smoke exposure and allergic airway inflammation

Jake K Nikota[1], Fernando M Botelho[2], Carla MT Bauer[1], Manel Jordana[2], Anthony J Coyle[4,5], Alison A Humbles[3] and Martin R Stampfli[2,5*]

Abstract

Background: While the presence of the chitinase-like molecule YKL40 has been reported in COPD and asthma, its relevance to inflammatory processes elicited by cigarette smoke and common environmental allergens, such as house dust mite (HDM), is not well understood. The objective of the current study was to assess expression and function of BRP-39, the murine equivalent of YKL40 in a murine model of cigarette smoke-induced inflammation and contrast expression and function to a model of HDM-induced allergic airway inflammation.

Methods: CD1, C57BL/6, and BALB/c mice were room air- or cigarette smoke-exposed for 4 days in a whole-body exposure system. In separate experiments, BALB/c mice were challenged with HDM extract once a day for 10 days. BRP-39 was assessed by ELISA and immunohistochemistry. IL-13, IL-1R1, IL-18, and BRP-39 knock out (KO) mice were utilized to assess the mechanism and relevance of BRP-39 in cigarette smoke- and HDM-induced airway inflammation.

Results: Cigarette smoke exposure elicited a robust induction of BRP-39 but not the catalytically active chitinase, AMCase, in lung epithelial cells and alveolar macrophages of all mouse strains tested. Both BRP-39 and AMCase were increased in lung tissue after HDM exposure. Examining smoke-exposed IL-1R1, IL-18, and IL-13 deficient mice, BRP-39 induction was found to be IL-1 and not IL-18 or IL-13 dependent, while induction of BRP-39 by HDM was independent of IL-1 and IL-13. Despite the importance of BRP-39 in cellular inflammation in HDM-induced airway inflammation, BRP-39 was found to be redundant for cigarette smoke-induced airway inflammation and the adjuvant properties of cigarette smoke.

Conclusions: These data highlight the contrast between the importance of BRP-39 in HDM- and cigarette smoke-induced inflammation. While functionally important in HDM-induced inflammation, BRP-39 is a biomarker of cigarette smoke induced inflammation which is the byproduct of an IL-1 inflammatory pathway.

Background

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide [1,2]. COPD is characterized as airflow limitation that is not fully reversible, progressive in nature, and associated with an abnormal inflammatory response in the lung to noxious particles or gases such as those contained within cigarette smoke [3]. The cellular components of this inflammatory response are characteristically macrophages, neutrophils, and CD8+ T lymphocytes [4-9]. A number of mediators released by these cells likely play a critical role in airflow obstruction because of their potential to induce mucus hypersecretion and alveolar destruction. Although recent studies have implicated members of the IL-1 family of cytokines in the inflammatory pathways activated by cigarette smoke

* Correspondence: stampfli@mcmaster.ca
[2]Department of Pathology and Molecular Medicine, Centre for Gene Therapeutics, McMaster University, Hamilton, Ontario, Canada
Full list of author information is available at the end of the article

[10,11], much ambiguity remains. Understanding the cellular and molecular mechanisms of cigarette smoke induced inflammation will shed light on disease pathogenesis and identify future therapeutic targets.

It is well understood that family-18 glycosyl hydrolases such as the chitinase-like molecule YKL-40 and the murine homologue breast regression protein (BRP)-39 are upregulated in a variety of inflammatory conditions [12-14]. Two members of this family of enzymatically active and inactive chitinases, acidic mammalian chitinase (AMCase) and BRP-39 have been shown to be crucial in murine models of allergic inflammation. Specifically, BRP-39 and AMCase have been shown to be a requirement for allergic sensitization in ovalbumin (OVA) and house dust mite (HDM) models of allergic airways disease [15,16]. Additionally, YKL-40 was found to be significantly elevated in smokers without COPD and further elevated in smokers with diagnosed COPD [17,18]. Moreover, human macrophages stimulated with YKL-40 produced the neutrophil chemoattractant IL-8, providing evidence that chitinases such as BRP-39 may contribute to the inflammatory response elicited by cigarette smoke. Studies in animal models, however, are needed to investigate the functional relevance and mechanism of induction of chitinases in distinct pulmonary inflammatory diseases. In murine models, cigarette smoke causes neutrophil infiltration into the lungs similar to smoke-induced inflammation in humans [19-22]. Thus, murine models may be utilized to investigate the importance of BRP-39 in cigarette smoke-induced inflammatory processes relative to the already established importance of BRP-39 in models of allergic airway disease.

In this study we sought to determine the relevance of BRP-39, in the inflammatory response elicited by cigarette smoke and house dust mite. We identify BRP-39 as a biomarker, but not a mediator, of subacute cigarette smoke-induced inflammation and identify IL-1R1 mediated pathways as critical for the induction of BRP-39. In contrast, BRP-39 was required for the expression of allergic airway inflammation. Our study shows a differential requirement for BRP-39 in cigarette smoke-induced inflammation and models of allergic asthma.

Methods

Animals

Female inbred C57BL/6, BALB/c mice and outbred CD1 mice (6-8 wk old) were purchased from Charles River Laboratories (Montreal, PQ, Canada). BRP-39 deficient mice, developed on a BALB/c background, and their wild type (WT) littermates were bred at Medimmune LLC, Gaithersburg, MD, USA. IL-13 deficient mice on a BALB/c background (kindly provided by A McKenzie, MRC lab, Cambridge England [23]) were bred at McMaster University. IL-1R1 knock out (KO) and IL-18 KO mice on a C57BL/6 background were obtained from The Jackson Laboratories (Bar Harbour ME, USA). All mice were maintained under specific pathogen-free conditions in an access-restricted area, on a 12-h light-dark cycle, with food and water provided *ad libitum*. The Animal Research Ethics Board of McMaster University approved all experiments.

Cigarette smoke exposure protocol

C57BL/6, BALB/c, and CD1 mice as well as IL-13, IL-18, IL-1R1, and BRP-39 KO mice were exposed to cigarette smoke using a whole body smoke exposure system (SIU-48, Promech Lab AB (Vintrie, Sweden)) as described in detail previously [19]. Mice were exposed to 12 2R4F reference cigarettes with filters removed (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) for a period of approximately 50 minutes, twice daily, for four days. This exposure period followed an initial acclimatization period whereby mice were accustomed to smoke exposure chamber over a three-day period. Control animals were exposed to room air only.

HDM exposure protocol

WT C57BL/6 and BALB/c mice as well as IL-13, IL-1R1, and BRP-39 KO mice were exposed to HDM using a protocol that was described in detail previously [24]. Briefly, animals were anesthetized with isoflurane (Abbott Laboratories, Saint-Laurent, Quebec, Canada) using a rodent anesthetic machine (Penlon Limited Abingdon, England) and inoculated intranasally with 25 μg of HDM extract (Greer Laboratories, Lenoir, NC, USA) in 10 μl of saline, 5 days/week for two consecutive weeks.

OVA Challenge Protocol

WT BALB/c and BRP-39 KO mice were placed into a plexiglass chamber and exposed to 1% (w/v) OVA (Grade V, Sigma-Aldrich, Oakville, ON, Canada) in sterile saline for 20 minutes daily as described previously [25]. The aerosol was generated using a Bennet twin nebulizer at a flow rate of 10 L/min. Exposure to OVA occurred after the second of the two daily cigarette smoke exposures. Two weeks of smoke exposure were utilized when establishing OVA sensitization. For the *in vivo* recall challenge, mice were exposed to aerosolized OVA for 20 minutes on three consecutive days.

Collection of specimens

Mice were anesthetized with isoflurane and euthanized by exsanguination prior to excision of the lungs. The trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). Prior to BAL, the right lobe of

the lung was tied off and placed in ice cold PBS for generating homogenates or preparing lung single cell suspensions. Bronchoalveolar lavage (BAL) fluid was collected after instilling the left lungs with 0.25 ml of ice cold 1x phosphate-buffered saline (1x PBS), followed by 0.2 ml of 1x PBS (6). Total cell numbers were counted using a haemocytometer. Cytospins were stained with Hema 3 (Biochemical Sciences Inc., Swedesboro, New Jersey, USA). 500 cells were counted per cytospin to identify mononuclear cells, neutrophils, and eosinophils. Following BAL, lungs were fixed at 30 cm H_20 pressure in 10% formalin for histological assessment.

Chitinase ELISAs

Lungs were homogenized in 1 mL PBS using a Polytron PT 2100 homogenizer (Kinematica, Switzerland). AMCase and BRP-39 levels were assessed by enzyme linked immune-sorbent assay (ELISA). The assay utilized anti-BRP-39 or anti-AMCase monoclonal antibodies for capture and respective biotinylated polyclonal antibodies for development (Medimmune LLC). Streptavidin conjugated horse radish peroxidase (HRP) (R&D Systems, Mineapolis, MN) and tetramethylbenzidine (BioFX Laboratories Owings Mills, MD) provided the enzymatic reaction and 2 fold dilutions beginning at 1000 ng and 100 ng of recombinant AMCase and BRP-39 respectively (Medimmune LLC), provided the standard for quantification. To control for variability in protein concentration between homogenate samples, Bradford assay (Bio Rad, Hercules, CA) was conducted to determine the total protein of the sample. Chitinase levels were expressed as percent of total protein.

Immunohistochemistry

Sections (4 μm) were cut from formalin-fixed, paraffin-embedded lung tissues. Antigens were retrieved by incubating tissue sections for 45 minutes in 0.01 M citrate buffer prior to incubation for 1 hour with primary anti-BRP-39 polyclonal rabbit antibody (Medimmune LLC) diluted in UltrAb diluent (Thermo Fisher Scientific, Waltham, MA) at 7 μg/mL. Recombinant AMCase at a concentration of 1 μg/mL (Medimmune LLC) was incubated for 1 hr with the primary antibody to control for cross reactivity with the similarly structured AMCase. IHC was developed with anti-rabbit Dakocytomation HRP (Dako, Glostrup, Denmark) and counterstained in a modified Mayer's Hematoxylin solution.

Flow cytometric analysis

Lung mononuclear cells were isolated as previously described [26]. Briefly, lungs were collected in 1x phosphate-buffered saline (PBS) and cell suspensions were generated by mechanical mincing and collagenase digestion. Debris was removed by passage through nylon mesh and cells were resuspended in 1x PBS containing 0.3% bovine serum albumin (Invitrogen, Burlington, ON, Canada) or in RPMI supplemented with 10% FBS (Sigma-Aldrich, Oakville, ON, Canada), 1% L-glutamine, and 1% penicillin/streptomycin for intracellular staining (Invitrogen, Burlington, ON, Canada). 1×10^6 lung mononuclear cells were washed once with 1x PBS/0.3% bovine serum albumin (BSA) and stained with primary antibodies directly conjugated to fluorochromes for 30 minutes at 4°C. 10^5 live events were acquired on an LSR II (BD Biosciences) flow cytometer and data analyzed with FlowJo analysis software (TreeStar Inc. and Standford University, Palo Alto, California). The following antibodies were used for flow cytometric analysis: FITC-conjugated anti-CD11c, PE-conjugated anti-CD11b, PE-Alexa Flour 610-conjugated anti-CD4 (Invitrogen), PE-cy5-conjugated anti-CD19, PE-cy7-conjugated anti-CD69, APC-conjugated anti-MHC class II, Alexa700-conjugated anti-Gr-1 (Invitrogen), APC-Alexa750-conjugated anti-CD8 (Invitrogen), Pacific Blue-conjugated anti-CD3. All antibodies were purchased from BD Biosciences (San Jose, California) or eBioscience (San Diego, California) unless otherwise indicated.

For intracellular flow cytometric analysis, whole lung cells were cultured for 4.5 hours in the presence of phorbol myristate acetate (PMA) and ionomycin (Sigma, St. Louis, MO, USA). Intracellular staining for cytokines was performed using BD cytofix/cytoperm and BD perm/wash reagents with GolgiStop as recommended by BD Pharmingen. Intracellular cytokine staining was performed using following antibodies: FITC-conjugated anti-T1/ST2 (MD Bioproducts), PE-conjugated anti-IL-5, PE Cy 5-conjugated anti-CD86, PE Cy 5.5-conjugated anti-CD11c, APC-conjugated anti-MHC II, Alexa Fluor 700-conjugated anti-Gr-1 (Invitrogen). All antibodies were purchased from BD Biosciences (San Jose, California) or eBioscience (San Diego, California) unless otherwise indicated. Isotype controls were utilized for each stain and are demonstrated in Additional File 1.

Statistical analysis

Data are expressed as means ± SEMs. Statistical analysis was performed with SPSS statistical software version 17.0 (Chicago, IL, USA). Univariate General Linear Model was used to assess significance; t-tests were subsequently used for 2-group comparison. Normal distribution could not be assumed for neutrophil and eosinophil data and Mann-Whitney U tests were utilized for these comparisons. Differences were considered statistically significant when $p < 0.05$. All statistically significant findings were repeated and data shown are representative of 2 experiments.

Results

Cigarette smoke-induced inflammation and expression of chitinases and chitinase-like molecules

To investigate the impact of cigarette smoke exposure on chitinase expression, BALB/c, C57BL/6, and CD1 mice were exposed to cigarette smoke twice daily for a 4 day period. Mice were sacrificed 18 hours after their last smoke exposure. Figure 1A shows the BAL cellular profile. We observed an increased total cell number in smoke- compared to room air-exposed mice in all three strains of mice. While all of the examined strains demonstrated significantly increased numbers of neutrophils in the BAL, neutrophilia was most robust in CD1 mice and least pronounced in C57BL/6 mice.

Since chitinase expression can be induced by cigarette smoke in humans [17], we sought to measure BRP-39 and AMCase expression in lung homogenates of room air- and cigarette smoke-exposed BALB/c, C57BL/6, and CD1 mice. We observed a statistically significant increase in the chitinase-like molecule BRP-39 after smoke exposure in all mouse strains (Figure 1B). The highest baseline levels of BRP-39 and most dramatic increase in BRP-39 levels were observed in CD1 mice. In contrast to BRP-39, the enzymatically active AMCase was not increased after 4 days of smoke exposure in any of the examined mouse strains (Figure 1B). Both AMCase and BRP-39 were significantly upregulated after 2 weeks of HDM exposure (Figure 1C), confirming previous reports [15,16,27,28].

Localization of BRP-39 expression after cigarette smoke exposure

To investigate the cellular source of BRP-39 expression, we performed immunohistochemistry on formalin fixed lung tissues from cigarette smoke- and room air-exposed BALB/c mice. We observed increased BRP-39 expression in the airway epithelium following smoke exposure, although low baseline expression of BRP-39 was visible in the epithelium of room air-exposed mice (Figure 2A). Analysis of lung parenchyma revealed positive staining in alveolar macrophages in tissues from smoke-exposed mice (Figure 2B). The signal was BRP-39-specific; lung tissues from 4 day smoke-exposed mice stained with a rabbit IgG isotype control antibody and 4 day smoke-exposed BRP-39 KO mice stained with anti-BRP39 antibodies showed no signal (Representative pictures are shown in Figures 2A and 2B).

BRP-39 induction is IL-1 dependent after subacute cigarette smoke exposure

Previous studies have implied that IL-13 is necessary to induce pulmonary BRP-39 production in models of allergic airway inflammation [15,29]. To investigate the role of IL-13 in the cigarette smoke mediated induction

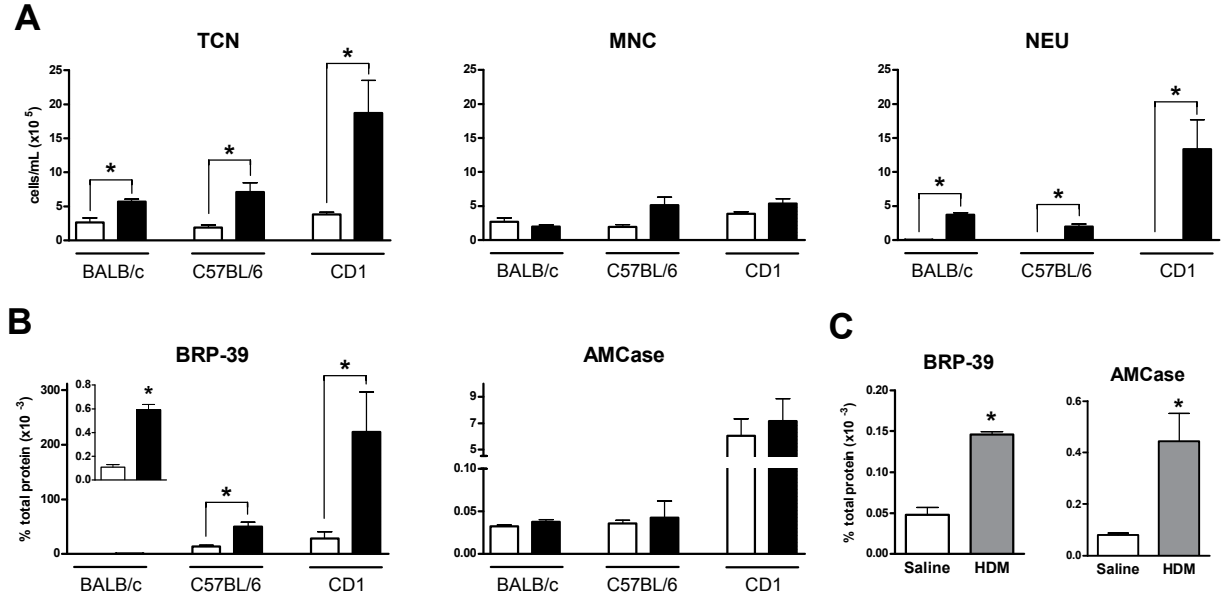

Figure 1 ***Cigarette smoke and HDM induce chitinase expression in the lung***. BALB/c, C57BL/6, and CD1 mice were exposed to room air (white bar) or cigarette smoke (black bar) for four days. (A) Total cell numbers (TCN), mononuclear cells (MNC), and neutrophils (NEU) in the BAL fluid were obtained. (B) BRP-39 and AMCase levels were assessed by ELISA. (C) BALB/c mice were challenged with saline (white bars) or HDM (grey bars) for 2 weeks and AMCase and BRP-39 levels were assessed by ELISA in lung homogenates. n = 5, data shown are representative of two separate experiments, * indicate $P < 0.05$.

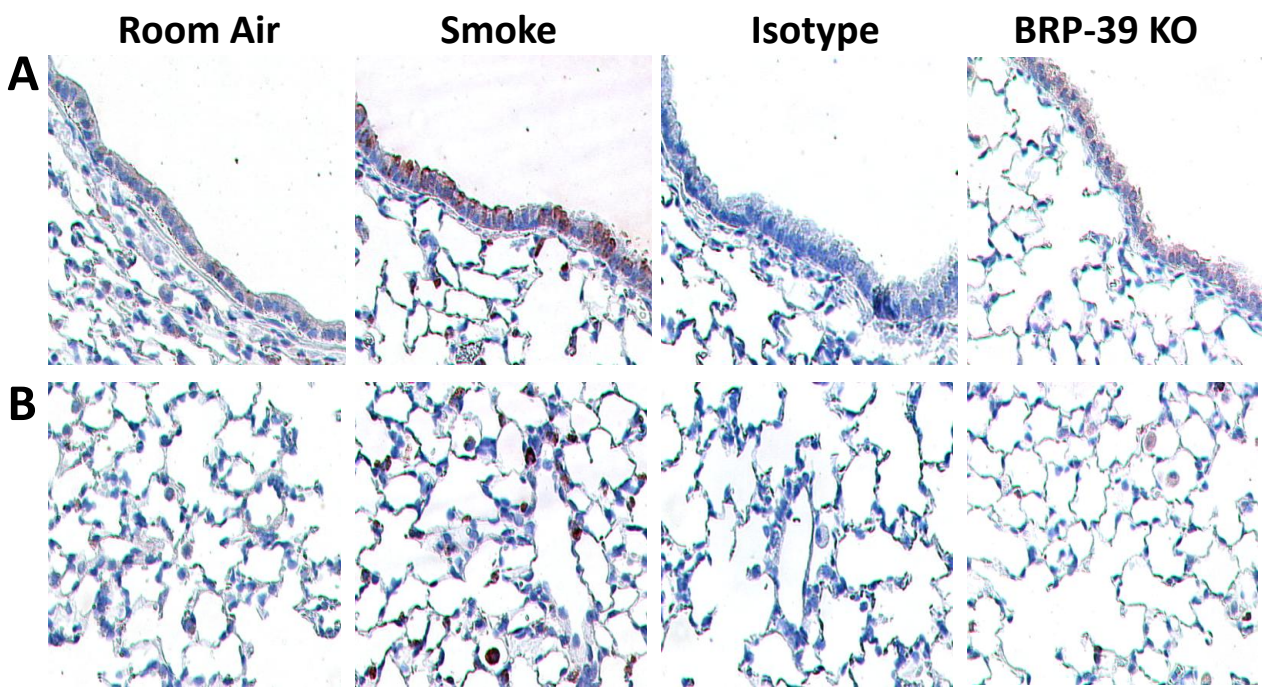

Figure 2 ***BRP-39 is induced in lung epithelium and alveolar macrophages***. BALB/c mice were room air or cigarette smoke-exposed for 4 days. BRP-39 expression was visualized in lung tissues by immunohistochemistry. Images represent BRP-39-stained lung sections from room air and cigarette smoke-exposed mice, IgG isotype stained tissue sections from smoke-exposed mice, and BRP-39 stained tissue sections from smoke-exposed BRP-39 KO mice. Representative BRP-39 expression in (A) airway epithelium and (B) lung parenchyma are shown.

of BRP-39, IL-13 deficient and BALB/c control mice were smoke-exposed and BRP-39 levels were determined in lung homogenates by ELISA. Figure 3A shows that there was no difference in the cellular profile in regards to total cells, mononuclear cells, or neutrophils in the BAL as well as no difference in BRP-39 levels between smoke exposed IL-13 deficient and WT mice.

IL-1R1 and IL-18 have been shown to be crucial components in the neutrophilic inflammation elicited by cigarette smoke [10,11,30]. We therefore investigated whether IL-1R1 and IL-18 may be responsible for BRP-39 induction in this model. Mice deficient in IL-1R1, and age matched C57BL/6 mice were exposed to cigarette smoke. Analysis of BAL fluid revealed a significant attenuation of cigarette smoke induced neutrophilia in IL-1R1 KO (Figure 3B). BRP-39 expression was also abrogated in these experiments with significantly reduced BRP-39 induction in smoke exposed IL-1R1 KO mice. The same experiments were performed with IL-18 deficient and age match C57BL/6 mice (Figure 3C). Smoke-exposed IL-18 KO mice showed no significant reduction in neutrophilic inflammation when compared to smoke-exposed WT mice and no impairment in BRP-39 induction was observed. Immunohistochemistry showed a loss of BRP-39 signal in alveolar macrophages and airway epithelial cells in smoke exposed IL-1R1 KO compared to WT mice (Figure 3D). These data suggest that BRP-39 is induced by inflammatory mechanisms that are integral to the neutrophil inflammation elicited by cigarette smoke.

HDM induced BRP-39 expression is IL-13 and IL-1 independent

Though IL-13 is redundant to the inflammatory process and induction of BRP-39 in a model of smoke exposure, we sought to investigate whether IL-13 was essential for the induction of BRP-39 in models of allergic airway inflammation. Thus IL-13 KO and BALB/c control mice were exposed to 2 weeks of HDM. As previously reported in models of allergic airway inflammation [31], IL-13 KO mice mount a dramatically decreased eosinophilic response to HDM (Figure 4A). We observed similar expression of BRP-39 in IL-13 KO and WT control mice, inferring a redundant role for IL-13 in the induction of BRP-39 by HDM.

To determine if IL-1 is equally a critical component of BRP-39 induction in models of allergic airway inflammation, IL-1R1 KO mice were HDM exposed for a 2 week period. No significant change was observed in IL-1R1 KO mice in terms of BAL total cells, mononuclear cells, and eosinophils when compared to WT controls (Figure 4B). No detectable levels of BAL neutrophils were observed in

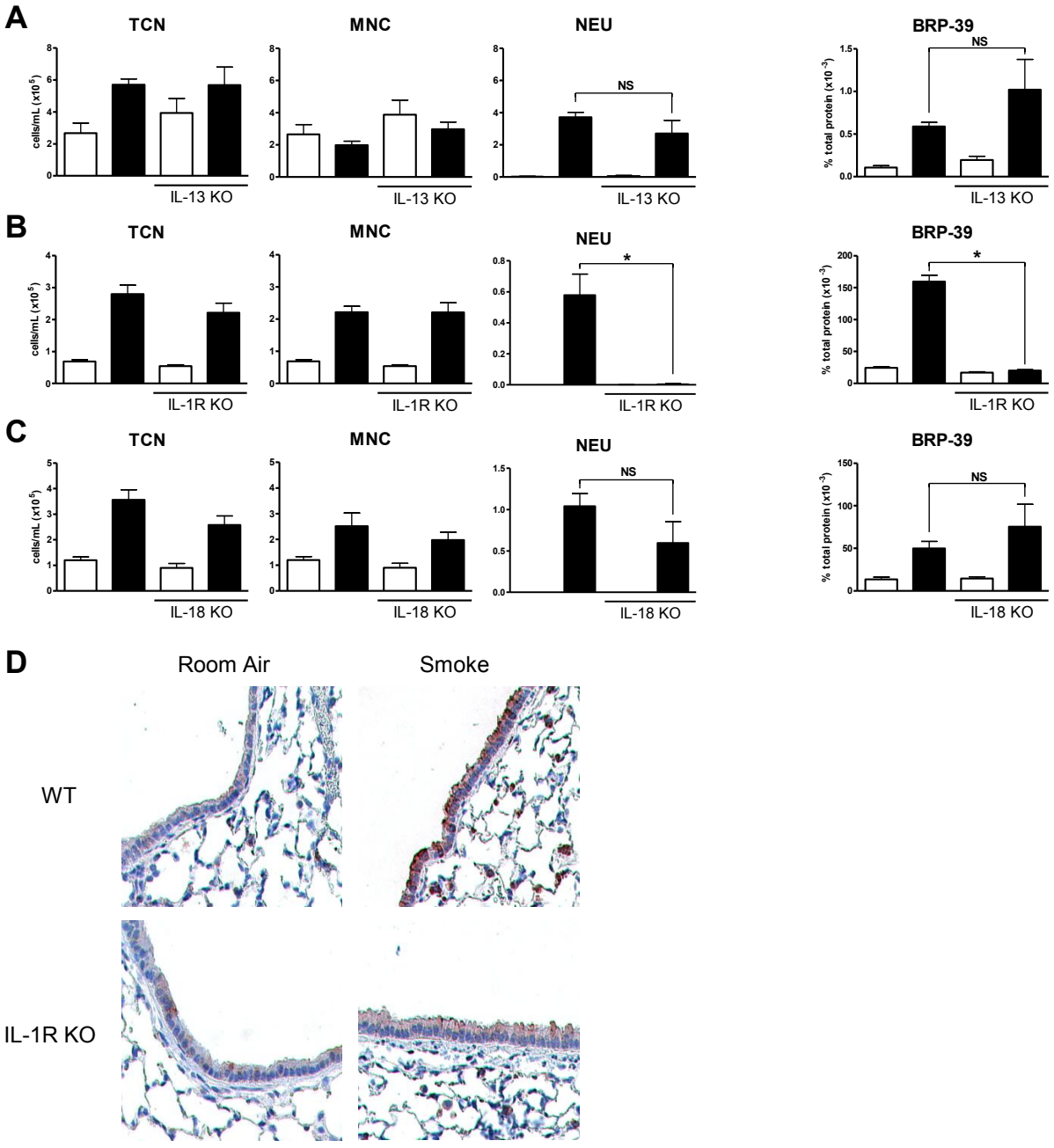

Figure 3 ***Cigarette smoke induced BRP-39 production is IL-1 dependent***. WT BALB/c and IL-13 KO mice were room air (white bars) or cigarette smoke-exposed (black bars). (A) Data show total cell numbers (TCN), mononuclear cells (MNC), and neutrophils (NEU) in the BAL as well as BRP-39 expression in lung homogenates. WT C57BL/6 and IL-1R1 KO (B) or IL-18 KO (C) mice were room air or cigarette smoke-exposed with the same corresponding readouts. (D) Immunohistochemistry was performed to identify the localization of BRP-39 expression in WT and IL-1R1 KO mice. n = 5, data shown in B are representative of 2 separate experiments, * indicate $P < 0.05$.

these experiments (data not shown). Despite changes to the inflammatory phenotype, IL-1R1 KO mice demonstrated no change in BRP-39 expression (Figure 4B). Therefore, BRP-39 induction by cigarette smoke is IL-1 dependent but BRP-39 induction by HDM is IL-1 independent.

BRP-39 is redundant in the inflammatory response to cigarette smoke

Having demonstrated that BRP-39 upregulation and neutrophil lung infiltration are IL-1 dependent phenomena, we sought to determine the relevance of BRP-39 to cigarette smoke-induced inflammation. BRP-39 KO

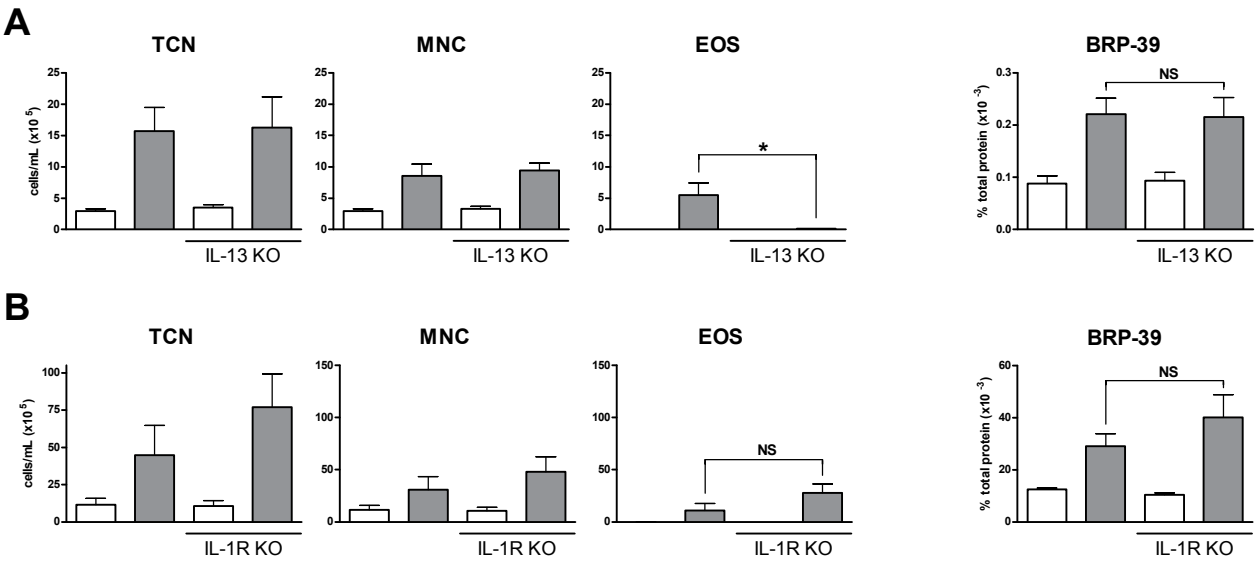

Figure 4 ***HDM induced BRP-39 is IL-13 and IL-1 independent***. WT BALB/c and IL-13 KO mice were saline (white bars) or HDM (grey bars) exposed for 10 days. (A) Data show total cell numbers (TCN), mononuclear cells (MNC), and eosinophils (EOS) in bronchoalveolar lavage fluid as well as BRP-39 expression in lung homogenates. (B) The same readouts are shown for IL-1R1 KO mice receiving HDM exposure. n = 5-10, data shown in B are representative of 2 separate experiments, * indicate $P < 0.05$.

mice were exposed to cigarette smoke and cellular inflammation was assessed in the BAL (Figure 5A). We observed similar total cell, mononuclear cell, and neutrophil counts in the BAL of WT and KO animals. Analysis of tissue neutrophils by flow cytometry revealed no significant differences between smoke-exposed WT and BRP-39 KO mice (Figure 5B). Previous characterization of the smoke exposure system utilized by this study confirmed an increase in dendritic cells and activation of CD4 T cells after smoke exposure [19]. Similar to tissue neutrophils, we observed no difference in dendritic cell numbers or CD4 T cell activation via flow cytometric analysis (Figure 5B). To confirm the veracity of the BRP-39 KO mice, BRP-39 expression was assessed in these mice by ELISA and no BRP-39 was detectable in the KO mice (data not shown). These data suggest that BRP-39 is redundant in the inflammatory response elicited by cigarette smoke.

BRP-39 is not required for cigarette smoke dependent allergic sensitization

Studies by Lee *et al* showed that BRP-39 plays a crucial role in processes leading to allergic sensitization to OVA and HDM [15]. To reproduce these previous findings, we exposed BALB/c and BRP-39 KO mice to HDM for 2 weeks (Figure 6A). In this model, we also observed a decrease in total cells, mononuclear cells and eosinophils in the BAL of BRP-39 KO mice when compared to their WT controls. We and others have previously reported that cigarette smoke has adjuvant properties allowing for allergic mucosal sensitization to OVA under conditions that otherwise induce inhalation tolerance [25,32]. To investigate whether BRP-39 is critical for cigarette smoke's adjuvant properties, BRP-39 KO and WT control mice were concurrently exposed to cigarette smoke and aerosolized OVA for 2 weeks. Mice were rested for 1 month prior to 3 consecutive days of OVA rechallenge. No differences were observed between BRP-39 KO mice and WT controls in terms of the BAL inflammatory profile (Figure 6B). We observed similar numbers of mononuclear cells and eosinophils in the BAL of BRP-39 and WT mice. Flow cytometric analysis of lung preparations further revealed no difference in numbers of Th2 cells (as assessed by T1/ST2 and IL-5 signal) and DC activation (as assessed by CD86+ signal on CD11c+, MHC II+ cells) between BRP-39 KO and WT mice (Figure 6C), suggesting that BRP-39 is not required for allergic sensitization in the context of cigarette smoke exposure.

Discussion

Though the induction of BRP-39 is observed in a wide variety of inflammatory conditions and has been debated as a biomarker of certain disease states, relatively little investigation into its relevance in inflammatory responses has been made; necessitating additional study with *in vivo* models (reviewed in [33]). Thus, the objective of this study was to determine the expression and relevance of the chitinases BRP-39 and AMCase in cigarette smoke-induced airway inflammation and

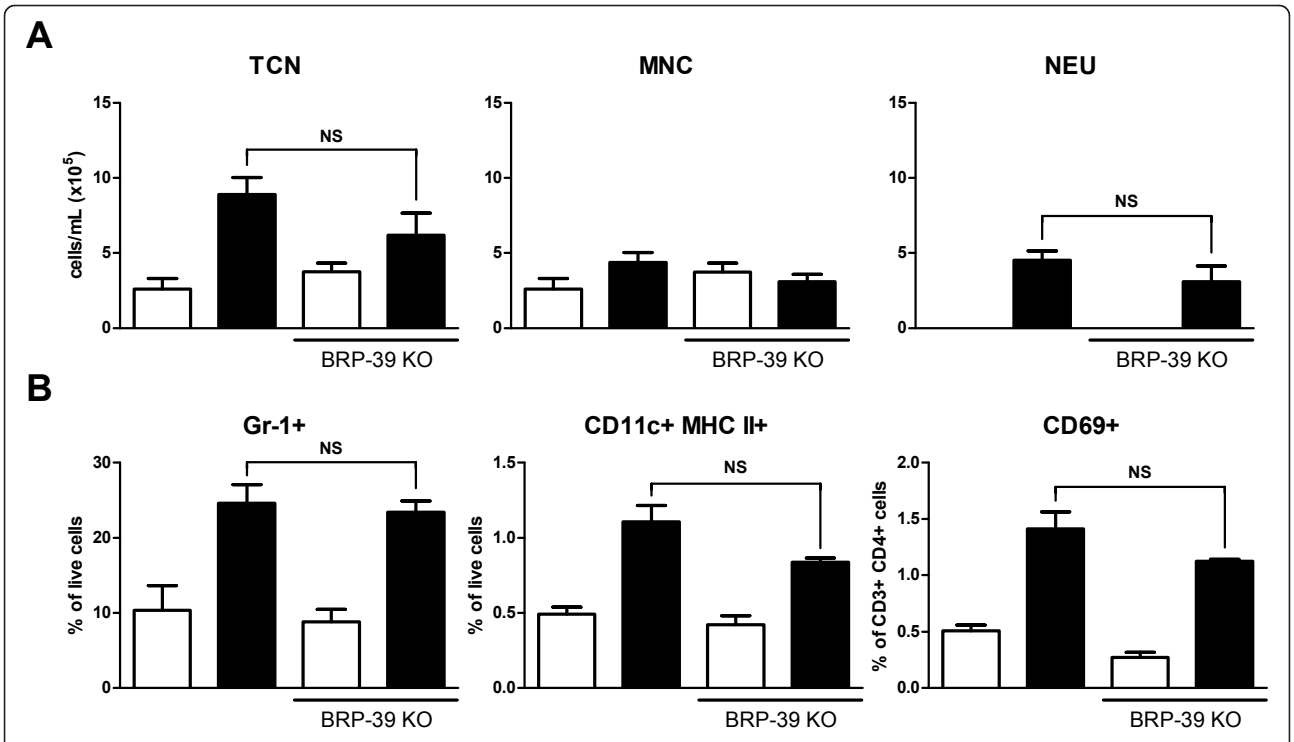

Figure 5 ***Cigarette smoke induced inflammation is not affected by BRP-39 deficiency***. BRP-39 KO and BALB/c WT mice were room air (white bars) or smoke (black bars) exposed for four days. (A) Data show total cell numbers (TCN), mononuclear cells (MNC), and neutrophils (NEU) in BAL fluid. (B) Flow cytometric analysis of lung digests for the presence of neutrophils (Gr-1+), dendritic cells (CD11c+ MHCII+), and CD4 T cell activation (CD69+). n = 5, data shown are representative of 2 separate experiments,* indicate $P < 0.05$.

contrast this to HDM-induced allergic inflammation because of previously established chitinase expression in allergic airways disease.

To pursue this study, we utilized a murine whole body cigarette smoke exposure system. Mice were exposed to cigarette smoke for 4 consecutive days. This time point was chosen based on previous time course experiments to determine when a robust inflammatory response could first be reliably detected (data not shown). Though this time point is ideal for assessing cellular inflammation, the smoke exposure period is not long enough to measure lung destruction characteristic of emphysema. The inflammation induced is largely neutrophilic in nature, an observation similar to that described in COPD patients [34,35]. As further validation of this model, we previously reported levels of carboxyhemoglobin (a measurement of the saturation of hemoglobin with carbon monoxide) and cotinine (a metabolic product of nicotine) similar to the human reference [19]. Similarly, the HDM model utilized a 2 week time point as this has been previously established as the earliest time point to observe robust eosinophilic inflammation [36], while prolonged exposure is required to induce airway remodeling. Thus, the focus of both models is the inflammatory response, which is believed to drive, at least in part, the pathogenesis of COPD and asthma.

The increase in BRP-39 expression after smoke exposure is a robust event observed across inbred strains and outbred stock. This induction is in agreement with clinical observations of increased YKL-40 expression levels in smokers and COPD patients. Unlike models of allergic airway inflammation where both AMCase and BRP-39 have been shown to be elevated [15,16], increased expression levels of AMCase were not observed following smoke exposure, thus distinguishing the chitinase expression profile elicited by cigarette smoke from the one elicited by allergens.

The induction of BRP-39 and the infiltration of cells into the lungs were concurrent phenomena after 4 days of cigarette smoke exposure. IHC on lung sections implicated epithelial cells and macrophages as the primary producers of BRP-39 in this model, which is in agreement with the YKL40 expression pattern in humans and other smoke exposure models [17,18]. Others have found that neutrophils are capable of producing YKL-40 in humans [37]; however, no evidence in our model suggests that this prominent inflammatory cell type is contributing to BRP-39 production. Regardless of the relevance of BRP-39 in disease pathology, its

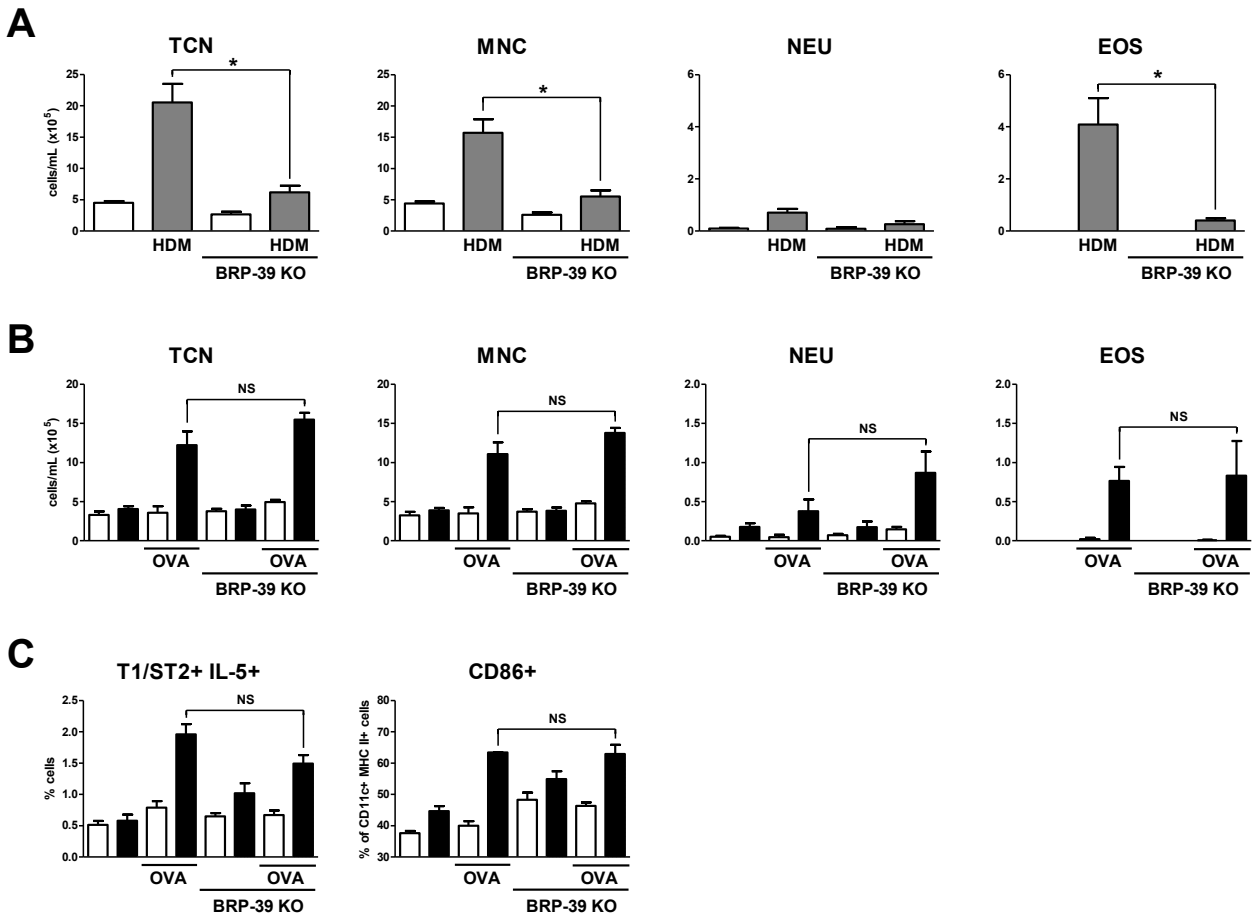

Figure 6 ***BRP-39 is not required for cigarette smoke induced allergic sensitization***. BALB/c and BRP-39 KO mice were saline (white bars) or HDM (grey bars) exposed for 10 days. (A) Data show total cell numbers (TCN), mononuclear cells (MNC), neutrophils (NEU), and eosinophils (EOS) in bronchoalveolar lavage fluid. In separate experiments, BRP-39 KO and BALB/c WT mice were room air (white bars) or smoke (black bars) exposed for 2 weeks and concurrently exposed to nebulized OVA. Upon rechallenge following a month of smoke and OVA exposure cessation, cellular inflammation was assessed. (B) Data show total cell numbers (TCN), mononuclear cells (MNC), neutrophils (NEU), and eosinophils (EOS) in the BAL fluid. (C) Lung digests were also generated and analyzed by flow cytometry for the presence of Th2 cells (T1ST2+ IL-5+) and activated dendritic cells (CD86+). n = 5, data in B and C are representative of 2 separate experiments, * indicate $P < 0.05$.

expression is closely associated with the inflammatory response and BRP-39 remains a biomarker of inflammatory disease.

Following the initial observation of BRP-39 induction in allergic disease, Th2 mechanisms were postulated as being responsible for driving this process [15,29]. Th2 responses are believed to be crucial for parasitic defense and the induction of enzymes with the potential to break down the protective sheaths of parasitic nematodes would be of great efficacy to such responses. The finding that enzymatically active AMCase is induced in an IL-13 dependent manner in Th2 driven inflammation reinforced this hypothesis [16]. Though Th2 cytokines, including IL-13, have been detected in the smoke exposure model utilized in this study [19], IL-13 KO mice revealed that BRP-39 induction by cigarette smoke is IL-13 independent. This is not entirely surprising as IL-13 does not appear to be a critical mediator of inflammation in the smoke exposure system for its deficiency also has no effect on cellular inflammation. Conversely, it was rather unexpected that in HDM-induced allergic inflammation; which is Th2-driven, IL-13 was unnecessary for the induction of BRP-39; in other words BRP-39 induction was unaltered and yet eosinophilic inflammation was markedly attenuated. These results are at variance with previous work that implicated BRP-39 as a crucial inflammatory component in similar HDM models [15]. This represents a significant finding and expands on previous work by Lee *et al* in which IL-13 dependence for BRP-39 induction in allergic airway inflammation was strongly implied by experiments where transgenic amounts of IL-13 had been over-expressed in the lungs

[15]. The experiments by Lee et al, however, did not utilize an IL-13 KO strain and as such these data only demonstrate that IL-13 is able to induce BRP-39 and not whether IL-13 is essential for BRP-39 induction. Our data show that although IL-13 is capable of inducing BRP-39 expression, it is redundant in models of cigarette smoke- and allergen-induced airway inflammation in the induction of BRP-39.

IL-1 has been implicated *in vitro* in BRP-39 induction [38]. The IL-1R1 KO mice were chosen for this reason and because IL-1R1 deficiency was sufficient to attenuate smoke-induced neutrophilic inflammation. The observation that smoke-exposed IL-1R1 KO mice did not up-regulate expression of BRP-39 suggests a crucial role of IL-1 in this phenomenon. This provides further evidence that the induction of BRP-39 is closely tied to inflammatory pathways. Further investigation of the importance of IL-1 in the induction of BRP-39 in allergic inflammation revealed that IL-1R1 was not crucial in the HDM model, highlighting the different inflammatory pathways engaged by these two models. Our data which confirms the importance of BRP-39 in HDM-induced inflammation imply that BRP-39, in the context of allergy, is part of an immune inflammatory pathway crucial to mononuclear cell and eosinophil recruitment that is not dependent on IL-1 or IL-13.

Recently Matsuura *et al* have implicated IL-18 as a mechanistic component of BRP-39 induction in a murine model of smoke exposure [18]. These data complement previous experiments that implicate IL-18 as a crucial component of cigarette smoke-induced inflammation [10]. Our data generated in IL-18 KO mice suggest that IL-18 is redundant in the inflammatory response and in the induction of BRP-39 which was confirmed by experiments with IL-18 receptor KO mice (data not shown). This discrepancy could be the result of different smoke exposure conditions as Matsuura *et al* utilized a nose only smoke exposure apparatus characterized by Shapiro *et al* [39], as opposed to a whole body smoke exposure system. A more likely explanation of the discrepancy is the length of smoke-exposure, as our study exposed mice to smoke for four days while Matsuura *et al* exposed mice to smoke for a month to determine the mechanistic relevance of IL-18. The four day time point was chosen for this study because experiments showed a greater induction of BRP-39 at subacute time points when compared to the chronic setting (data not shown). These findings taken in context with the data from IL-1R1 KO mice imply a timeline for cigarette smoke induced inflammation where IL-1 inflammatory pathways are more important early on in disease progression with IL-18 mediated pathways engaged after sustain cigarette smoke stimuli.

Evidence such as the stimulation of cells with YKL-40 inducing inflammatory chemokines has implied a role for this YKL-40 and BRP-39 in cellular inflammation [17,38], yet BRP-39 deficiency did not lead to significantly attenuated lung-infiltrating cell types after smoke exposure. The redundant nature of BRP-39 in this inflammatory response represents the most striking finding of this study and again contrasts the work by Matsuura *et al* [18]. As stated before, this is likely the result of the different durations of smoke exposure as Matsuura *et al* did not witness reduced inflammation in smoke-exposed BRP-39 KO mice until at least 3 months of smoke-exposure. This implicates BRP-39 in the survival of inflammatory cells in a chronic inflammatory setting and not in the initial recruitment of cells to the lungs. The lack of significant difference in tissue neutrophils, DCs, and CD4 T cell activation more specifically reinforces the redundant nature of BRP-39 in the early stages of cigarette smoke-induced inflammation.

Another striking conclusion of these experiments was that although BRP-39 has been shown to be crucial for allergic sensitization, it is redundant in the adjuvant properties of cigarette smoke. This implies a different mechanism of sensitization when cigarette smoke is utilized as an adjuvant. This is not an unprecedented assertion as HDM models of allergic sensitization and models of cigarette smoke induced OVA sensitization have been shown to utilize different inflammatory pathways [40]. Lee *et al* postulated that the attenuation of allergic responses in BRP-39 deficient mice was due to an increase in apoptosis of a key mediating cell type [15]. Apoptosis was not assessed in this study but if there was increased apoptosis in BRP-39 deficient animals it was not sufficient to impede sensitization or decrease the amount of activated DCs, implying that an increase in apoptosis may not be sufficient to interrupt sensitization when alternate pathways are driving sensitization. This is likely the case when cigarette smoke is utilized as an adjuvant.

Conclusions

In conclusion, these results demonstrate that BRP-39 is a biomarker of cigarette smoke- and allergen-induced inflammation. Its induction by cigarette smoke is IL-1R1 dependent, which is unique from BRP-39 induction in HDM-induced allergic inflammation which is both IL-1R1 and IL-13 independent. Despite the fact that BRP-39 is induced by an inflammatory agent, BRP-39 is itself redundant in cigarette smoke-induced inflammation. Also, despite being a crucial mediator of allergic sensitization in widely utilized models of airway inflammation, BRP-39 is not crucial for the adjuvant properties of cigarette smoke. This study highlights the inflammatory

mechanism elicited by cigarette smoke to induce BRP-39 expression which is unique from allergic inflammation as well as the function of BRP-39 in subacute smoke exposure and cigarette smoke induced allergic sensitization.

Additional material

Additional File 1: *Isotype controls for flow cytometry data*. The appropriate isotype controls are shown in flow cytometry pseudo-dot plots of data generated from for the lung digests of 4 day smoke exposed lungs (A,C,D) and smoke- and OVA-exposed mice after 1 month of cessation and 3 days of rechallenge with OVA (B,E). Histogram data (C-E) contrasts positive stain (black line) with the appropriate isotype control (solid grey line).

Acknowledgements
The authors gratefully acknowledge the expert technical support of Joanna Kasinska and Sussan Kianpour, the secretarial assistance of Marie Colbert, and Gordon Gaschler for discussion and preparation of the manuscript. Additional technical assistance was provided by Jagbir Khinda, Pamela Shen, Ashling Kelly, Kristen Lambert, and Gabriel Nikota. This study was funded in part by the Canadian Institutes of Health Research (CIHR) and MedImmune LLC.

Author details
[1]Medical Sciences Graduate Program, McMaster University, Hamilton, ON, Canada. [2]Department of Pathology and Molecular Medicine, Centre for Gene Therapeutics, McMaster University, Hamilton, Ontario, Canada. [3]MedImmune LLC, Gaithersburg, MD, USA. [4]Pfizer, Cambridge, MA USA. [5]Department of Medicine, McMaster University, Hamilton, Ontario, Canada, L8N 3Z5.

Authors' contributions
JKN conducted mouse experiments, aided in experiment design, performed IHC and ELISAs, participated in flow cytometric analysis and drafted the manuscript. FMB participated in mouse experiments and conducted flow cytometry. CMTB participated in mouse experiments and manuscript preparation. MJ, AJC, and AAH participated in the design of the study helped to draft the manuscript. MRS conceived and designed the study and aided in drafting the manuscript. All authors read and approved the manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 15 September 2010 Accepted: 7 April 2011
Published: 7 April 2011

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doi:10.1186/1465-9921-12-39
Cite this article as: Nikota *et al.*: Differential expression and function of breast regression protein 39 (BRP-39) in murine models of subacute cigarette smoke exposure and allergic airway inflammation. *Respiratory Research* 2011 **12**:39.

CHAPTER THREE

Cigarette smoke primes the pulmonary environment to IL-1alpha/CXCR-2 dependent nontypeable Haemophilus influenzae-exacerbated neutrophilia in mice

This article has been submitted to the Journal of Immunology and is conditionally accepted contingent of minor revisions.

The experiments conducted in chapter two reinforced previously published findings that identified the importance of IL-1R1 in the recruitment of innate immune cells to a cigarette smoke-exposed lung (120). Our lab expanded upon these data by identifying IL-1α as the crucial ligand in this signaling pathway. Our lab had also established a model of cigarette smoke-exposure and NTHi infection that was characterized by exaggerated recruitment of innate immune cells, particularly neutrophils (202). This presented a unique opportunity, as the mechanism of bacteria-exacerbated inflammation in the context of cigarette smoke had not previously been reported. In this study, we investigated the IL-1 signaling pathway in this process.

Dr. Stampfli, Pamela Shen, and I were responsible for the experimental design and the interpretation of results. Pamela Shen and I contributed equally to the data generated. I prepared the final manuscript and Pamela Shen and Dr. Martin Stampfli edited it. Dr. Mathieu Morrisette and Kimberly Fernandes provided technical assistance in the completion of experiments, and Dr. Abraham Roos generated additional experimental data. Drs. Derek Chu and Nicole Barra also assisted with specific experimental procedures. Yoichiro Iwakura, Roland Kolbeck, and Alison A Humbles provided critical reagents and advice.

TITLE

Cigarette smoke primes the pulmonary environment to IL-1alpha/CXCR-2 dependent nontypeable *Haemophilus influenzae*-exacerbated neutrophilia in mice.

Running Title

Cigarette smoke primes pulmonary IL-1 responses to bacteria

Authors

Jake K Nikota*[,†], Pamela Shen*[,†], Mathieu C Morissette[‡], Kimberly Fernandes[‡], Abraham Roos[‡], Derek K Chu[†], Nicole G Barra[†], Yoichiro Iwakura[§], Roland Kolbeck[¶], Alison A Humbles[¶], Martin R Stampfli[‡,‖].

* Authors contributed equally.

Affiliations

[†]Medical Sciences Graduate Program, McMaster University, Hamilton, Ontario, Canada; [‡]Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, McMaster University, Hamilton, Ontario, Canada; [§]Center for Experimental Medicine and Systems Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [¶]MedImmune LLC, One MedImmune Way, Gaithersburg, MD, USA; [‖]Department of Medicine, Firestone Institute for Respiratory Health at St. Joseph's Healthcare, McMaster University, Hamilton, Ontario, Canada

Corresponding Author Martin R. Stämpfli,

McMaster University

Department of Pathology and Molecular Medicine

1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

Phone: 905 525 9140 x22493

Email: stampfli@mcmaster.ca.

ABSTRACT

Cigarette smoke has a broad impact on the mucosal environment with the ability to alter host defense mechanisms. Within the context of a bacterial infection, this altered host response is often accompanied by exacerbated cellular inflammation, characterized by increased neutrophilia. The current study investigated the mechanisms of neutrophil recruitment in a murine model of cigarette smoke exposure and, subsequently, a model of both cigarette smoke exposure and bacterial infection.

We investigated the role of IL-1 signaling in neutrophil recruitment and found that cigarette smoke-induced neutrophilia was dependent on IL-1α produced by alveolar macrophages. In addition to being the crucial source of IL-1α, alveolar macrophages isolated from smoke-exposed mice were primed for excessive IL-1α production in response to bacterial ligands. To test the relevance of exaggerated IL-1α production in neutrophil recruitment, a model of cigarette smoke exposure and nontypeable *Haemophilus influenzae* (NTHi) infection was developed. Mice exposed to cigarette smoke elaborated an exacerbated CXCR2-dependent neutrophilia in response to NTHi. Exacerbated neutrophilia was dependent on IL-1α priming of the pulmonary environment by cigarette smoke as exaggerated neutrophilia was dependent on IL-1 signaling. These data characterize a novel mechanism of cigarette smoke priming the lung mucosa towards greater IL-1driven neutrophilic responses to bacteria, with a central role for the alveolar macrophage in this process.

INTRODUCTION

Cigarette smoking has become one of the greatest health concerns of the 21st century (1–3). Despite declining smoking rates in developed nations, global smoking prevalence has reached epidemic levels with nearly 20% of the world's adult population considered a smoker (4). Clinical evidence suggests that pulmonary infections in smokers are significantly more severe than in non-smokers (1–3). The effects of cigarette smoke during pulmonary infection may be life threatening to vulnerable populations such as individuals suffering from Chronic Obstructive Pulmonary Disease (COPD) (5). Ninety percent of COPD cases are attributed to chronic cigarette consumption and the progressive loss of lung function that characterizes COPD is periodically exacerbated by microbial infections (6). Understanding the extent to which cigarette smoke modifies host defense mechanisms and predisposes an individual for exacerbated infectious episodes will be crucial knowledge for future health care strategies.

Cigarette smoke is an unusual stimulus for the lung mucosal environment, with both immune activating and immune suppressing characteristics. Cigarette smoke exerts damaging and pro-inflammatory effects in the lungs, but can also directly suppress innate and adaptive immune processes (7–10). Studies in animal models have demonstrated that exposure to cigarette smoke exacerbates inflammatory responses elicited by several different bacterial agents, including nontypeable *Haemophilus influenzae* (NTHi), *Pseudomonas aeruginosa*, and *Streptococcus pneumonia* (11–14). In all studies, the cellular composition of the bacteria-exacerbated inflammatory response was neutrophilic in nature. This exacerbated neutrophilia seemed to contrast findings that suggest that cigarette smoke suppresses the expression of proinflammatory mediators such as TNF-α in response to bacterial stimuli (15, 16). A more detailed examination of the inflammatory processes in a murine model of NTHi-exacerbated,

cigarette smoke-induced inflammation yielded the observation that certain well characterized inflammatory mediators were suppressed, while other pro-inflammatory factors, not normally induced by NTHi, were now being expressed (14). This altered phenotype was observed specifically at the level of the alveolar macrophage; a critical orchestrator of immune responses in the lung (17). Though the altered lung phenotype is now being characterized, the specific mediators of neutrophilic inflammation within this altered response have not been identified.

Animal models have identified the importance of IL-1R1 signaling to neutrophilic inflammation elicited by cigarette smoke (18–20). The cytokines IL-1α and IL-1β activate IL-1R1 (21). Precursor IL-1α is biologically active as a 31kDa protein (22). In contrast, IL-1β is initially synthesized as pro-IL-1β (also 31kDa), which is biologically inactive and requires proteolytic cleavage. Additional mechanisms involved in the neutrophil recruitment process include the neutrophil associated chemokine receptor CXCR2, which is activated in response to CXCL-1, -2, and -5 (23, 24). Neutrophils are a source of molecular mediators which contribute to the irreversible loss of lung function associated with chronic smoking and COPD, such as proteases which can break down the extra cellular matrix of the lung (25–27). Neutrophil accumulation is associated with the development of emphysematous lung destruction often observed in COPD patients (28). IL-1 mediated neutrophil accumulation is an important aspect of cigarette smoke-induced inflammation that may be exacerbated in the context of a subsequent bacterial infection.

In the current study, we sought to investigate the role of IL-1 signaling in cigarette smoke-induced neutrophil accumulation and investigate the role of IL-1 in the context of a bacterial exacerbation of cigarette smoke-induced inflammation. We found that cigarette smoke-induced neutrophilia was dependent on IL-1α produced by alveolar macrophages. In addition to

stimulating the production IL-1α, cigarette smoke predisposed alveolar macrophages to produce exacerbated levels of IL-1α in response to bacterial stimuli. We then investigated the importance of a cigarette smoke-exposed lung environment primed for exacerbated IL-1α production in an *in vivo* model of NTHi infection. This model elucidated an exacerbated neutrophilic response to NTHi in smoke-exposed mice that was dependent on IL-1α. Additionally, the important neutrophil-attracting IL-1-dependent chemokine was epithelial cell-derived CXCL5. These data demonstrate the important role of IL-1 signaling in the altered inflammatory response elicited in a smoke-exposed lung, providing mechanistic insight into this potentially pathogenic phenomenon.

MATERIALS AND METHODS

Animals

6-8 week old female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Saint-Constant, PQ, Canada) and Jackson Laboratories (Bar Harbor, Maine, USA). Mice deficient in CXCR2 on a BALB/c background and mice deficient in IL-1R1 on a C57BL/6 background were purchased from the Jackson Laboratories. IL-1α Knock Out (KO) mice and IL-1β KO mice were on a C57BL/6 background and bred in house (29). Mice were housed under specific pathogen-free conditions with *ad libitum* access to food and water and subjected to a light-dark cycle of 12 hours. All experiments were approved by the Animal Research Ethics Board at McMaster University.

Cigarette smoke exposure

A whole body cigarette smoke exposure system (SIU-48, Promech Lab AB (Vintrie, Sweden)) was utilized. Mice were exposed to 12 3R4F reference cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) with filters removed, for 50 minutes, twice daily, for four days or 5 days per week for 8 weeks. Details of the exposure protocol were previously reported (30). Control mice were exposed to room air only.

Preparation of nontypeable *Haemophilus influenzae* (NTHi)

The nontypeable *Haemophilus influenzae* strain 11P6 (kindly provided by Dr. S. Sethi, VA Medical Research, Buffalo, NY, USA) was utilized. This clinical strain of NTHi was isolated from the sputum of a COPD patient during acute exacerbation. NTHi was initially grown on chocolate agar plates containing 1% Isovitalex (BD Biosciences, Franklin Lakes, NJ, USA). Colonies of NTHi were then grown to log phase in 10mL of brain-heart infusion (BHI) broth (Difco, Fisher Scientific, Ottawa, ON, Canada) supplemented with Hemin and nicotinamide

adenine dinucleotide (NAD) (Sigma, Oakville, ON, Canada). The inoculated BHI + Hemin + NAD broth was maintained on a rotary shaker at 37^0C until an OD value of 0.7-0.8 was obtained at a 600nm wavelength. CFU was predicted from the OD value based on a previously generated standard curve. Before alveolar macrophage stimulation and infection of mice, NTHi was washed three times with phosphate-buffered saline (PBS), re-suspended, and diluted to a ratio of 10CFU/cell (for alveolar macrophage culture) and 10^6 CFU/35μL (for mouse infection).

BAL and differential cell counting and generation of lung homogenates

Prior to BAL, the right lung was tied off and homogenized in 2 mL PBS using a Polytron PT 2100 homogenizer (Kinematica, Switzerland) at 21,000-25,000 RPM for 3-6 seconds. BAL fluid was collected after instilling the lungs first with 250 μL of ice cold PBS, then with 200 μL of PBS. Total cell number in the BAL was determined using a haemocytometer. Cytospins were prepared and stained with Hema 3 (Biochemical Sciences Inc., Swedesboro, NJ, USA). 500 cells were counted per cytospin for determination of percent mononuclear cells (MNCs) and percent neutrophils (NEU). Differential cell counts were calculated using this percentage and the total cell number (TCN).

Macrophage isolation and culture

BAL fluid was collected after instilling the whole lung with 1 mL of ice cold PBS. This process was repeated 5 times for maximal recovery of macrophages. BAL cells were re-suspended in 500 μL fresh PBS. Alveolar macrophages were identified and counted using a haemocytometer. BAL cells were re-suspended in RPMI supplemented with 10% FBS (Sigma-Aldrich, Oakville, ON, Canada), 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% β-mercaptoethanol (Invitrogen, Grand Island, NY, USA) and cultured in polystyrene flat-bottomed 96 well plates at 50,000 alveolar macrophages per well. Cells were incubated at 37^0C and 5% CO_2 for 1 hour to

facilitate adherence. Non-adherent cells were removed by washing three times with warm PBS. Cells were cultured for 24 hours with RPMI alone, in the presence of 1 μg/mL Pam3CSK4, 1 μg/mL LPS (Invivogen, San Diego, CA, USA), or 10CFU/cell of NTHi. Cell supernatants were collected and stored at -20°C for the measurement of TNF-α, IL-1α, and IL-1β protein levels by ELISA. Additionally, RNA was isolated from alveolar macrophages after 4 hours of LPS stimulation using the RNeasy mini Kit with optional DNase step (Qiagen, Mississauga, ON, Canada). cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen) and Taqman real time RT qPCR was carried out with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Target gene expression was normalized to that of the housekeeping gene GAPDH in the same sample and expressed as fold increase over the control un-stimulated group.

Cytokine measurements

IL-1α, IL-1β, CXCL1, CXCL2, CXCL5, and CXCL7 protein levels in lung homogenates were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's instructions.

Immunohistochemistry

Following the BAL, lungs were fixed at 30 cm H_20 pressure in 10% formalin for histological assessment. After a minimum of 24 hour formalin-fixation, lungs were paraffin-embedded and 4 μm slices were cut. Tissues were treated for 45 minutes in 0.01 M citrate buffer to retrieve antigen and incubated for 1 hour with either anti-IL-1α polyclonal goat antibody or anti-CXCL5 polyclonal rabbit antibody (R&D Systems) diluted in UltrAb diluent (Thermo Scientific, Rockford, IL, USA) at 7 μg/mL. Immunohistochemistry was developed with goat on rodent

HRP probe (Biocare, Concord, CA, USA) or anti-rabbit Dakocytomation HRP (Dako Canada, Burlington, ON, Canada) and counterstained in Meyer's solution.

Lung slice culture

Lung slices were generated as previously described (18). Briefly, lungs were inflated with 37°C agarose (Sigma Aldrich) prepared to 2% in Hank's buffered saline solution (HBSS) supplemented with HEPES (Sigma Aldrich). The agarose was allowed to cool. Lung lobes were dissected and maintained in an ice-cold HBSS solution prior to and during slicing. 120 µm thick slices were generated using a vibratome (Leica Microsystems, Concord, ON, Canada). Approximately 40 slices were isolated from each mouse lung. RNA was isolated and RT qPCR was performed as described for alveolar macrophage cultures.

Lung function measurement

Forced oscillation measurements were performed using the FlexiVent ventilator system (SCIREQ, Montreal, Canada). Mice were sedated and anaesthetized with 10 mg/kg xylazine (Bayer Healthcare, Berlin, Germany) and 30 mg/kg sodium pentobarbital (Ceva, Lenexa, KS, USA), both given by intraperitoneal injection. Mice were then immobilized by intraperitoneal injection of 10 mg/kg rocuronium bromide (Omega Laboratories, Montreal, Canada), whereupon each mouse underwent tracheostomy using a blunted 18-gauge needle. Oxygen saturation and heart rate were continuously monitored using an infrared pulse oxymeter (Biox 3700; Ohmeda, Boulder, CO). The animal was next connected to a computer-controlled Flexivent ventilator. All mice were ventilated with 150 breaths/minute, with an applied pressure limit of 30 cmH_2O. A snapshot perturbation maneuver was performed with forced oscillation perturbation consequently applied. Maximal pressure-regulated pressure-volume (PVr-P) loops were finally generated to obtain maximal vital (total) lung capacity and static compliance. Following data collection,

animals were removed from the ventilator and immediately euthanized via terminal exsanguination.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was carried out using SPSS Software (IBM, Armonk, NY, USA). Levene's test for equality of variances was used to adjust for differences in data variability between groups. The Univariate General Linear Model was used and independent t-tests were applied subsequently for 2-group comparisons. Differences with $P<0.05$ were considered statistically significant. Inflammatory cell count data for key experiments was pooled for from two independent experiments.

RESULTS

Cigarette smoke induces IL-1α from a hematopoietic cell-type that drives neutrophilia

To model cigarette smoke-induced inflammation, C57BL/6 mice were cigarette smoke-exposed for 4 days. We have previously shown that 4 days of cigarette smoke exposure is sufficient to elicit lung neutrophilic inflammation (30). Cigarette smoke exposure led to a significant increase in IL-1α and IL-1β in the lung homogenates (Figure 1A and B). IL-1α KO and IL-1β KO mice were exposed to cigarette smoke for 4 days, and despite both IL-1α and IL-1β being upregulated in wild type animals, only IL-1α deficiency resulted in significantly reduced neutrophilia where the mean neutrophil counts was reduced from $1.080x10^5$ ($\pm 2.028x10^4$ SEM) cells/mL in the WT mice to 1182 (±988 SEM) cells/mL in the IL-1α KO mice (Figure 1A and B). This reduction in neutrophil infiltration was associated with a decrease in the neutrophil recruiting chemokines CXCL1 and CXCL5 (Figure 1A). Interestingly IL-1β deficiency had no effect of neutrophilia and resulted in less CXCL1 but not significantly less CXCL5, indicating that CXCL5 is a better biomarker of neutrophilic inflammation in this experimental system (Figure 1B).

Immunohistochemistry has previously shown that alveolar macrophages stain positive for IL-1α in a 4 day smoke exposure protocol (14). To assess whether the critical source of IL-1α is a hematopoietic cell, we generated bone marrow chimeric mice with IL-1α KO and wild type mice, as depicted in Figure 1C. After 4 days of smoke exposure, mice deficient in IL-1α expression in their hematopoietic cells had an almost complete abrogation in pulmonary neutrophil infiltration (Figure 1C). In contrast, when IL-1α was deficient in radio-resistant structural cells, there was no significant reduction in neutrophilia. These data in conjunction with our previous findings provide strong evidence that the alveolar macrophage is crucial to the neutrophilic response to cigarette smoke through the production of IL-1α.

Cigarette smoke leads to the accumulation of alveolar macrophages primed to produce IL-1α

Due to our finding that IL-1α derived from alveolar macrophages drives smoke-induced neutrophilia, we sought to further investigate the phenotype of this cell within the context of cigarette smoke. In addition to the accumulation of neutrophils, cigarette smoke exposure led to the expansion of the monocyte/macrophage population in the lung lumen, as evident from the accumulation of these cells in BAL fluid (Figure 2A). In addition to an increased number of monocytes/macrophages in the BAL, alveolar macrophages were observed to have an altered phenotype. The morphology of these cells changed in the smoke-exposed pulmonary environment, as smoke-exposed alveolar macrophages were larger, had an irregular shape, and contained large vesicle structures (Figure 2B) (14).

In our current study, we demonstrate that alveolar macrophages isolated from cigarette smoke-exposed mice spontaneously produced IL-1α when placed in culture (Figure 2C). Smoke-exposed macrophages also produced significantly more IL-1α in response to stimulation with the TLR2 and TLR4 ligands Pam3CSK4 and LPS, as well as the live bacteria NTHi (Figure 2C). The increased IL-1α was regulated at the transcriptional level as IL-1α mRNA was significantly increased in smoke-exposed macrophages placed in medium alone or following LPS stimulation (Figure 2D). While alveolar macrophages from cigarette smoke-exposed mice were primed to produce more IL-1α in response to bacterial stimuli, these same macrophages produced significantly less TNF-α in response to Pam3CSK4, LPS, and NTHi (Figure 2E). IL-1β was measured in the supernatants of macrophages from smoke-exposed mice as well, and although IL-1β signal could be detected, the levels were below the limit of the assay to produce quantifiable results (Figure 2F). These data suggest that cigarette smoke increases the number of

monocytes/macrophages in the lung environment and primes alveolar macrophages to produce exaggerated levels of IL-1α in response to bacterial ligands.

NTHi infection exacerbates cigarette smoke-induced pulmonary inflammation and the neutrophil attracting chemokine CXCL5

We have established the importance of the IL-1α/IL-1R1 signaling axis in cigarette smoke-induced neutrophilic inflammation and that cigarette smoke primes alveolar macrophages to produce excessive amounts of IL-1α in response to bacteria. These data strongly imply a role for IL-1 signaling in the context of cigarette smoke and bacterial infection. In order to investigate this, we established a model where BALB/c mice were infected with NTHi after 8 weeks of smoke exposure. Cigarette smoke-induced cellular inflammation was significantly increased by bacterial infection (Figure 3A); the hallmark of this exacerbated inflammation was a significant increase in neutrophils.

CXCR2 ligands have previously been shown to be important in neutrophil recruitment in an animal model of cigarette smoke-induced inflammation, as a CXCR2 inhibitor attenuated neutrophilia (24). To assess the role of CXCR2 in bacterial exacerbation of cigarette smoke-induced inflammation, CXCR2 KO mice were exposed to 8 weeks of cigarette smoke followed by NTHi infection. Though the NTHi-exacerbated neutrophilia was not as dramatic in this experiment, it was still statistically significant and CXCR2 deficiency resulted in a significant reduction in the neutrophils observed in the BAL (Figure 3B). To determine the CXCR2 ligands involved, we assessed CXCL1, CXCL2, and CXCL5 in BALB/c mice exposed to cigarette smoke followed by NTHi infection. CXCL1 and CXCL2 were increased by NTHi infection in room air-exposed mice, and this increase was, interestingly, close to significantly attenuated in smoke-exposed mice (Figure 3C for CXCL1, data not shown for CXCL2). Though we do not

completely understand the reduced CXCL1 levels as neutrophilia increased, CXCL5 seems to be the crucial CXCR2 ligand as CXCL5 was the only CXCR2 ligand assessed that was both induced by NTHi and further increased in the smoke-exposed and NTHi-infected group of mice (Figure 3C).

Our previous data suggest the importance of structural cells in neutrophil recruitment (31), such as epithelial cells. It would be an intuitive supposition that these epithelial cells could be a source of neutrophil recruiting chemokines such as CXCL5. Immunohistochemistry utilizing an anti-CXCL5 antibody identified epithelial cells of the airways and alveoli as being a potential source of CXCL5 (Figure 3D). Though some staining of alveolar macrophages was observed with CXCL5 immunohistochemistry, we were unable to measure CXCL5 in *ex vivo* cultures of macrophages from mice that were exposed to cigarette smoke for 8 weeks and stimulated for 24 hours with NTHi (data not shown). To demonstrate that, although the macrophage is not producing CXCL5, the lung tissue is primed for its production by cigarette smoke, CXCL5 was measured in precision cut lung slices, an ex vivo culture system where all lung cell types are present. Increased CXCL5 mRNA expression was detected in lung slices generated from 8 week smoke-exposed mice compared to room air-exposed mice following stimulation with NTHi (Figure 3E). These data suggest that the source of CXCL5 was the lung epithelium and that it is transcriptionally upregulated by smoke and NTHi. Our findings establish that NTHi exacerbates cigarette smoke-induced neutrophilia in a CXCR-2 dependent manner and that the most relevant CXCR2 ligand in this system is likely CXCL5.

Cigarette smoke-exacerbated neutrophilia is dependent on IL-1 signaling

After establishing the importance of IL-1 signaling in driving cigarette smoke-induced neutrophilia and characterizing a model of bacterial exacerbation of cigarette smoke-induced

neutrophilia, we sought to investigate the role of IL-1 signaling in the smoke and NTHi experimental system with IL-1R1 KO mice. The exacerbated cellular inflammation elicited by cigarette smoke and NTHi was significantly attenuated in IL-1R1 KO mice (Figure 4A). The attenuated inflammation was evident on the level of total cell number, monocytes/macrophages, and, neutrophils. Of note, IL-1R1 deficiency did not impact the cellular profile in room air exposed mice following NTHi infection. These data strongly imply that neutrophil recruitment to the lungs observed in our smoke exposure model is dependent on IL-1 signaling and that this signaling axis is further activated upon NTHi challenge.

To study mechanisms of attenuated neutrophilia, we assessed CXCR2 chemokine expression in the BAL of IL-1R1 mice exposed to cigarette smoke followed by NTHi infection (Figure 4B). While CXCL1 expression was increased, CXCL5 was significantly reduced in IL-1R1 KO mice. These observations demonstrate the importance of IL-1 signaling for the induction of CXCL5, which represents a biomarker of neutrophilia as both CXCL5 and neutrophilia were attenuated in IL-1R1 KO mice.

NTHi exacerbation of cigarette smoke-induced inflammation is IL-1α dependent

We have demonstrated that cigarette smoke exposure primes alveolar macrophages to produce exacerbated levels of IL-1α in response to bacteria and that IL-1 signaling is important in an *in vivo* model of cigarette smoke and bacterial inflammation. We subsequently sought to investigate the specific role of IL-1α in this *in vivo* system. In our bacterial exacerbation model, IL-1α levels were significantly increased by the combination of cigarette smoke and NTHi when compared to either stimulus alone (Figure 5A). This differed from the observed levels of IL-1β in this model in which NTHi induced IL-1β but this induction was not significantly greater in the smoke and NTHi samples (Figure 5A). We next utilized immunohistochemistry to examine the

source of IL-1α (Figure 5B). The cell type that demonstrated the highest signal was the alveolar macrophage, consistent with our findings in Figure 2. These findings suggest that IL-1α, and not IL-1β, drives the exacerbated response. To test this hypothesis, IL-1α and IL-1β KO mice were smoke-exposed and subsequently infected with NTHi, and cellular inflammation was assessed in BAL fluid. IL-1α deficiency led to a significant decrease in the total cells observed in the BAL, and this reduction was the result of decreases in both monocyte/macrophage and neutrophil recruitment to the lungs (Figure 5C). This differed from the BAL cell counts in IL-1β KO mice which did not elaborate a reduced inflammatory response (Figure 5D). These data demonstrate a role for IL-1α in driving NTHi-exacerbated responses *in vivo* through the priming of alveolar macrophages.

Disrupting IL-1 signaling may increase NTHi burden

To investigate the implications of disrupted IL-1 signaling on the clearance of bacteria from the lungs, bacterial burden was assessed in lung homogenates generated from 8 week smoke-exposed, NTHi-infected mice. Bacterial burden was significantly increased in smoke-exposed mice that were deficient in IL-1R1 and CXCR2 (Figure 6). Of note, bacterial burden was only increased in IL-1R1 KO mice that were cigarette smoke-exposed. Bacterial burden was not significantly increased in IL-1α KO mice. To investigate the consequences of decreased bacterial clearance on chemokine expression CXCL1, CXCL2, and CXCL5 were measured in the BAL from the CXCR2 KO experiment (Supplementary Figure 1). The increased NTHi burden was accompanied by a marked increase in these chemokines in CXCR2 KO mice. These data suggest that the increased cellular inflammation in cigarette smoke-exposed mice aids in the control of bacterial burden.

Repeated NTHi infection can exacerbate cigarette smoke-induced lung pathology

Chronic cigarette smoke induced-inflammation is believed to drive lung pathology associated with COPD and, specifically, IL-1 driven inflammatory mechanisms have been implicated in this process (6, 20). The current study describes a model of exacerbated IL-1-dependant inflammation in a model of smoke exposure. To test whether this exacerbated inflammatory response can induce measurable changes in lung physiology over time, a model of long-term cigarette smoke exposure and repeated NTHi infection was utilized (Figure 7A). Using Flexivent technology, pressure-driven pressure volume curves were generated, and cigarette smoke-exposed lungs had a greater volume at lower pressures when compared to room air-exposed mice and this increase was even greater in lungs from smoke–exposed mice that received repeated NTHi infections (Figure 7B). For a statistical quantification, these data were used to calculate the compliance of the lungs (Figure 7C). The combined stimulus of smoke and NTHi led to a significantly more compliant lung than cigarette smoke and control PBS. This data suggests that exacerbated inflammation in this model is accompanied by changes to the lung physiology and supports previous observations that NTHi leads to greater levels of the collagen breakdown product hydroxyproline in smoke exposed mice (14), indicative of extracellular matrix destruction.

DISCUSSION

Cigarette smoke's impact on immune inflammatory processes elicited by pulmonary infection affects a large population of individuals worldwide. It is of particular concern to COPD patients as microbial-driven exacerbations are a significant health concern, yet the mechanisms of these disease exacerbations are not well understood. Most experimental work studies either cigarette smoke-induced inflammation or bacterial infection in isolation without extrapolating these results to experimental models that combine infectious agents and cigarette smoke. The objective of the current study was to investigate the cellular and molecular mechanisms that contribute to bacterial exacerbation of cigarette smoke-induced inflammation. This approach elucidated a novel mechanism by which cigarette smoke can elicit an exacerbated inflammatory response to bacteria.

To address the objectives of this study, we utilized a murine model of cigarette smoke exposure and intranasal infection with NTHi. Our smoke exposure system elicits neutrophilic inflammation. This was a central readout of our study due to the importance of chronic neutrophilia in the pathogenesis of COPD (32, 33). Our experimental system also reproduces other clinical hallmarks associated with cigarette smoking, such as airspace enlargement and changes in ventilation and perfusion following prolonged cigarette smoke exposure (34). The observation that carboxyhemoglobin and cotinine levels measured in our system are comparable to human smokers further validates this experimental system (30). In order to model an infection that is clinically relevant in the context of a COPD patient, the strain of NTHi chosen to be utilized in this study was isolated from a COPD patient experiencing an exacerbation (35). It is for these reasons that we feel this experimental approach can provide meaningful insight into the

inflammatory pathways engaged by cigarette smoke and bacteria that will be relevant to a COPD patient experiencing an exacerbation due to a bacterial infection.

The importance of IL-1 is increasingly being shown to be a major signaling factor in initiating immune inflammatory responses to cigarette smoke, but the biologically relevant source has not been definitively identified. Additionally, there is evidence of the clinical relevance of IL-1 signaling as increased levels of both IL-1α and IL-1β have been observed in human smokers (18). In this study, we demonstrate that cigarette smoke-induced neutrophilia is IL-1α-dependent and redundant of IL-1β. This observation is supported by findings by us and others, showing an IL-1α dependency for neutrophil recruitment in response to cigarette smoke exposure (18, 36). We have previously reported that alveolar macrophages produce IL-1α in response to cigarette smoke based on observations from immunohistochemistry of lung samples(31); however, these findings did not provide any indication as to how important alveolar macrophage-derived IL-1α was in the process of neutrophilic inflammation. Using bone marrow chimeric mice, we demonstrate that hematopoietic cells are the critical source of IL-1α. The data presented in the current study are novel and conclusively demonstrates that the relevant source of IL-1α is a hematopoietic cell-type. This finding can be considered in conjunction with our previous histology data to conclude that neutrophilic inflammation elicited by cigarette smoke is dependent on IL-1α produced by a population of alveolar macrophages. It has previously been hypothesized that alveolar macrophages are orchestrators of inflammation within the context of COPD (17). A recent study has shown that depleting macrophages prevents lung damage and the loss of lung function elicited by cigarette smoke (37). The findings of our current study reinforce the crucial role of the alveolar macrophage in cigarette smoke-induced inflammation and identify IL-1α as the key signaling molecule for this cell-type's action. Specifically, our data

suggest that cigarette smoke primes the lung, and specifically the alveolar macrophage, to exacerbated responses to bacterial stimuli (Outlined in Figure 7).

We have observed that cigarette smoke-induced IL-1α leads to the production of the neutrophil attracting chemokine CXCL5, however, the IL-1α/CXCL5 signaling axis is not unique to cigarette smoke-induced inflammation as this pathway is also involved in sterile inflammation (38). The importance of IL-1α in the inflammatory response to sterile tissue damage and cell death has been validated in multiple model systems (38–40). Similar to cigarette smoke, macrophages are the sensors of necrotic debris in models of sterile inflammation and are the crucial producers of IL-1α (41). Experimental models of cigarette smoke exposure have been known to induce apoptosis and necrosis (42, 43). It is currently unclear how the insult of cigarette smoke is sensed and the inflammatory response of cigarette smoke may be driven in part by the sensing of cigarette smoke-induced apoptosis/necrosis and the release of damage associated molecular patterns (DAMPs). The findings of the current study suggest that cigarette smoke-induced inflammation may involve overlap with components of the sterile inflammatory response and that prolonged exposure to a sterile inflammatory stimulus may have implications for subsequent bacterial infection.

A key observation of this study is that cigarette conditions the lung environment to a fundamentally altered response. These findings are of critical importance; as it suggests that molecular pathways engaged in a healthy, non-inflamed tissue may be distinct to inflammatory pathways engaged in an inflamed tissue, such as the environment generated by cigarette smoke. Our findings suggest that smoke-exposed macrophages take a pragmatic approach and favor further activation of the IL-1α pathway upon challenge with a secondary stimulus that would normally engage different signaling and effector pathways. Molecularly, IL-1R1 signaling

pathways were redundant in NTHi-induced inflammatory processes in room air-exposed control mice. Contrasting this observation, cigarette smoke exposure primed the lungs to an exaggerated inflammatory response that involved distinct molecular signaling pathways; IL-1R1-dependency was only observed in cigarette smoke-exposed mice. This represents a novel finding and suggests that mucosal immune responses are shaped by the immune inflammatory history of the tissue. This general phenomenon could explain the exacerbated inflammatory response observed in our experimental model. Together, our observations establish the critical importance of the lung mucosal environment in determining inflammatory responses engaged by environmental agents.

The findings of the current study provide some of the first mechanistic insight into the mediators that drive bacterial exacerbations of cigarette smoke-induced inflammation. These findings form the basis for future studies to dissect the relative importance of bacterial exacerbation in the pathology elicited by cigarette smoke; as exacerbations are associated with a greater loss of lung function in COPD (44–46). Work has begun in this area, as a previous study demonstrated that repeated challenge with the viral stimulus poly I:C led to increased airspace enlargement (47). A recent study that utilizes cigarette smoke exposure and two administrations of heat killed NTHi demonstrated that both stimuli were necessary to induce measurable increases in pathology as measured by airspace enlargement and an increase in lung compliance (48). This study characterized exacerbated inflammation in their model, but did not examine potential mechanisms. Our study also utilizes the flexivent system to measure changes in lung physiology which has been established as capable of detecting differences in a model of cigarette smoke exposure (37). Here we add to these studies and show that bacterial infection can accelerate cigarette smoke-induced changes to lung physiology. Our focus on IL-1 signaling and

neutrophilia synergizes with previous work that suggests these to be important in driving lung pathology in smoke-exposure models. Churg *et al* have shown that mice deficient in IL-1R1 have attenuated airspace enlargement after 6 months of cigarette smoke exposure and also observed the IL-1-dependency of neutrophilia in the context of cigarette smoke (20). Our bacterial exacerbation model demonstrates that NTHi infection increases the levels of IL-1α and neutrophil numbers in the lung. We have provided the proof-of-principle data which shows that repeated exacerbation of these inflammatory mechanisms led to measurable differences in lung function and future studies will examine this in greater detail.

The increased inflammation observed in cigarette smoke-exposed mice following infection with NTHi may not be entirely detrimental to the host as there may be a role for the increased macrophage and neutrophil numbers in the control of bacterial burden. The data presented in Figure 6 suggests that in the absence of IL-1R1 and CXCR2 bacterial clearance was compromised, but this increased bacterial burden was only observed in the smoke-exposed groups. It is possible that enhanced cellular inflammation is necessary to compensate for other aspects of host defense that are impaired by cigarette smoke. It has been established that cigarette smoke attenuates the phagocytic ability of macrophages, and this impairment has been specifically demonstrated in the context of NTHi (49). In addition to phagocytic activity, cigarette smoke also compromises the production of antimicrobial peptides and disrupts the integrity of the epithelial barrier. Exaggerated cellular recruitment may be necessary for bacterial clearance to compensate for these smoke-induced host defense deficiencies. Additionally, the increased bacteria burden likely results in increased sensing of the bacteria which could explain the increased levels of CXCL1 observed in smoke and NTHi infected IL-1R1 KO mice and the increased CXCL1, CXCL2, and CXCL5 observed in CXCR2 KO mice. Disrupting the clearance

mechanisms could be expected to lead to increased cytokine and chemokine production as the lung attempts to mount an unsuccessful inflammatory response against a stimulus that is not being eliminated and thus continues to propagate the inflammatory response.

Enhanced bacterial burden may seem like a barrier to the efficacy of targeting of IL-1 signaling for therapeutic purposes. This same phenomenon of enhanced bacterial burden was observed when corticosteroids were utilized as a treatment in our smoke exposure and NTHi infection model (14). In COPD patients, the treatment of an acute exacerbation of COPD with a steroid is recommended to be supplemented with antibiotic treatment whenever an infection is suspected (6, 50). The combined antibiotic and corticosteroid treatment is associated with better patient outcomes and a lower mortality (51). Our data would suggest that future therapeutic strategies that target IL-1 signaling may need to follow the recommended practices as corticosteroid treatment and be accompanied by antibiotic treatment to compensate for host defense mechanisms that are attenuated by cigarette smoke.

The murine model possesses some unique characteristics in terms of the differences between the mouse strains utilized. We have previously compared the inflammatory response between BALB/c and C57BL/6 mice (14, 30), and there are differences in the response of the different strains to cigarette smoke. BALB/c mice have a more neutrophilic response, while C57BL/6 mice have more monocyte recruitment. This trend was observed again in the current study, as the exacerbation of cigarette smoke induced cellular inflammation was predominantly neutrophilic in nature and less so in C57BL/6 mice. This highlights the disadvantage of using multiple strains, however, even though it was not as dramatically increased in C57BL/6 mice, NTHi-induced neutrophilia was still significantly exacerbated by cigarette smoke. Investigating the differences between the two strains may be of interest in future studies as it could help to

identify what factors in an outbred population may predispose to exacerbated responses. Another strain difference observed in this study was the bacterial burden in smoke exposed mice. We have previously published the phenomenon of increased clearance of NTHi in a smoke exposed lung due to an increase in NTHi-specific antibodies induced by cigarette smoke (14, 52). In the current study we observed this increased clearance for the BALB/c strain, but not C57BL/6 mice. A possibly explanation for this is that we have changed the supplier of C57BL/6 mice since the initial study, however, we have not measured NTHi-specific antibody expression in these mice as this was not the focus of the current study. When interpreting results from experiments employing a murine smoke exposure system, it is important to consider the strain of mice utilized.

The data presented in the current study expand the understanding of the role of IL-1 signaling in cigarette smoke-induced inflammation and elucidates the importance of this signaling axis in the context of a bacterial infection. The crucial source of IL-1α is a hematopoietic cell, reinforcing the perceived importance of the alveolar macrophage in orchestrating the inflammatory response elicited by cigarette smoke. In addition to driving inflammation, cigarette smoke changes the phenotype of alveolar macrophages, priming these cells to produce exacerbated IL-1α in response to bacterial stimulus. *In vivo* infection with NTHi results in greater IL-1α levels in smoke-exposed mice and this was accompanied by IL-1R1 dependent CXCL5 production and exacerbated neutrophil recruitment. The current study highlights an important phenotypic change to a mucosal environment with important ramifications to host defense mechanisms in a model relevant to exacerbations of COPD.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical support of Joanna Kasinska, the secretarial assistance of Marie Bailey, and Dr. Abraham Roos for discussion and preparation of the manuscript.

DISCLOSURE

The work described herein was funded in-part by the CIHR and MedImmune LLC; JKN holds an Ontario Graduate Scholarship; MCM holds a Canadian Thoracic Society Fellowship. The funders had no role in data collection and analysis, decision to publish, or preparation of the manuscript.

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FOOTNOTES

This study was funded in part by the Canadian Institutes of Health Research (CIHR) and MedImmune LLC .

Abbreviations used in this article: BAL, bronchoalveolar lavage; COPD, Chronic Obstructive Pulmonary Disease; MNCs, mononuclear cells; NEU, neutrophils; NTHi, nontypeable Haemophilus influenzae; TCN, total cell number

FIGURE LEGENDS

Figure 1. *Cigarette smoke-induced neutrophilia is dependent on IL-1α derived from a hematopoietic cell type.* Wild type C57BL/6, IL-1α and IL-1β KO mice were exposed to room air or cigarette smoke for 4 days and sacrificed 18 hours post smoke exposure. IL-1α and IL-1β were measured in lung homogenates. CXCL1 and CXCL5 levels were assessed in broncho-alveolar lavage (BAL) fluid. Neutrophils (NEU) were counted in BAL fluid. (A) represents BAL neutrophil counts and inflammatory mediator expression in IL-1α KO mice and (B) represents data from IL-1β KO mice. (C) IL-1α chimeric mice were generated as shown and smoke-exposed for 4 days. Neutrophils were assessed in BAL. Data represent mean ± SEM, n=4-10. Statistical analysis was performed using General Linear Model followed by individual t-test. * indicates $P<0.05$.

Figure 2. *Cigarette smoke alters alveolar macrophage phenotype and primes alveolar macrophages to produce IL-1α.* (A) Mononuclear cells (MNC) were assessed in the BAL of C57BL/6 mice exposed to room air or cigarette smoke for 4 days and 8 weeks. (B) The altered morphology of alveolar macrophages from 8 week smoke-exposed mice was determined by H&E stained cytospins. C57BL/6 mice were smoke-exposed for 8 weeks and alveolar macrophages were isolated by adherence. Alveolar macrophages were stimulated with media (RPMI), Pam3CSK4, LPS, or NTHi for 24 hours. (C, E, F) TNF-α, IL-1α, and IL-1β were assessed by ELISA in culture supernatants. (D) IL-1α mRNA levels were measured by RT qPCR after 4 hours of LPS stimulation. Data represent mean ± SEM, and statistical analysis was performed using General Linear Model followed by individual t-test. * indicates $P<0.05$, n=5.

Figure 3. *NTHi exacerbates cigarette smoke-induced inflammation and neutrophil-recruiting CXCL5 expression.* (A) BALB/c mice were exposed to room air or cigarette smoke for 8 weeks and subsequently challenged with 10^6 CFU of NTHi. Mice were sacrificed 12 hours post infection. Data show total cell numbers (TCN), mononuclear cells (MNC), and neutrophils (NEU) in BAL fluid. (B) CXCR2 KO mice on a BALB/c background were taken through the same protocol and neutrophilia was assessed in the BAL. (C) CXCL1 and CXCL5 were assessed by ELISA in BAL fluid from BALB/c mice. (D) CXCL5 was assessed in lung tissue by immunohistochemistry. (E) Precision cut lung slices were generated from 8 week smoke-exposed C57BL/6 mice and stimulated for 6 hours with NTHi, and CXCL5 mRNA was assessed by RT qPCR. Data represent mean ± SEM, and statistical analysis was performed using General Linear Model followed by individual t-test. * indicates $P<0.05$, n=5-10 (n=3 for lung slice experiments).

Figure 4. *Cigarette smoke-exacerbated neutrophilia is dependent on IL-1 signaling.* Wild type C57BL/6 and IL-1R1 KO mice were exposed to 8 weeks of cigarette smoke and infected with 10^6 CFU of NTHi. (A) Cellular inflammation was assessed in BAL fluid and (B) CXCL1 and CXCL5 were measured in the BAL fluid by ELISA. Data represent mean ± SEM, and statistical analysis was performed using General Linear Model followed by individual t-test. * indicates $P<0.05$, n=5-10.

Figure 5. *NTHi exacerbation of cigarette smoke-induced inflammation is dependent on IL-1α but not IL-1β.* (A) IL-1α and IL-1β levels were measured by ELISA in lung homogenates from mice exposed to 8 weeks of cigarette smoke and 10^6 CFU of NTHi. (B) Immunohistochemistry

was performed on formalin fixed paraffin embedded lung tissue samples from the same experiments to stain for IL-1α. (C) IL-1α KO mice and (D) IL-1β KO mice were exposed to room air or cigarette smoke for 8 weeks and subsequently challenged with 10^6 CFU of NTHi. Mice were sacrificed 12 hours post infection. Data show total cell numbers (TCN), mononuclear cells (MNC), and neutrophils (NEU) in the BAL fluid. Data represent mean ± SEM, and statistical analysis was performed using General Linear Model followed by individual t-test. * indicates $P<0.05$, n=5-10.

Figure 6. *IL-1R1 and CXCR2 KO mice have exacerbated bacterial burden.* IL-1R1, CXCR2, and IL-1α KO mice were cigarette smoke-exposed for 8 weeks and subsequently challenged with 10^6 CFU of NTHi. Twelve hours after challenge, mice were sacrificed and lung homogenates plated onto chocolate agar plates. CFU were counted 48 hours later. Data are expressed as mean ± SEM, and statistical analysis was performed using General Linear Model followed by individual t-test. * indicates $P<0.05$, n=5-10.

Figure 7. *Repeated NTHi infection leads to a more compliant lung in smoke-exposed mice.* (A) C57BL/6 mice were exposed to cigarette smoke for 10 weeks and given 10^6 CFU of NTHi intranasally every 2 weeks for 4 administrations. (B) Pressure volume curves and (C) compliance data were generated. Data are expressed as mean ± SEM, and statistical analysis was performed using General Linear Model followed by individual t-test. * indicates $P<0.05$, n=5-10.

Figure 8. *The inflammatory-priming effects of cigarette smoke.* Cigarette smoke induces IL-1α that elicits CXCR2 chemokines to recruit neutrophils to the lung environment. Cigarette smoke exposure changes the phenotype of alveolar macrophages, priming them for exacerbated IL-1α responses to bacteria such as NTHi. This exacerbated IL-1α response is characterized by greater CXCL5 production and increased neutrophilia *in vivo*.

Figure 1

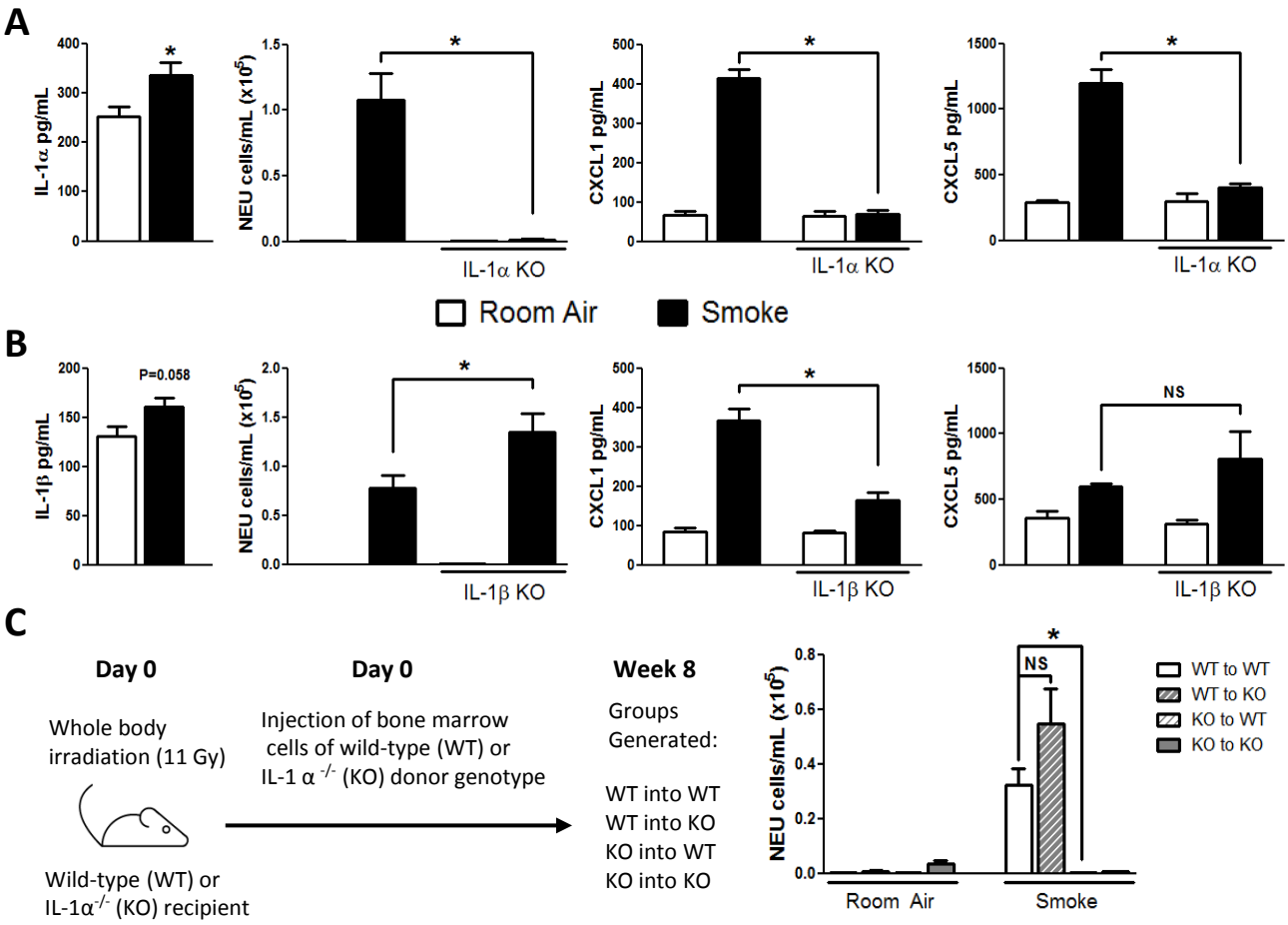

A
IL-1α pg/mL
NEU cells/mL (x10^5)
CXCL1 pg/mL
CXCL5 pg/mL
IL-1α KO
Room Air
Smoke
B
IL-1β pg/mL
P=0.058
NEU cells/mL (x10^5)
CXCL1 pg/mL
CXCL5 pg/mL
NS
IL-1β KO
C
Day 0
Whole body irradiation (11 Gy)
Wild-type (WT) or IL-1α-/- (KO) recipient
Day 0
Injection of bone marrow cells of wild-type (WT) or IL-1 α -/- (KO) donor genotype
Week 8
Groups Generated:
WT into WT
WT into KO
KO into WT
KO into KO
NEU cells/mL (x10^5)
NS
Room Air
Smoke
WT to WT
WT to KO
KO to WT
KO to KO

Figure 2

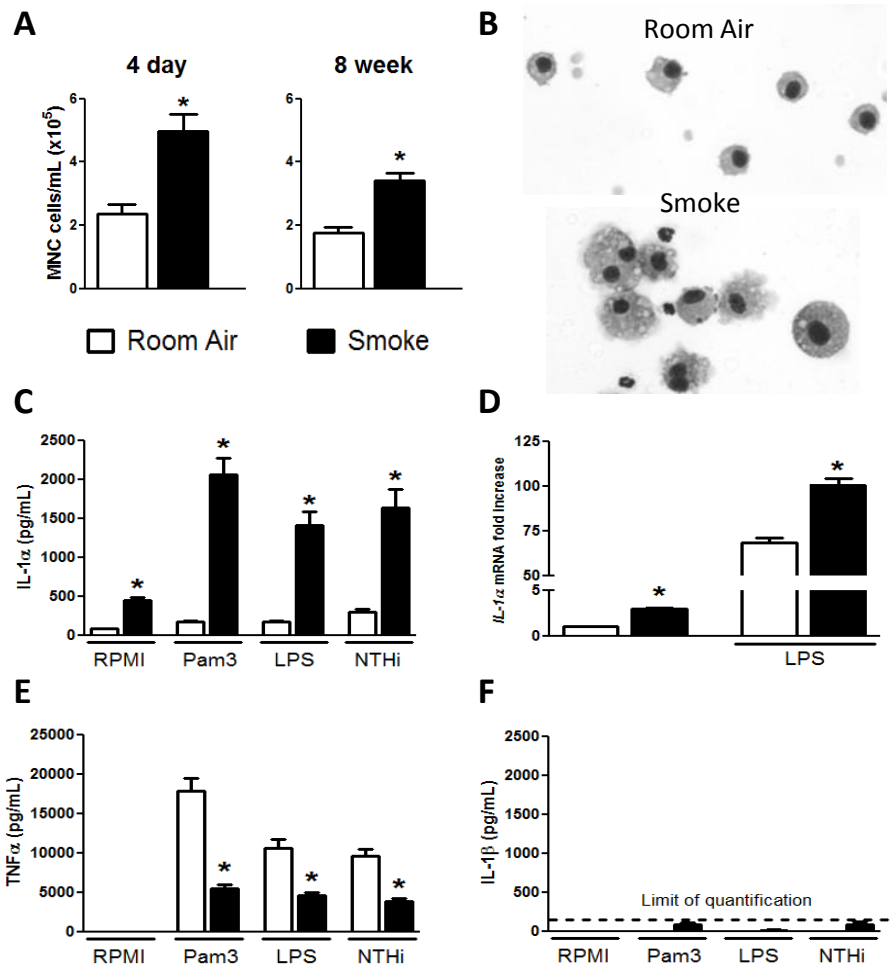

A
4 day
8 week
MNC cells/mL (x10^5)
Room Air
Smoke
B
Room Air
Smoke
C
IL-1α (pg/mL)
RPMI
Pam3
LPS
NTHi
D
IL-1α mRNA fold increase
LPS
E
TNFα (pg/mL)
RPMI
Pam3
LPS
NTHi
F
IL-1β (pg/mL)
Limit of quantification
RPMI
Pam3
LPS
NTHi

Figure 3

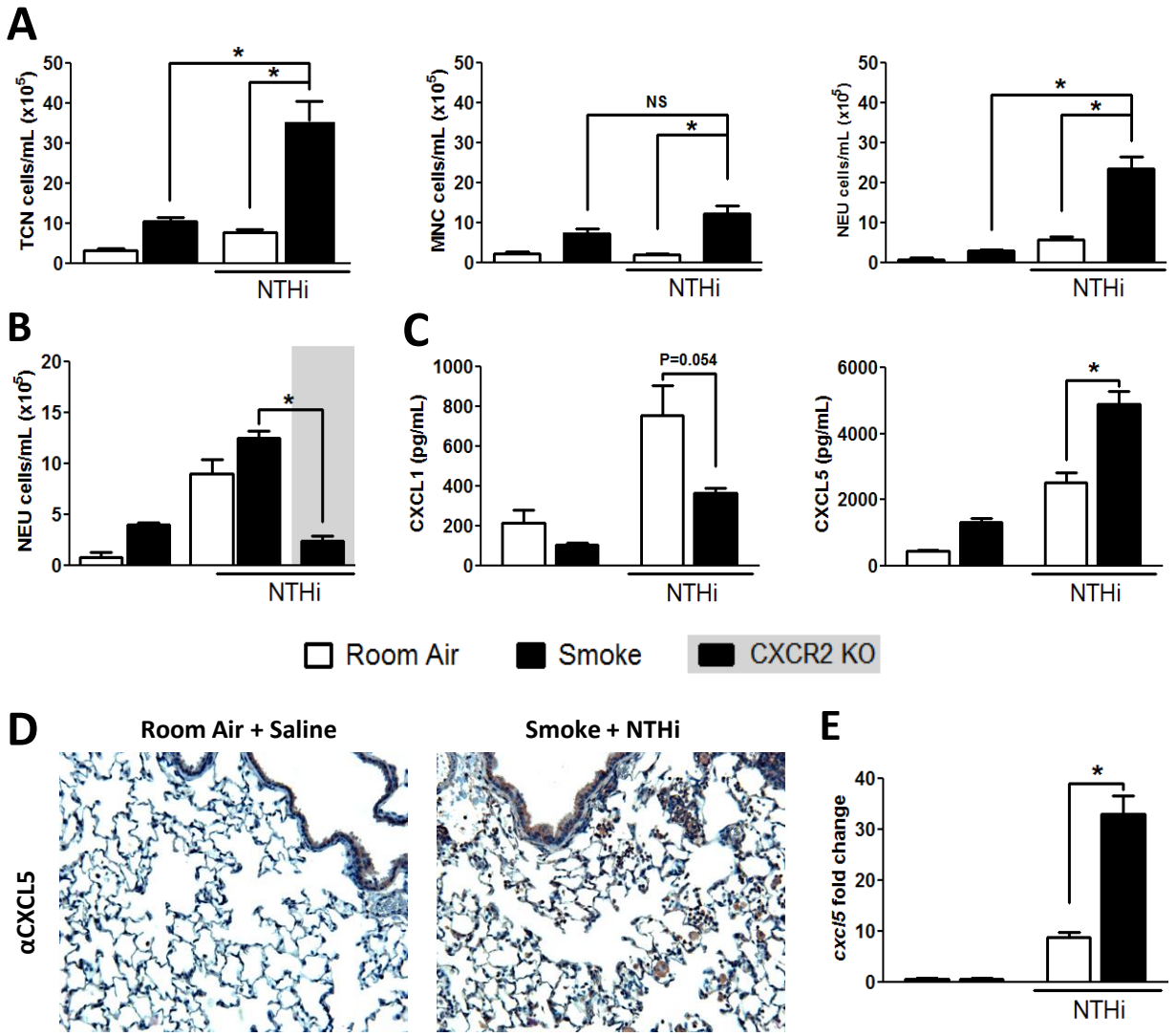

A
TCN cells/mL (x10^5)
MNC cells/mL (x10^5)
NEU cells/mL (x10^5)
NS
NTHi
B
NEU cells/mL (x10^5)
C
CXCL1 (pg/mL)
P=0.054
CXCL5 (pg/mL)
Room Air
Smoke
CXCR2 KO
D
Room Air + Saline
Smoke + NTHi
αCXCL5
E
cxcl5 fold change

Figure 4

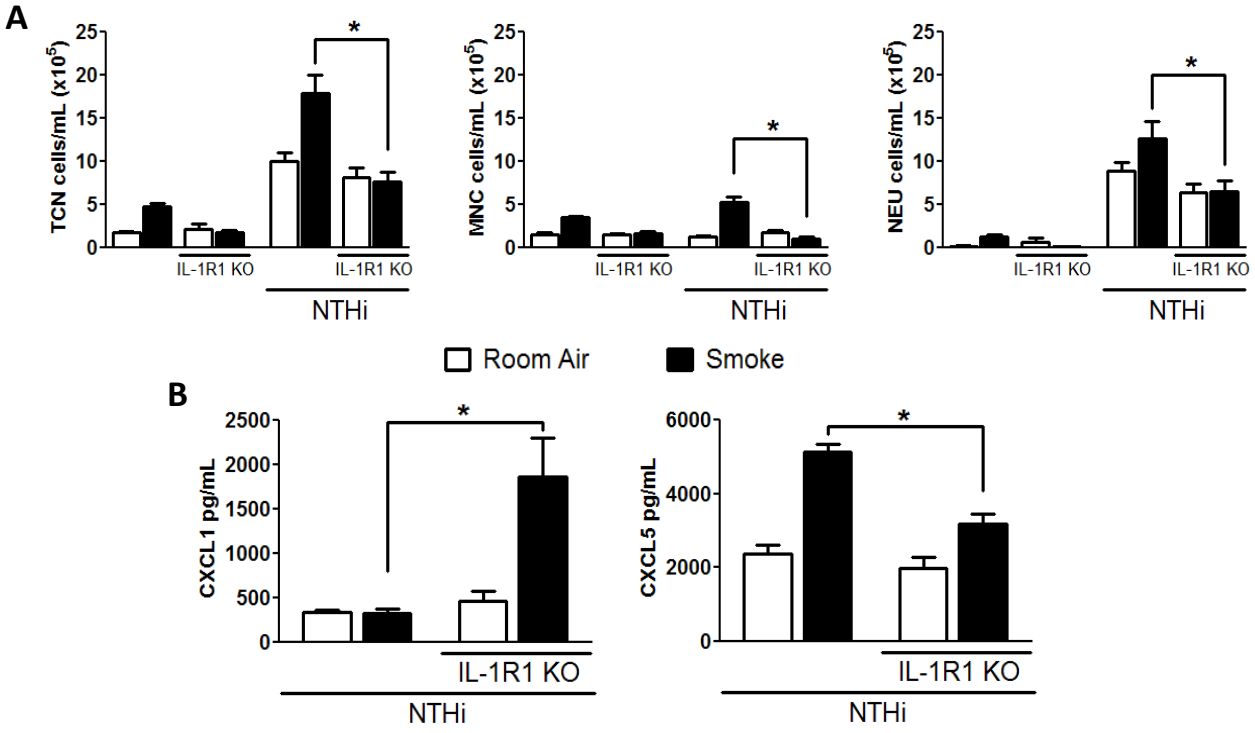

A
TCN cells/mL (x10^5)
MNC cells/mL (x10^5)
NEU cells/mL (x10^5)
0
5
10
15
20
25
*
IL-1R1 KO
IL-1R1 KO
NTHi
Room Air
Smoke
B
CXCL1 pg/mL
0
500
1000
1500
2000
2500
CXCL5 pg/mL
0
2000
4000
6000
IL-1R1 KO
NTHi

Figure 5

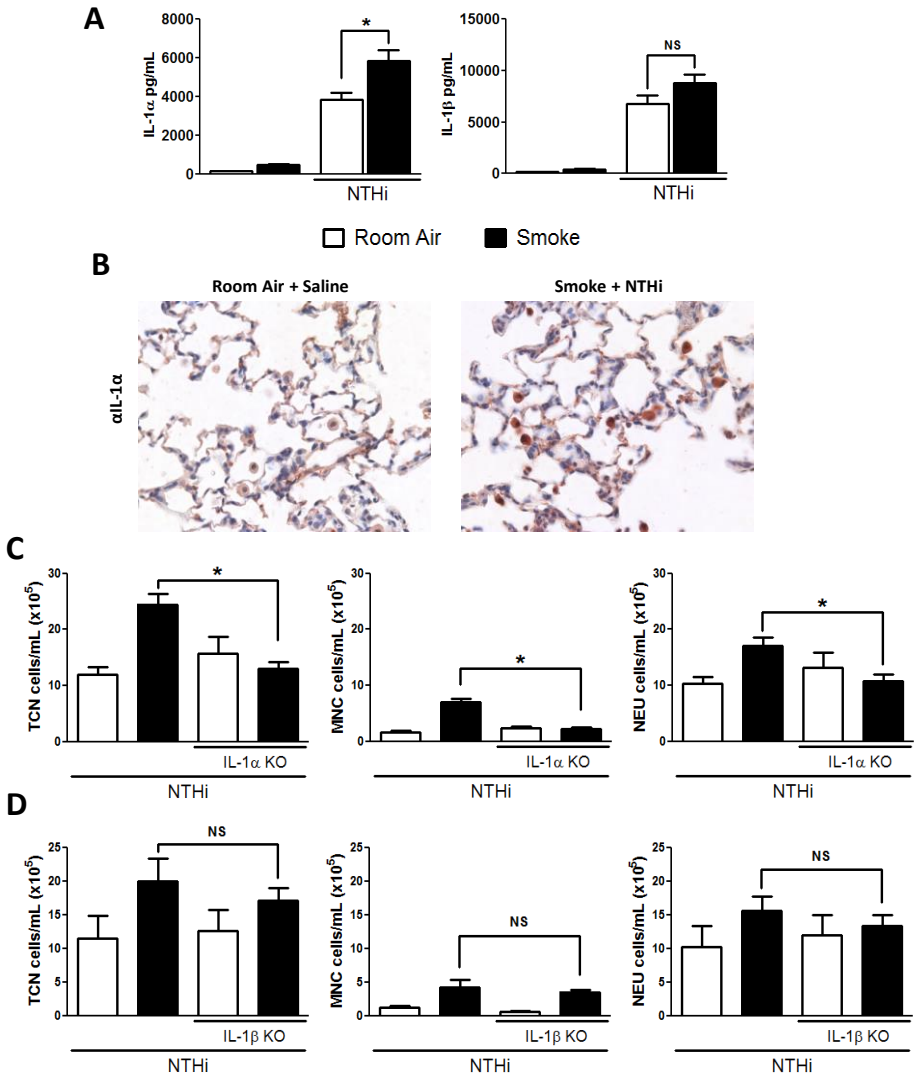

A
IL-1α pg/mL
8000
6000
4000
2000
0
*
NTHi
IL-1β pg/mL
15000
10000
5000
0
NS
NTHi
Room Air
Smoke
B
Room Air + Saline
Smoke + NTHi
αIL-1α
C
TCN cells/mL (x10^5)
MNC cells/mL (x10^5)
NEU cells/mL (x10^5)
IL-1α KO
NTHi
D
IL-1β KO
NTHi
NS

Figure 6

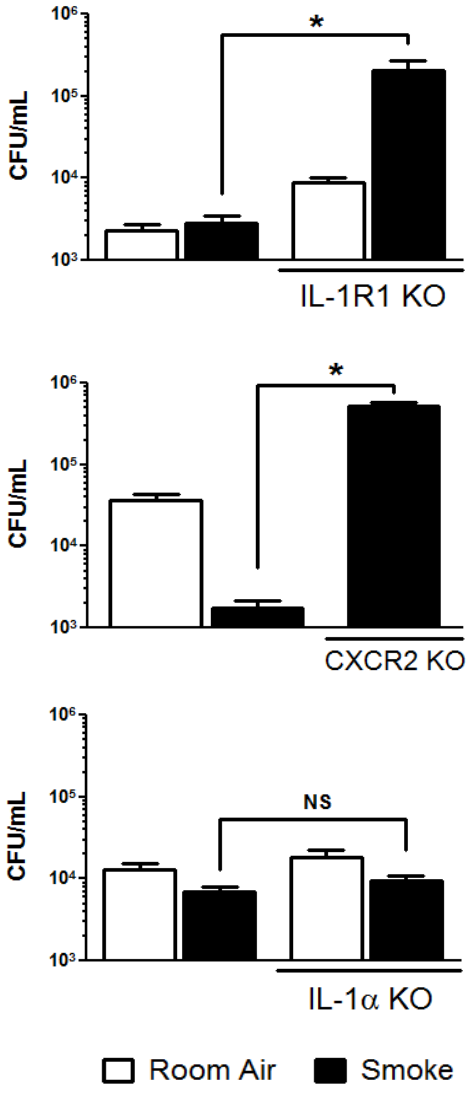

CFU/mL
10^6
10^5
10^4
10^3
*
IL-1R1 KO
*
CXCR2 KO
NS
IL-1α KO
Room Air
Smoke

Figure 7

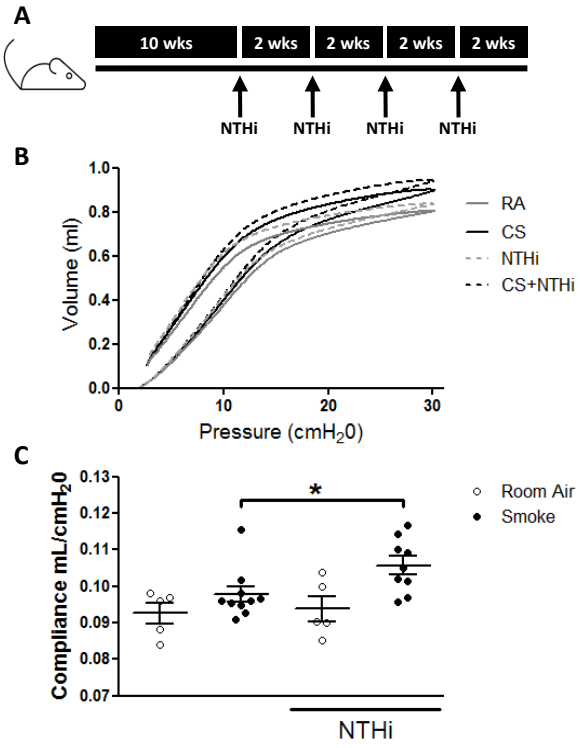
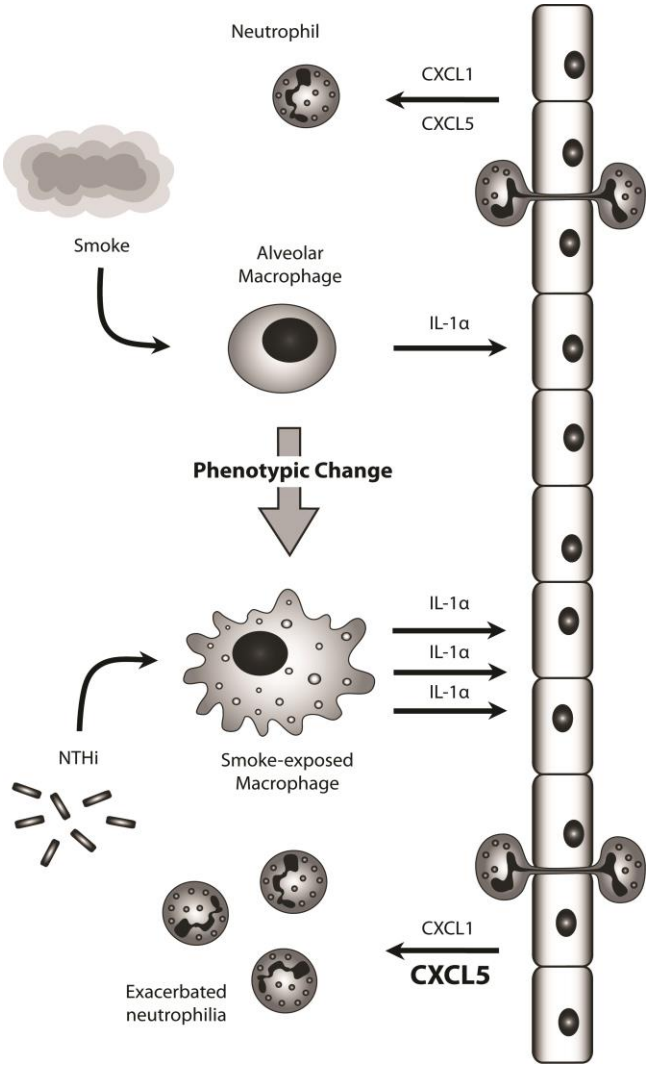
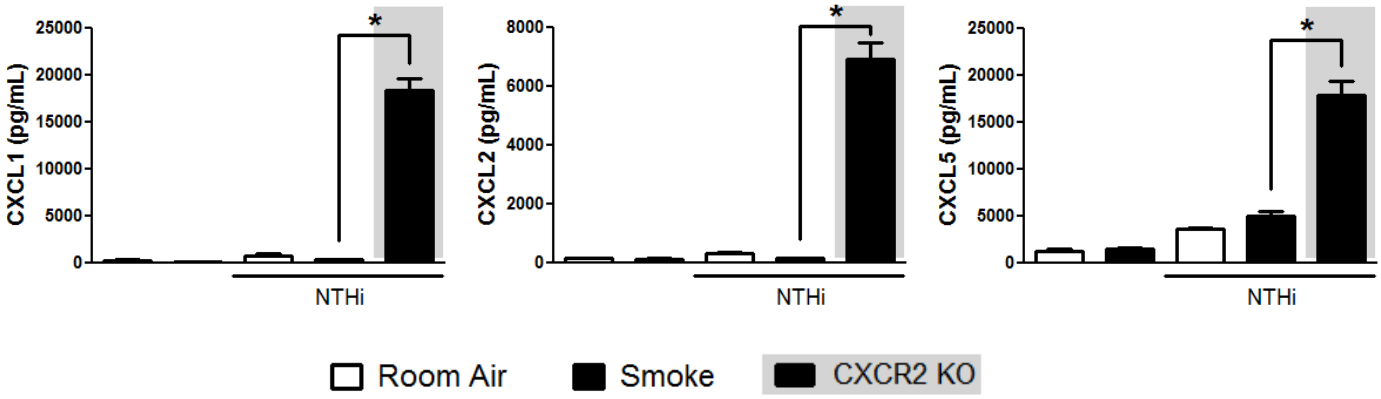

A
10 wks
2 wks
2 wks
2 wks
2 wks
NTHi
NTHi
NTHi
NTHi
B
Volume (ml)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Pressure (cmH20)
RA
CS
NTHi
CS+NTHi
C
Compliance mL/cmH20
0.13
0.12
0.11
0.10
0.09
0.08
0.07
*
Room Air
Smoke
NTHi

Figure 8


Neutrophil
CXCL1
CXCL5
Smoke
Alveolar
Macrophage
IL-1α
Phenotypic Change
IL-1α
IL-1α
IL-1α
NTHi
Smoke-exposed
Macrophage
CXCL1
CXCL5
Exacerbated
neutrophilia



Supplementary Figure 1. *CXCR2 KO exacerbates CXCR2 ligand expression in NTHi-infected smoke-exposed mice.* BALB/c and CXCR2 KO mice were exposed to room air or cigarette smoke for 8 weeks and subsequently challenged with 10^6 CFU of NTHi. Mice were sacrificed 12 hours post infection. CXCL1, CXCL2, and CXCL5 were assessed by ELISA in BAL fluid. Data represent mean ± SEM, and statistical analysis was performed using General Linear Model followed by individual t-test. * indicates $P<0.05$, n=5

CHAPTER FOUR

Transcriptomic analysis of the cigarette smoke-altered bacterial response in murine alveolar macrophages

This study is complete and beginning the submission process.

One of the more intriguing findings from the previous study was the discovery that cigarette smoke primed alveolar macrophages to produce an excessive amount of inflammatory mediators in response to bacterial ligands. This gives the macrophage a central role in the cigarette smoke-altered response to bacteria and the exacerbated inflammation observed during this response. The altered inflammatory mediator profile of a bacteria-stimulated smoke-exposed macrophage has been reported (202), but the extent of the changed phenotype is currently unknown. To contribute to the understanding of this altered phenotype, we performed a whole genome expression analysis of macrophages isolated from room air- and smoke-exposed animals and stimulated them *ex vivo* with NTHi. We focused on the comparison between NTHi-stimulated macrophages from smoke-exposed mice and NTHi-stimulated macrophages from room air control mice. This allowed us to directly compare the differences in the response to NTHi due to cigarette smoke.

Dr. Stampfli and I were responsible for the experimental design and interpreting the results. I carried out the analysis of the gene array, performed the necessary follow-up experiments, and prepared the article. Dr. Gaschler generated the initial biological samples utilizes in the gene array, while Pamela Shen and Dr. Abraham Roos provided technical assistance with experimental procedures. Drs. Sabina Halappanavar, Carole Yauk, and Andrew Williams were involved in the execution of the gene array and the initial statistical analysis.

Title

Transcriptomic analysis of the cigarette smoke-altered bacterial response in murine alveolar macrophages

Authors

Jake K Nikota[1,2], Sabina Halappanavar[3], Andrew Williams[3], Carole Yauk[3], Gordon Gaschler[1,2], Pamela Shen[1,2], Abraham Roos[2], Martin R Stampfli[2,4].

Affiliations

[1]Medical Sciences Graduate Program, McMaster University, Hamilton, Ontario, Canada; [2]Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, McMaster University, Hamilton, Ontario, Canada; [3]Environmental and Radiation Health Sciences Directorate, Health Canada, Ottawa, Ontario, Canada; [4]Department of Medicine, Firestone Institute for Respiratory Health at St. Joseph's Healthcare, McMaster University, Hamilton, Ontario, Canada

Corresponding Author

Martin R. Stämpfli,

McMaster University

Department of Pathology and Molecular Medicine

1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

Phone: 905 525 9140 x22493

Email: stampfli@mcmaster.ca.

Introduction

Cigarette smoke is an unusual stimulus for the mucosal environment of the lung. With respect to immune function, cigarette smoke has been shown to activate damaging proinflammatory mechanisms while suppressing both innate and adaptive immune processes (1–4). The damaging inflammatory response induced by cigarette smoke is believed to be the main etiological factor in the pathogenesis of chronic obstructive pulmonary disease (COPD) (5). Concurrently, the suppression of host defense mechanisms in a smoke-exposed lung is also relevant to COPD as the progressive loss of lung function that characterizes COPD is periodically exacerbated by bacterial and viral infection (5). Exacerbations of COPD represent the greatest burden of the disease on health care systems, and exacerbations are often life threateningly severe (6). Additionally, smokers with or without COPD experience more frequent and severe infections (7–10), a probable repercussion of the suppression of host defense mechanisms. With over one billion smokers in the world (11), understanding the impact of cigarette smoke on bacterial host defense mechanisms will inform health care strategies for a large segment of the global population.

Pulmonary host defense mechanisms are not simply suppressed by cigarette smoke, but rather skewed towards a unique immune response. This altered phenotype is particularly evident in the context of bacterial infection, where inflammatory mechanism are both activated and inhibited. Studies in animal models have demonstrated that exposure to cigarette smoke exacerbates cellular inflammation by several different bacterial agents, including nontypeable *Haemophilus influenzae* (NTHi), *Pseudomonas aeruginosa*, and *Streptococcus pneumonia* (12–15). While cellular inflammation is exacerbated, cigarette smoke suppresses the expression of inflammatory mediators such as TNF-α (16, 17). These findings have been expanded upon to

demonstrate that the suppression of some inflammatory mediators was accompanied by the increased expression of a different set of pro-inflammatory genes, such as MCP-1, that were not normally induced by the bacterial stimulus (15).

A key mediator of immune responses in the lung is the alveolar macrophage (AM). This cell type is often the first leukocyte to encounter pulmonary insults and, in addition to phagocytic clearance, directs subsequent immune responses through inflammatory cytokine production (18, 19). Due to their central role in initiating immune responses within the lung, macrophages have been proposed to be the orchestrators of COPD (20). In murine models, the lung destruction associated with chronic smoke exposure is lessened by macrophage depletion (21), and the inflammatory mediators produced by alveolar macrophages are crucial mediators of leukocyte recruitment in response to cigarette smoke. (22)(Chapter 3). The immunosuppressive effects of cigarette smoke are also present in the macrophage as smoke attenuates the bacterial phagocytic ability of macrophages (23). Additionally, the skewed bacterial host defense elicited by cigarette smoke is observable in the cytokine profile elicited in smoke-exposed AMs after bacterial stimulation (20). These data imply that cigarette smoke induces a novel macrophage phenotype, and characterizing this phenotype will provide novel insight into the immune modulating effects of cigarette smoke.

To determine how cigarette smoke alters the bacterial host response, AMs were isolated from smoke-exposed mice and stimulated with NTHi. Gene expression of the whole-genome was determined by microarray on RNA isolated from these AMs. The combination of the two stimuli leads to a unique transcriptome in each of the four experimental groups. The NTHi-stimulated AMs from room air- and smoke-exposed mice were directly compared and Gene Ontologies (GO) and canonical pathways were enriched, revealing that cell cycle/cell division

biological functions were increased while immune response-related biological functions were both increased and decreased. PCR confirmed the altered gene expression and helped characterize a subset of genes associated with cell division and cytokine production, and identified the additive effect of smoke and NTHi with certain cytokines. Predictions of upstream regulators of this unique macrophage profile indicated that smoke-exposed macrophages were in an environment filled with growth factors, and measurements in bronchoalveolar lavage (BAL) fluid confirmed this. Our findings provide an extensive characterization of the unique phenotype of a smoke-exposed AM and how it's response to a bacterial stimulus is altered.

Results

Cigarette smoke and NTHi stimulation elicit unique transcriptomic phenotypes

Utilizing a murine smoke exposure model, it has previously been established that cigarette smoke leads to an altered AM phenotype, with both visible and functional differences (15). The altered morphology of AMs is evident as these smoke-exposed macrophages appear larger with foam-like internal structures (Fig 1A). To investigate the functional differences of a cigarette smoke-exposed AM in the context of a bacterial infection, mice were exposed to cigarette smoke for 8 weeks and AMs were subsequently isolated and stimulated with NTHi for 6 hours before RNA was isolated for analysis (Fig 1B). This produced four distinct experimental groups: unstimulated AMs cultured from room air control mice, unstimulated AMs cultured from smoke-exposed mice, AMs from room air control mice cultured with NTHi, and AMs from smoke-exposed mice cultured with NTHi.

In order to comprehensively characterize the distinct response of the smoke-altered macrophage to NTHi, a microarray analysis was performed on these four groups. The microarray identified 3288 genes that were differentially expressed relative to each experimental group. Initially the data were analyzed for a global impact of cigarette smoke and NTHi infection on macrophages by cluster analysis (Fig 1C). Macrophages were isolated from room air- or smoke-exposed mice with 5 biological replicates per group, and each sample was clustered together into its respective group. Interestingly, NTHi-stimulated samples clustered closer together than unstimulated samples, yet all NTHi-stimulated samples from smoke-exposed AMs clustered distinctly from NTHi stimulated samples from room air-control AMs. These data establish 4 distinct gene expression patterns corresponding with each experimental group.

The specific objective of this study is to identify and characterize the different responses to NTHi between AMs from room air- and smoke-exposed mice. For this reason subsequent analysis was performed on the fold change difference between smoke-exposed AMs stimulated with NTHi and room air-control AMs stimulated with NTHi. This is the relevant comparison to best analyze significant differences in the bacterial host response induced by smoke exposure. In order to be considered significantly altered, expression of a given gene must exceed a fold change of greater than the threshold set at 1.5 to eliminate minimally differentiated genes. In addition, the fold change had to have a false discovery rate-adjusted p value of greater than 0.05. With these parameters in place, 1165 genes were found to be differentially expressed between NTHi-stimulated AMs from room air- and smoke-exposed mice.

AMs from smoke-exposed mice enriches genes in GO and canonical pathways associated with the cell cycle and immune function in response to NTHi

To gain an appreciation for the biological functions associated with the altered gene expression between room air- and smoke-exposed-AMs stimulated with NTHi, a GO analysis was performed to compare the differences between these two groups. Using NextBio software we enriched 1438 GO processes out of the data set. This consisted of 840 biological processes that were increased and 598 biological processes that were decreased. The top 20 rankings of increasing and decreasing GO processes are presented in detail in Supplementary tables 1 and 2. To visualize this large amount of data, these GO processes were organized into pie charts (Figure 2). Any GO processes where all of the enriched genes were contained in another GO process were excluded from the visualization.

The top increased GO groups enriched were *cell cycle* (GO:0007049) and *cell division* (GO:0051301) with the third most enriched GO being *chromosome* (GO:0005694). This represented an interesting finding that the highest ranked biological processes are tied to cell division, indicating that cigarette smoke exposure may have a proliferative effect on AMs. Other GO's of interest to bacterial host defense included regulation of *immune system process* (GO:0002376), *immune system development* (GO:0002520), and *leukocyte activation* (GO:0002520). These data indicate that cigarette smoke increases some immunological mechanisms of AMs in response to NTHi.

When examining the decreased GO biological groups that were enriched out of the dataset, most of the top 20 ranked were related to immune function. *Immune response* (GO:0006955), *immune effector process* (GO:0002252), *regulation of lymphocyte activation* (GO:0051249), *response to other organism* (GO:0051707), and *cytokine activity* (GO:0005125) were all enriched in the list of decreasing biological groups. Also of interest, the GO *Regulation of mononuclear cell proliferation* (GO:0032944) was decreased which adds to the evidence indicating that AMs from smoke-exposed mice are in a proliferative state. These data indicate that there are many immune functions decreased between NTHi stimulated AMs from cigarette smoke-exposed mice in comparison to room air controls.

The GO analysis indicated that there are genes involved with cell division that are increasing and genes involved with immune function that are both increasing and decreasing. To go into more detail about the specific biological pathways induced by NTHi stimulation that are activated or suppressed by cigarette smoke, we utilized NextBio software to analyze the Molecular Signatures Database of canonical pathways altered by cigarette smoke in the context of NTHi stimulation. This identified 440 differentially enriched pathways with 178 increasing

and 262 decreasing. To simplify the data set the top 20 increasing and top 20 decreasing pathways were identified (Table 1 and 2). The top increasing pathways were dominated by biological functions associated with the cell cycle and mitosis. The top enriched increasing pathways associated with immune function included *cytokine receptor signaling*, *genes involved in Chemokine receptors bind chemokines*, *IL12 and Stat4 Dependent Signaling Pathway in Th1 Development*. The decreasing canonical pathways included many associated with immune function such as the *cytosolic DNA-sensing pathway*, *NOD-like receptor signaling pathway*, *cells and molecules involved in the local inflammatory response*, *signal transduction through IL1R*. Of note, 7 of the 12 decreasing pathways associated with immune function included the gene for IL-6 (*Il6*). Canonical pathway enrichment confirmed the GO analysis in finding the increase in cell division pathways and increasing and decreasing immunological pathways.

Cigarette smoke induces a transciptome indicative of increased cell division genes in AMs

Analysis of the biological processes and canonical pathways that differ between NTHi stimulated AMs from smoke-exposed mice versus room air-control mice highlighted groups of genes associated with a proliferative AM phenotype. Examining the individual gene expression values from the microarray for the *cell division* GO illustrates the upregulation of the genes involved in this biological process (Figure 3A). To confirm the findings of the gene array and to examine cell division-related gene expression in this model, real time qPCR was utilized to confirm the expression of the 5 most differentially expressed genes in the cell division GO. RNA was isolated from AMs generated using the same experimental conditions as the microarray. Expression of the genes for proline-serine-threonine phosphatase interacting protein 1 (*Pstpip1*), budding uninhibited by benzimidazoles (*Bub1*), spindle and kinetochore-associated protein (*Ska1*), microtubule-associated serine/threonine kinase-like protein (*Mastl*), and

survivin/baculoviral inhibitor of apoptosis repeat-containing 5 (*Birc5*) gene expression were assessed and each of these genes was significantly increased by cigarette smoke over the background of NTHi infection (Figure 3B), thus reproducing the findings of the gene array. Interestingly, the increased expression of these cell division-associated genes was significantly increased by cigarette smoke alone and there was no significant interaction between cigarette smoke and NTHi with the exception of Pstpip1 whose induction by cigarette smoke was significantly less in NTHi stimulated AMs. These data indicate that cigarette smoke leads to an increase in proliferation of AMs which is predominantly unaffected by NTHi stimulation.

The genes altered by cigarette smoke consist of genes induced by smoke alone and genes induced by the combined stimulus

Detailed examination of the genes associated with cell division indicate that at least part of the cigarette-smoke altered response to NTHi can be explained as the phenotype of a smoke-exposed AM regardless of NTHi stimulation. To gain an appreciation for the extent of the gene expression in our analysis that is expected in an AM from smoke-exposed mice regardless of NTHi stimulation, Venn analysis was performed on the significantly altered genes identified between NTHi stimulated AMs from smoke-exposed mice and room air control mice and the significantly altered genes in unstimulated AMs from smoke-exposed mice (Figure 4). Of the 733 genes increased by cigarette smoke in NTHi stimulated AMs, 505 of these would be expected to be increased by cigarette smoke alone and 228 genes were uniquely increased by the combined stimuli. Of the 432 decreased genes 235 of those genes were decreased in smoke-exposed macrophages that were not stimulated with NTHi and 197 genes were only decreased in the context of both cigarette smoke exposure and NTHi stimulation. Cumulatively these data indicate that the altered phenotype of the smoke-exposed AM's response to NTHi encompasses

genes that are differentially expressed following smoke exposure alone and a unique group of genes that are only differentially expressed in the context of the combined stimulus of smoke and NTHi.

Cigarette smoke activates and suppresses the AM cytokine response to NTHi

AMs are crucial orchestrators of inflammatory responses in the lung, communicating with other cells through the production of cytokines. We sought to examine in greater detail how cigarette smoke affects the cytokine response to NTHi. Examining the GO for cytokines identified genes whose expression was increased or decreased by cigarette smoke exposure in NTHi stimulated AMs (Table 3). The values for induction of the genes by smoke exposure in unstimulated AMs and the induction values for NTHi stimulation alone are also presented for comparison. Significantly upregulated cytokines included a large induction of the gene for IL-12p40 (*Il12b*), as well as the induction of the chemokines IP-10/CXCL10 (*Cxcl10*), MCP-1/CCL2 (*Ccl2*), TARC/CCL17 (*Ccl17*), MIP-1β/CCL4 (*Ccl4*), and MIP-1α/CCL3 (*Ccl3*). Many of these cytokines are produced by AMs when stimulated by NTHi, but the gene expression is significantly increased by cigarette smoke. Decreasing cytokines include genes associated with acute inflammatory responses such as *Il6*, IL-1β (*Il1b*), KC/CXCL1 (*Cxcl1*), and 3 members of the TNF superfamily. *Il6* and *Cxcl1* are of particular interest because these genes are induced by over 100 fold by NTHi, implying that they represent an important aspect of the host response to NTHi under normal homeostatic conditions.

To confirm the microarray findings and examine the cytokine expression in greater detail, RNA was isolated from AMs generated using the same experimental conditions as the gene array and real time qPCR was utilized to confirm the expression of the 6 most upregulated and the 5

most downregulated cytokine genes in the microarray. With respect to the increased genes, *Il12b*, *Cxcl10*, and *Ccl2* were increased in NTHi stimulated AMs from smoke-exposed mice when compared to NTHi-stimulated AMs from control mice or unstimulated AMs from smoke-exposed mice (Figure 5A). The osteopontin/secreted phosphoprotein 1 gene (*Spp1*) was significantly increased by cigarette smoke in NTHi stimulated AMs, though not as robustly as other identified cytokines. The growth and differentiation factor-3 gene (*Gdf3*) was increased in NTHi stimulated AMs from smoke-exposed mice when compared to AMs from control mice, but this induction was significantly less than the expression of *Gdf3* in unstimulated AMs from smoke-exposed mice. The gene for Inhibin beta-A (*Inhba*) was significantly downregulated by smoke in NTHi-stimulated AMs, which did not reproduce the findings of the microarray. With respect to down regulated genes, the NTHi induction of IL-7 (*Il7*), GROγ/CXCL3 (*Cxcl3*), and *Il6* were all significantly decreased by cigarette smoke, with the most dramatic decreases observed with Il6 and *Cxcl3* (Figure 5B). The tumor necrosis factor ligand superfamily member 18 gene (*Tnfsf18*) was significantly decreased by cigarette smoke; however it was not significantly increased by NTHi to begin with. The gene for IL-36γ (*Il1f9*) was significantly increased by cigarette smoke in NTHi-stimulated AMs which did not replicate the findings of the gene array. The qPCR data identified *Il12b*, *Cxcl10*, and *Ccl2* as cytokine genes that are primed by cigarette smoke to be highly expressed in response to NTHi, and *Cxcl3* and *Il6* as genes that are heavily suppressed by cigarette smoke when stimulated by NTHi in AMs.

Immune responses in the lungs also produce cytokines from other cellular sources that direct AM function, and the response of AMs to cytokines may be affected by changes in the expression of these cytokine receptors. The GO for cytokine receptors was examined in Table 4. Interestingly, there were significant increases in the expression of the genes for the IL-18 and IL-

7 receptor as well as several chemokine receptors. The changes in cytokine receptor expression are more independent of NTHi stimulation and appear to be a result of cigarette smoke alone. These data suggest that smoke-exposed AMs may respond differently to cytokine signaling, possibly contributing to their altered phenotype.

Upstream regulator analysis predicts a pulmonary environment rich in growth factors

The microarray has been useful in providing a comprehensive characterization of the cigarette smoke-altered response to NTHi in AMs; however, the multiplex data can provide insight into the mechanism responsible for the altered phenotype. Ingenuity Pathway Analysis (IPA) provides the capacity to predict upstream regulators of gene expression pattern and a list of possible molecules that could be responsible for the gene expression changes observed in the array was generated. From this analysis, the soluble extracellular mediators were selected and displayed in (Table 5). The focus was placed on soluble mediators because this best represents the pulmonary environment that could give rise to the specific phenotype of the smoke-exposed AM. Interestingly, all of the potential upstream regulators predicted by these criteria were growth factors. To confirm the presence of some of these predicted growth factors in the environment of the smoke-exposed AM, the top 3 z-scored upstream regulators were measured on the protein level. BAL was collected from mice smoke exposed for 4 days and GM-CSF (transcribed from *Csf2*), VEGF, and HGF were measured by ELISA (Figure 6). GM-CSF, VEGF, HGF, and M-CSF were all significantly increased in the BAL of smoke-exposed mice. These data indicate that a large contributing factor to the phenotype of the smoke-exposed AM is the growth factor rich lumen of a smoke-exposed lung.

Discussion

Understanding how cigarette smoke alters bacterial host defense could have implications for the healthcare management of a large segment of the global population. The objective of this study was to provide a broad characterization of how cigarette smoke changes the response to a common bacterial agent. Additionally, this work provides a more detailed characterization of an experimental system that is used to model cigarette smoke-related disease. This also represents the first time that AMs from smoke-exposed mice, stimulated with NTHi, have been examined on the whole-genome transcriptomic level. NTHi is the most common cause of bacterial exacerbations of COPD (24), and these findings will have implications for the treatment of COPD exacerbation and the management of bacterial infections in smokers.

The objectives of this study were addressed with a murine model of cigarette smoke exposure and *ex vivo* stimulation of AMs with NTHi. The smoke exposure apparatus has previously been shown to mimic clinical aspects of cigarette smoke exposure observed in human smokers. Carboxyhemoglobin and cotinine levels measured in our system are comparable to values observed in human smokers (25). Our experimental system also reproduces other clinical hallmarks associated with cigarette smoking, such as airspace enlargement and changes in ventilation and perfusion following prolonged cigarette smoke exposure (26). The strain of NTHi chosen for *ex vivo* stimulation was selected for its clinical relevance to COPD as it was isolated from a COPD patient experiencing an exacerbation (27). We feel that these experimental conditions are optimal for examining how cigarette smoke alters the bacterial host-response in AMs.

Microarrays are useful tools to gain a broader understanding of gene expression in experimental systems, and such studies have begun to evaluate the transciptome of smoke-exposed AMs. Microarrays from clinical samples have characterized the unique activation state of AMs from smokers and COPD patients (28). This novel AM phenotype has also been compared to transgenic animal models of COPD (29), but these models induce lung pathology by transgene expression in the lungs, and AMs have not been examined in an animal model that incorporates cigarette smoke exposure. Additionally, current studies in humans have not incorporated stimulation with live bacteria to evaluate altered host defense mechanisms, possibly due to technical complications associated with having to culture the bacterial stimulus each time a clinical sample is collected. Lung tissue from a mouse model of smoke exposure and challenge with the bacterial ligand LPS has been characterized (30), but to our knowledge there has been no study isolating AMs from smoke-exposed mice and stimulating them with a live bacteria such as NTHi.

Investigations into the phenotype of smoke-exposed macrophages began with the observation that smoke-exposed AMs had a suppressed inflammatory phenotype. AMs isolated from human smokers produce less TNF-α and IL-6 when stimulated with LPS (31). A later study found that, in addition to suppressed TNF-α and IL-6 responses, AMs from human smokers produced less of the classic inflammatory cytokines, IL-1β, RANTES and IL-8 when stimulated with TLR2 and TLR4 agonists (32). These clinical findings have also been observed in animal models, as the suppression of TNF-α, IL-6, and IL-1β in response to bacterial ligands has been demonstrated in the smoke exposure model utilized in the current study (15, 16). When viewed in isolation, these findings seem intuitive. Cigarette smoke is a chronic stimulus capable of eliciting a damaging inflammatory response, thus the transition of lung resident leukocytes to

a more quiescent state could suppress inflammatory signaling as a means of minimizing damage in a situation of chronic stimulation.

Paradoxically, the decreased production of many inflammatory cytokines that form the hallmark of an acute inflammatory response is associated with even greater leukocyte trafficking to the smoke-exposed lung when stimulated with bacterial ligands. Animal models that incorporate cigarette smoke exposure and infection with bacterial agents, including NTHi, display exacerbated cellular inflammation that is primarily neutrophilic in nature (12–15). This increased inflammation is also observed in COPD patients experiencing an exacerbation (24, 33). One of the benefits of an extensive characterization of the smoke-exposed AM is the identification of inflammatory mediators that could be compensating for the inhibited inflammatory factors. The exacerbated expression levels of the genes for the chemokines CXCL10 and CCL2 could explain increased levels of T cell and monocyte recruitment to the lungs. Additionally, our analysis identified a dramatic increase in the *Il12b* gene. Il12b has been shown to be necessary for neutrophil homeostasis and neutrophil migration in a bacterial infection model (34, 35). However, the role of *Il12b* in neutrophil recruitment in this model is more complex as Il12b encodes IL-12p40, a subunit of both IL-12 and IL-23 and neither of these cytokine's other subunits were found to be increased by the array. Additionally, the role of IL-12 in the pulmonary recruitment of neutrophils may require IL-17 production from a cell type other than the macrophage (35). Regardless of these complications, Il12b and the other inflammatory mediators identified in this array will make for interesting and novel targets in future studies.

Cellular inflammation drives the pathology of COPD by inflammatory processes elicited by cigarette smoke and this damaging inflammation is further increased during exacerbations (5).

Evidence suggests that infectious episodes contribute to the progressive loss of lung function, as exacerbations are associated with a greater rate of lung function decline (36–38). Ganeson *et al* have shown that in animal models that incorporated smoke exposure and heat killed NTHi instillation, NTHi accelerates the pathology induced by cigarette smoke as assessed by airspace enlargement and an increase in lung compliance (39). Additionally, Beckett *et al* have demonstrated that depletion of macrophages reduces pathology in an murine model of smoke exposure (21). These data synergize well with the prevailing opinion that macrophages are orchestrators of cigarette smoke induced pathology (20). AMs and their secreted molecules have thus been considered important targets in the treatment of COPD and exacerbation of COPD. The current study indicates that a unique subset of genes may need to be targeted during a bacterial exacerbation, and, because of the unique response of smoke-exposed AMs, only experimental models that incorporate both smoke and bacterial stimuli will be useful in identifying future therapeutics.

Phenotypically, macrophages are often classified into a dichotomy of classically activated (M1) macrophages that promote inflammation and alternatively activated (M2) macrophages that are anti-inflammatory in nature and promote healing processes. Attempts to characterize the phenotype of the smoke-exposed AM have also been framed in this paradigm. Shaykheiv *et al* have addressed this question in human AMs, where a list of M1/M2 genes were identified and changes in gene expression in non-smokers, smokers , and smokers with COPD were compared (28). This study concluded that smoke-exposed AMs possessed a M1-suppressed partially-M2 phenotype. Our analysis of NTHi-stimulated AMs from smoke-exposed mice compared to NTHi stimulated AMs from control mice revealed a similar conclusion. There were 11 genes from the M1 list that were increased including *Il12b*, *Cxcl10*, and *Ccl4*, and 6 genes from the M1

list that were decreased including *IL6*, *IL1b*, and *Cxcl1*. With respect to M2-associated genes, 6 were increased including *Ccl17* and *Mmp2* and no M2 genes were decreased. The conclusion that smoke-exposed macrophages represent a phenotype unique from the M1/M2 paradigm that shares aspects of both cell-types was also confirmed by Hodge *et al* (40). There is also ambiguity as to the extent of the M1/M2 paradigm within the context of the AM as there is a lack of studies which examine M1/M2 activation in a macrophage population that has already differentiated into an AM.

An unexpected finding of our analysis was the identification of an increase in many genes associated with the cell cycle and cell division, implying that cigarette smoke induces a proliferative state in smoke-exposed AMs. An important distinction to make about the current analysis is that the AM population that we examined was isolated by adherence and represents a heterogenous mix of different phagocyte populations (Reviewed in (41)). The population of AMs consists of tissue resident macrophages and macrophages that derive from recruited monocytes. Tissue resident macrophages have been shown to proliferate during the resolution of an inflammatory response (42, 43). In situations of repeated inflammatory stimulus, mechanisms that initiate and resolve inflammation may be active simultaneously. The presence of a replicating macrophage population, partial M2 phenotype, and induction of growth factors observed in our model could be indicative of a lung environment attempting to resolve inflammation and activate healing processes. The increased expression of genes associated with cell division was also observed in the gene array analysis of whole lung tissue from smoke-exposed mice challenged with LPS (30). Our data coincides with this observation and provides the additional detail that the proliferative phenotype observed was likely contributed to, at least in part, by an AM population.

The prediction that the soluble up-stream mediators based on the gene expression in our system were entirely growth factors was an unexpected and novel finding of the analysis. These growth factors can explain both the increase in cell proliferation and the production of inflammatory mediators. Specifically, GM-CSF has been shown to be a significant contributor to the neutrophilic inflammation elicited by cigarette smoke (44, 45). There may also be a role for GM-CSF in the production of IL-12 as we have previously observed that GM-CSF blockage leads to significant reductions in IL-12 in a smoke exposure model (44). Some of the other growth factors identified may also contribute to inflammatory mediator production, such as VEGF which has been shown to be part of the inflammatory response in allergic airways disease (46–48). The current analysis indicates that cigarette smoke-induced growth factors are integral in shaping the phenotype of smoke-exposed AMs.

As the search for novel therapeutics targeting inflammation focuses on disrupting specific molecules and pathways, identifying clinical conditions that fundamentally change the mechanistic nature of an inflammatory response becomes a crucial endeavor. Cigarette smoking has been identified as such a condition and the current study characterizes how it alters the bacterial response in AMs. AMs from smoke-exposed mice acquire a proliferative phenotype and mount a unique inflammatory response to NTHi. Additionally, analysis of this phenotype indicates that it is likely the result of a lung environment rich in cigarette smoke-induced growth factors. This study provides a broad data set relevant to the treatment of exacerbations of COPD and the management of bacterial infections in smokers.

Materials and methods

Animals

6-8 week old female BALB/c mice were purchased from Charles River Laboratories (Saint-Constant, PQ, Canada) and Jackson Laboratories (Bar Harbor, Maine, USA). Mice were housed under specific pathogen-free conditions with *ad libitum* access to food and water and subjected to a light-dark cycle of 12 hours. All experiments were approved by the Animal Research Ethics Board at McMaster University.

Cigarette smoke exposure

A whole body cigarette smoke exposure system (SIU-48, Promech Lab AB (Vintrie, Sweden)) was utilized. Mice were exposed to 12 3R4F reference cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) with filters removed, for 50 minutes, twice daily, for 5 days per week for 8 weeks. Details of the exposure protocol were previously reported (25). Control mice were exposed to room air only.

Preparation of nontypeable *Haemophilus influenzae* (NTHi)

The nontypeable *Haemophilus influenzae* strain 11P6 (kindly provided by Dr. S. Sethi, VA Medical Research, Buffalo, NY, USA) was utilized. This clinical strain of NTHi was isolated from the sputum of a COPD patient during acute exacerbation. NTHi was initially grown on chocolate agar plates containing 1% Isovitalex (BD Biosciences, Franklin Lakes, NJ, USA). Colonies of NTHi were then grown to log phase in 10mL of brain-heart infusion (BHI) broth (Difco, Fisher Scientific, Ottawa, ON, Canada) supplemented with Hemin and nicotinamide adenine dinucleotide (NAD) (Sigma, Oakville, ON, Canada). The inoculated BHI + Hemin +

NAD broth was maintained on a rotary shaker at 37^0C until an OD value of 0.7-0.8 was obtained at a 600nm wavelength. CFU was predicted from the OD value based on a previously generated standard curve. Before alveolar macrophage stimulation and infection of mice, NTHi was washed three times with phosphate-buffered saline (PBS), re-suspended, and diluted to a ratio of 10CFU/cell (for alveolar macrophage culture) and 10^6 CFU/35μL (for mouse infection).

Macrophage isolation and culture

BAL fluid was collected after instilling the whole lung with 1 mL of ice cold PBS. This process was repeated 5 times for maximal recovery of macrophages. BAL cells were re-suspended in 500 μL fresh PBS. Alveolar macrophages were identified and counted using a haemocytometer. BAL cells were re-suspended in RPMI supplemented with 10% FBS (Sigma-Aldrich, Oakville, ON, Canada), 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% β-mercaptoethanol (Invitrogen, Grand Island, NY, USA) and cultured in polystyrene flat-bottomed 96 well plates at 50,000 alveolar macrophages per well. Cells were incubated at 37^0C and 5% CO_2 for 1 hour to facilitate adherence. Non-adherent cells were removed by washing three times with warm PBS. Cells were cultured for 6 hours with RPMI alone or 10CFU/cell of NTHi.

RNA isolation and RT qPRC

RNA was isolated from alveolar macrophages using the RNeasy mini Kit with optional DNase step (Qiagen, Mississauga, ON, Canada). cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen) and Taqman real time RT qPCR was carried out with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Target gene expression was normalized to that of the housekeeping gene GAPDH in the same sample and expressed as fold increase over the control un-stimulated group.

Microarray and analysis

Gene expression profiling was measured using the whole mouse gene expression 44K V2 microarray (Agilent Technologies, Santa Clara, CA, USA) and normalized using the *limma* package. Wilcoxon tests were used to detect genes differentially expressed between the four experimental groups. Genes were considered differentially expressed for Benjamini-Hochberg corrected p-value <0.05.

Cluster analysis of the gene array data was conducted with GeneSpring Software (Agilent Technologies) . GO and biological pathway analysis was performed with NextBio software to identify biological functions and canonical pathways, from the Molecular Signatures Database (MSigDB), enriched for genes induced by NTHi specifically in the context of cigarette smoke (NextBio, Cupertino, CA, USA). The upstream regulator analysis of the gene expression signature was performed with Ingenuity Pathway Analysis software, (IPA, Ingenuity Systems, Redwood City, CA, USA)

Cytokine measurements

GM-CSF, VEGF, and HGF protein levels in lung homogenates were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's instructions.

Statistical analysis (For data generated separately from the gene array)

Data are expressed as mean ± SEM. Statistical analysis was carried out using SPSS Software (IBM, Armonk, NY, USA). Levene's test for equality of variances was used to adjust for differences in data variability between groups. The Univariate General Linear Model was used

and independent t-tests were applied subsequently for 2-group comparisons. Differences with $P<0.05$ were considered statistically significant.

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Figure Legends

Figure 1 *Alveolar macrophages isolated from cigarette smoke-exposed mice show a distinct transcriptome following NTHi stimulation*. BALB/c mice were room air or cigarette smoke-exposed for 8 weeks. Alveolar macrophages were isolated and stimulated with NTHi for 6 hours. RNA was isolated for a whole genome microarray. (A) Displays representative cytospins of alveolar macrophages isolated from room air and cigarette smoke exposed mice. (B) Outlines the the experimental protocol utilized to generate RNA samples. (C) Cluster analysis of the differentially expressed genes identified by microarray.

Figure 2 *Go biological processes of NTHi stimulated AMs isolated from cigarette smoke-exposed mice compared to room air control mice*. Pie charts represent the top 20 most incresed and the top 20 most decreased GO processes based on the NextBio normalized score. Redundant GO processes, where all of the significant genes are contained in another GO, are not shown. Numbers appearing with the GO process represent the number of genes enriched in each process. n=5 mice per group, FDR adjusted $p<0.05$

Figure 3 *Cigarette smoke leads to an increase in genes associated with cell division*. (A) The fold change of genes significantly enriched in the cell division GO comparing NTHi stimulated AMs from smoke-exposed mice with NTHi stimulated AMs from room air control mice. (B) RT qPCR was performed to confirm the expression of the 5 most differentially expressed genes enriched in the cell division GO. RNA was isolated from AMs with the same experimental conditions as the microarray, and Pstpip1, Bub1, Sk1, Mastl, and Birc5 were assessed. Data represent mean ± SEM, n=4-5. * indicates $p<0.05$

Figure 4 *The cigarette smoke-altered NTHi response consists of genes differentially expressed only by the combined stimuli*. Venn analysis of common and uniquely expressed genes between the NTHi stimulated AMs from smoke-exposed mice compared to NTHi stimulus versus unstimulated AMs from smoke-exposed mice versus unstimulated AMs from control mice. Genes were filtered based on fold change (≥1.5) FDR adjusted $p<0.05$

Figure 5 *Cigarette smoke exacerbates and suppresses immune genes in response to NTHi*. RT qPCR was performed to confirm the expression of the 6 most upregulated genes and the 5 most down regulated genes enriched in the *Cytokine activity* GO. RNA was isolated from AMs with the same experimental conditions as the microarray. (A) The Il12b, Cxcl10, Ccl2, Gdf3, Spp1, Inhba (B) Il7, Cxcl3, Il6, Tnfsf18, and Il1f9 were assessed. Data represent mean ± SEM, n=4-5. * indicates $p<0.05$

Figure 6 *Cigarette smoke leads to an increase in growth factor expression*. BALB/c mice were some expose for 4 days and BAL was collected. The top 3 scoring soluble factors identified by IPA upstream regulator analysis; GM-CSF, VEGF, and HGF were measured by ELISA. Data represent mean ± SEM, n=5. * indicates $p<0.05$

Figure 1

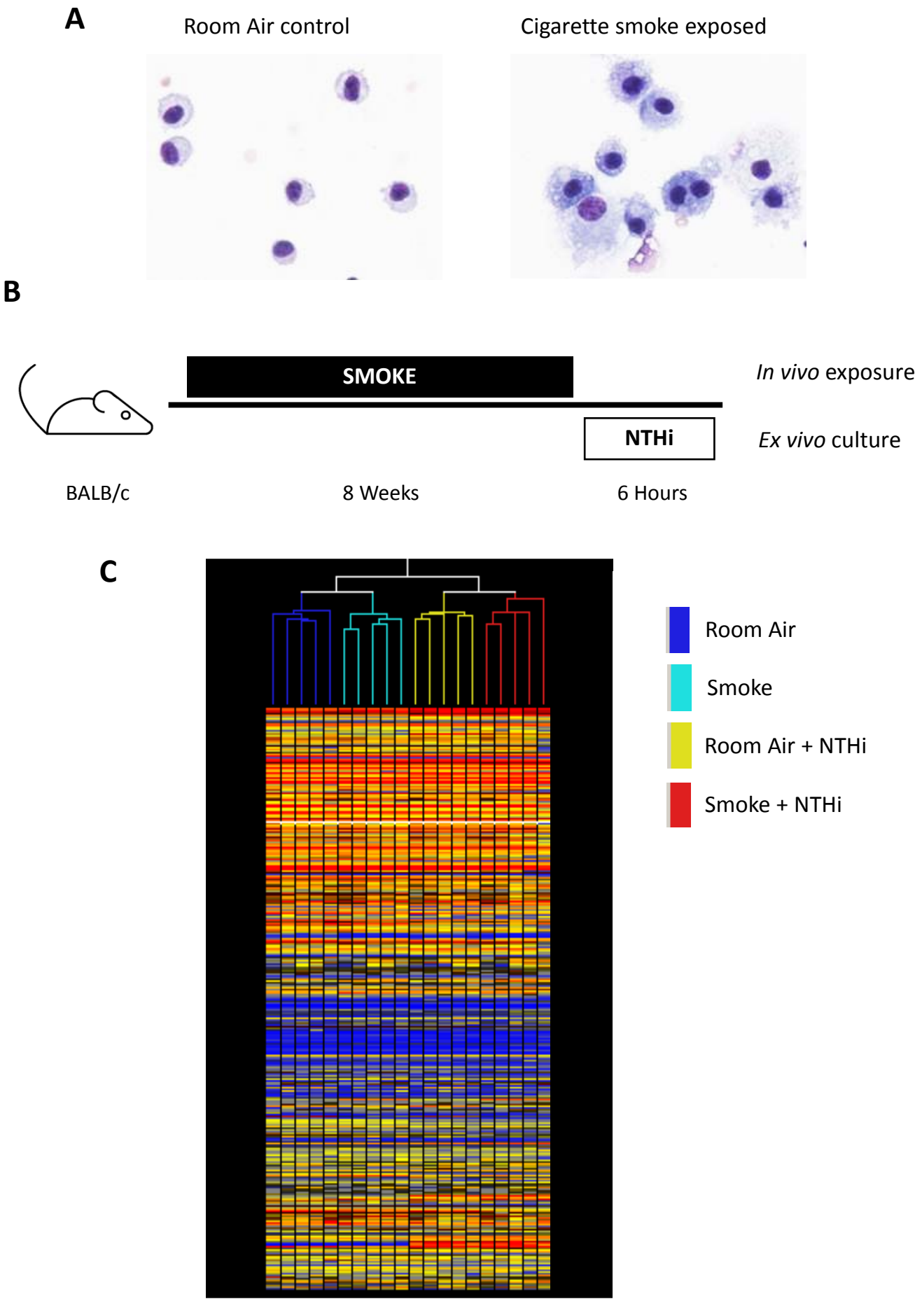

A
Room Air control
Cigarette smoke exposed
B
SMOKE
NTHi
In vivo exposure
Ex vivo culture
BALB/c
8 Weeks
6 Hours
C
Room Air
Smoke
Room Air + NTHi
Smoke + NTHi

Figure 2

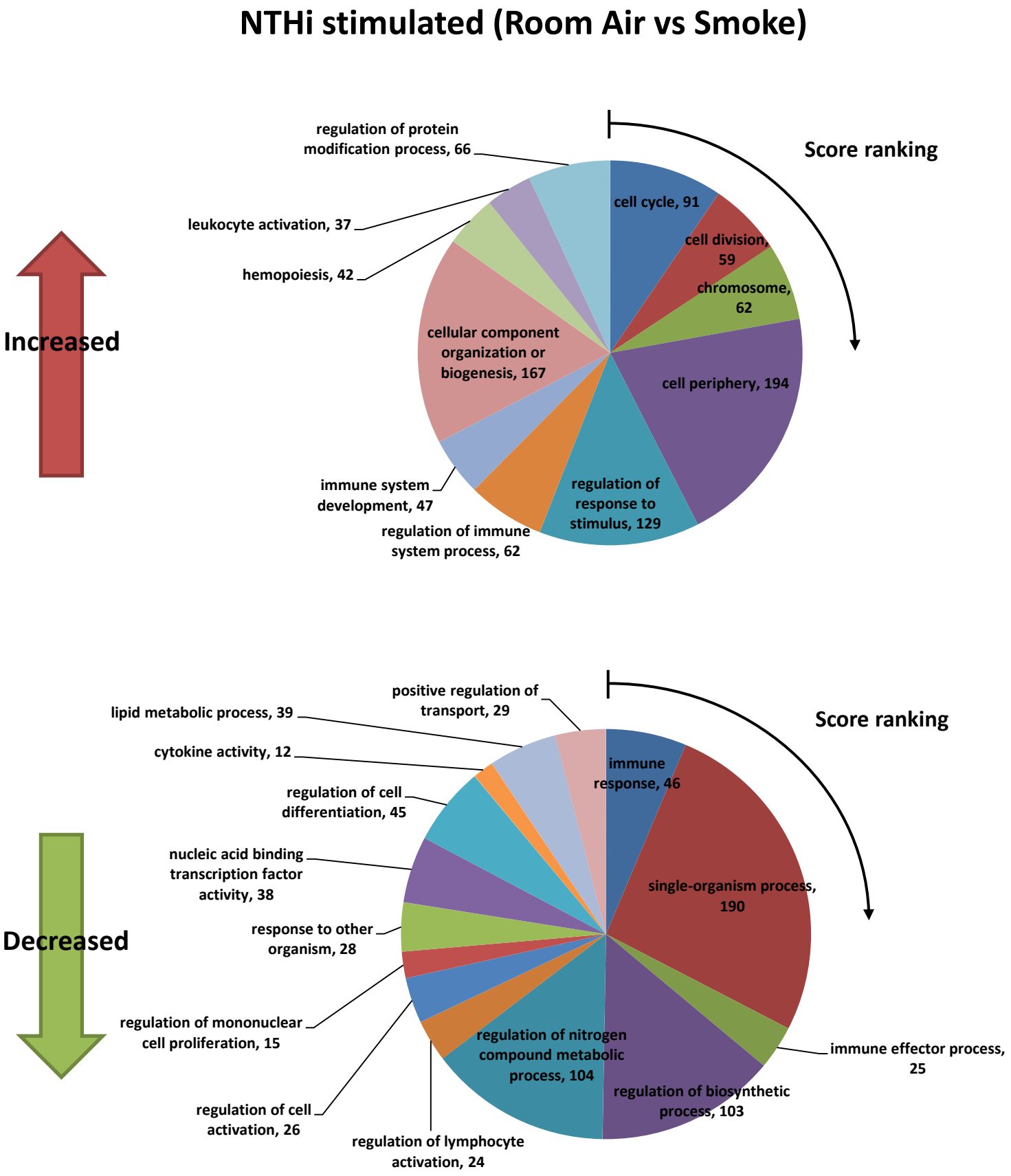

NTHi stimulated (Room Air vs Smoke)
Increased
Score ranking
regulation of protein modification process, 66
leukocyte activation, 37
hemopoiesis, 42
cell cycle, 91
cell division, 59
chromosome, 62
cellular component organization or biogenesis, 167
cell periphery, 194
immune system development, 47
regulation of immune system process, 62
regulation of response to stimulus, 129
Decreased
Score ranking
positive regulation of transport, 29
lipid metabolic process, 39
cytokine activity, 12
regulation of cell differentiation, 45
nucleic acid binding transcription factor activity, 38
response to other organism, 28
regulation of mononuclear cell proliferation, 15
regulation of cell activation, 26
regulation of lymphocyte activation, 24
immune response, 46
single-organism process, 190
immune effector process, 25
regulation of biosynthetic process, 103
regulation of nitrogen compound metabolic process, 104

Table 1

Rank	Canonical Pathway	NextBio Normalized score	Genes Enriched	P-value	Genes (**increased**, *decreased*)
			Cell Cycle / Mitosis		
1	Genes involved in Cell Cycle, Mitotic	43.57228469	46	1.20E-19	**Bub1, Ska1, Birc5, Cdkn2a, Spc25, Ccnd1,** *Ninl,* **Ndc80, Cenpk, Ccna2, Sgol1, Rrm2, Ccnb1, Casc5, Nek2, Nuf2, Mcm6, Spc24, Sgol2, Ube2c, Aurkb, Tsga14, Kif18a, Cenph, Cenpa, Cenpi, Cdk1, Wee1, Kntc1,** *Prkar2b,* **Kif20a, Plk1, Cdkn2d, Kif2c, Itgb3bp, Dhfr, Ccnb2, Kif23, Nsl1,** *Bub1b,* **Prim1,** *Ppp1cc, Kif2a,* **Orc1, Aurka, Cdca8,** *Dna2,* **Mcm2, Cenpq, Psmd8, Cdk4, Cdkn1a,** *Ppp2r5a*
2	Genes involved in Cell Cycle	43.42823867	51	1.40E-19	*Syne2,* **Bub1, Ska1, Birc5, Cdkn2a, Spc25, Ccnd1,** *Ninl,* **Ndc80, Cenpk, Oip5, Ccna2, Sgol1, Rrm2, Ccnb1, Casc5, Nek2, Nuf2, Mcm6, Spc24, Sgol2, Chek1, Ube2c, Aurkb, Brca1, Tsga14, Kif18a, Cenph, Cenpa, Cenpi, Cdk1, Wee1, Kntc1,** *Prkar2b,* **Kif20a, Plk1, Cdkn2d, Kif2c, Itgb3bp, Dhfr, Ccnb2, Kif23, Nsl1,** *Bub1b,* **Prim1,** *Ppp1cc, Kif2a,* **Orc1, Mis18bp1, Aurka, Cdca8, Hist2h2ac,** *Dna2,* **Mcm2, Cenpq, Psmd8, Cdk4, Cdkn1a,** *Ppp2r5a*
3	Genes involved in Mitotic Prometaphase	36.86844062	23	9.70E-17	**Bub1, Ska1, Birc5, Spc25, Ndc80, Cenpk, Sgol1, Casc5, Nuf2, Spc24, Sgol2, Aurkb, Kif18a, Cenph, Cenpa, Cenpi, Kntc1, Plk1, Kif2c, Itgb3bp, Nsl1,** *Bub1b, Ppp1cc, Kif2a,* **Cdca8, Cenpq,** *Ppp2r5a*
4	Genes involved in DNA Replication	34.38727856	32	1.20E-15	**Bub1, Ska1, Birc5, Spc25, Ndc80, Cenpk, Ccna2, Sgol1, Casc5, Nuf2, Mcm6, Spc24, Sgol2, Aurkb, Kif18a, Cenph, Cenpa, Cenpi, Kntc1, Kif20a, Plk1, Kif2c, Itgb3bp, Kif23, Nsl1,** *Bub1b,* **Prim1,** *Ppp1cc, Kif2a,* **Orc1, Cdca8,** *Dna2,* **Mcm2, Cenpq, Psmd8, Cdkn1a,** *Ppp2r5a*
5	Genes involved in Mitotic M-M/G1 phases	33.90842558	30	1.90E-15	**Bub1, Ska1, Birc5, Spc25, Ndc80, Cenpk, Sgol1, Casc5, Nuf2, Mcm6, Spc24, Sgol2, Aurkb, Kif18a, Cenph, Cenpa, Cenpi, Kntc1, Kif20a, Plk1, Kif2c, Itgb3bp, Kif23, Nsl1,** *Bub1b,* **Prim1,** *Ppp1cc, Kif2a,* **Orc1, Cdca8, Mcm2, Cenpq, Psmd8,** *Ppp2r5a*
6	Aurora B signaling	31.10422166	15	3.10E-14	**Bub1, Birc5, Ncapg, Ndc80, Sgol1, Stmn1, Ncaph, Aurkb, Racgap1, Cenpa, Kif20a, Kif2c, Kif23,** *Ppp1cc,* **Aurka, Cdca8**
7	FOXM1 transcription factor network	29.09671233	15	2.30E-13	**Esr1, Birc5, Cdkn2a, Ccnd1, Ccna2, Ccnb1, Nek2, Aurkb, Cenpa, Cdk1, Plk1, Cenpf, Ccnb2, Mmp2, Cdk4**
8	PLK1 signaling events	25.96271537	14	5.30E-12	**Bub1,** *Ninl,* **Ndc80, Sgol1, Ccnb1, Spc24, Tpx2, Ect2, Prc1, Cdk1, Wee1, Plk1, Kif20a,** *Pak1,* **Cenpe,** *Bub1b, Kif2a,* **Aurka**
16	Cell cycle	17.5648883	18	2.40E-08	**Cdkn1c, Bub1, Cdkn2a, Ccnd1, Ccna2, Ccnb1, Mcm6, Ttk, Chek1, Cdk1, Wee1, Plk1, Cdkn2d,** *Tgfb2,* **Ccnb2,** *Bub1b,* **Orc1, Mcm2, Cdkn1a, Cdk4**
			Immune function		
9	Cytokine-cytokine receptor interaction	24.84144456	28	1.60E-11	**Il18r1, Cxcr6,** *Tnfsf18,* **Il12b,** *Il7,* **Pdgfa, Cxcr5, Ccr7,** *Flt1,* **Ppbp,** *Cxcl3, Il6, Il1b,* **Lepr,** *Met, Tnfsf4,* **Cxcl10,** *Cxcl13,* **Inhba, Il7r, Tnfrsf25, Ccr6, Tnfrsf14, Ccl2, Tnfrsf13c, Kitl, Ccl17, Kit,** *Il1r1,* **Ccl4, Il2rb,** *Cxcl1,* **Ccl3, Csf1,** *Tnfsf13b,* **Lta, Ltb,** *Tgfb2, Cx3cl1,* **Tnfrsf13b, Tnfrsf18,** *Csf1r,* **Vegfb,** *Ifngr1, Tgfbr1, Bmpr2, Acvr1b, Fas*
13	Genes involved in Adaptive Immune System	19.7170784	39	2.70E-09	**Klrd1, Cd3d, Icos, Rasgrp3, Cd3g,** *H2-DMb1,* **Sell, Cd8b1, Lrr1, Spsb4, Cd79b,** *C3,* **Prkcq, Tnfrsf14, Itgb7, Ube2e2, Kif15, Kif4, Kif11, Amica1, Trib3, Cd96, Itk,** *Icam1,* **Ube2c, Fcgr4,** *Nfkbia,* **Kif22, Lck,** *Ap1s2, Vasp,* **Kif18a, Cd86, Racgap1, Blnk, Lat,** *Prkcb,* **Kif20a,** *Ctss, Pak1,* **Fcgr2b,** *Ikbkg,* **Kif2c,** *Cd74,* **Kif23, Ctsl,** *Itgal, Kif2a, H2-DMa, Akt3, H2-K1,* **Dynll2, Ctsc,** *Spsb1,* **Psmd8,** *Bcl10,* **Cdkn1a,** *Ppp2r5a*
14	Genes involved in Chemokine receptors bind chemokines	18.14158455	10	1.30E-08	**Cxcr6, Cxcr5, Ccr7, Ppbp,** *Cxcl3,* **Cxcl10,** *Cxcl13,* **Ccr6, Ccl2, Ccl17, Ccl4,** *Cxcl1,* **Ccl3,** *Cx3cl1*
15	Genes involved in Immune System	17.60840145	52	2.30E-08	**Klrd1,** *Il7,* **Cd3d, Icos, Rasgrp3, Pstpip1, Cd3g, Bcl2,** *Il6, Il1b, H2-DMb1,* **Il7r, Sell, Cd8b1, Lrr1, Cd79b, Spsb4,** *C3,* **Prkcq, Ccr6, Tnfrsf14, Itgb7, Mef2c, Ube2e2, Kif15, Kif4,** *Il1r1,* **Kif11, Amica1, Trib3, Cd96, Itk,** *Irf7,* **Il2rb,** *Icam1,* **Jun, Ube2c, Fcgr4,** *Nfkbia, Irf5,* **Kif22, Cfh, Lck,** *Ap1s2, Vasp,* **Cd86, Kif18a,** *Zbp1,* **Racgap1, Blnk, Dusp4,** *Irf1,* **Lat,** *Prkcb, Peli1,* **Cdk1, Kif20a,** *Ctss, Pak1,* **Fcgr2b,** *Ikbkg,* **Kif2c,** *Cd74,* **Cd14, Kif23, Ctsl,** *Itgal, Kif2a, H2-DMa, Akt3, H2-K1,* **Dynll2,** *Birc3, Nod2, Ifngr1, Tlr2,* **Ctsc,** *Spsb1,* **Bcl2l1,** *Nfkb2,* **Lgals3,** *Gbp2,* **Psmd8,** *Bcl10,* **Cdkn1a,** *Ppp2r5a*
17	IL12 and Stat4 Dependent Signaling Pathway in Th1 Development	16.96120034	6	4.30E-08	**Il18r1, Il12b, Cd3d, Stat4, Cd3g, Jun**
18	Genes involved in Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	16.76097789	10	5.30E-08	**Klrd1, Cd3d, Cd3g, Sell, Cd8b1,** *C3,* **Itgb7, Amica1, Cd96,** *Icam1,* **Fcgr4, Fcgr2b,** *Itgal, H2-K1*
20	Primary immunodeficiency	15.97831739	9	1.20E-07	**Cd3d, Icos, Il7r, Cd8b1, Tnfrsf13c,** *Aicda,* **Ada, Lck, Blnk,** *Ikbkg,* **Tnfrsf13b**
			Other		
10	Genes involved in Kinesins	24.27861715	10	2.90E-11	**Kif15, Kif4, Kif11, Kif22, Kif18a, Racgap1, Kif20a, Kifc5b, Kif2c, Kif23,** *Kif2a*
11	Genes involved in Hemostasis	20.93084885	37	8.10E-10	**Hbb-b1, P2ry1, Pdgfa, Itgb3, Ppbp, Ptk2, Mgll,** *Dgkg,* **Sell, Adra2a, Gata3, Pde2a, Prkcq, Kif15, Kif4, Kif11, Srgn, Amica1, Prkar1b,** *Irf7, Nfe2,* **Ptgir, Kif22,** *F11r,* **Lck, Angpt2, Kif18a, Racgap1,** *Tfpi, Irf1,* **Lat,** *Prkcb,* **Wee1,** *Prkar2b,* **Kif20a, Vcl,** *Dock9, Cav1,* **Kifc5b, Kif2c,** *Tgfb2,* **Kif23,** *Dock6, Tmsb4x, Itgal, Dok2, Kif2a, Prkce,* **Cd63,** *Akt3,* **Vegfb, Gna13, F3, Fn1, Plau,** *Ppp2r5a*
12	ATF-2 transcription factor network	20.80710103	14	9.20E-10	**Esr1, Bcl2,** *Il6,* **Dusp10, Ccnd1, Ccna2, Dusp8, Jun, Brca1,** *Cbfb, Tgfb2,* **Jdp2, Ddit3, Mmp2,** *Junb,* **Plau, Bcl2l1, Cdk4**
19	Genes involved in Factors involved in megakaryocyte development and platelet production	16.40166432	14	7.50E-08	**Hbb-b1, Gata3, Kif15, Kif4, Kif11, Prkar1b,** *Irf7, Nfe2,* **Kif22, Kif18a, Racgap1,** *Irf1,* **Wee1,** *Prkar2b,* **Kif20a,** *Dock9,* **Kifc5b, Kif2c, Kif23,** *Dock6, Kif2a*

Table 2

Rank	Canonical Pathways	NextBio Normalized score	Genes Enriched	P-value	Genes (**increased**, *decreased*)
	Immune function				
1	Leishmania infection	22.60315597	12	1.50E-10	**Il12b,** *Il1b, H2-DMb1, Ptgs2, C3,* **Jun, Fcgr4,** *Nfkbia, Prkcb, Tgfb2, Itga4, H2-DMa, Nfkb1, Ifngr1, Tlr2*
2	Cytosolic DNA-sensing pathway	16.52233536	8	6.70E-08	*Il6, Il1b,* **Cxcl10, Ccl4,** *Irf7, Nfkbia, Zbp1, Trex1, Ikbkg, Nfkb1*
3	Chemokine signaling pathway	14.38266092	13	5.70E-07	**Cxcr6, Cxcr5, Ccr7, Ppbp,** *Cxcl3,* **Ptk2, Cxcl10,** *Cxcl13,* **Ccr6, Ccl2, Ccl17, Ccl4, Itk,** *Cxcl1,* **Ccl3,** *Nfkbia, Adrbk2, Prkcb, Pak1, Ikbkg, Cx3cl1, Akt3, Nfkb1, Prex1, Plcb3*
4	TNF-alpha/NF-kappa B signaling complex (CHUK, KPNA3, NFKB2, NFKBIB, REL, IKBKG, NFKB1, NFKBIE, RELB, NFKBIA, RELA, TNIP2)	14.08248854	5	7.70E-07	*Nfkbia, Ikbkg, Nfkb1, Nfkb2, Relb*
5	NOD-like receptor signaling pathway	13.77425465	8	1.00E-06	**Pstpip1,** *Il6, Il1b,* **Ccl2,** *Cxcl1, Nfkbia, Ikbkg,* **Card6,** *Nfkb1, Birc3, Nod2*
6	Cells and Molecules involved in local acute inflammatory response	13.45014986	5	1.40E-06	*Il6, C3, Icam1, Itgal, Itga4*
7	Signal transduction through IL1R	12.88055326	6	2.50E-06	*Il6, Il1b, Il1r1,* **Jun,** *Nfkbia, Tgfb2, Nfkb1*
11	TNF-alpha/Nf-kappa B signaling complex (RPL6, RPL30, RPS13, CHUK, DDX3X, NFKB2, NFKBIB, REL, IKBKG, NFKB1, MAP3K8, RELB, GLG1, NFKBIA, RELA, TNIP2, GTF2I)	12.09324127	5	5.60E-06	*Nfkbia, Ikbkg, Nfkb1, Nfkb2, Relb*
12	Graft-versus-host disease	11.5509635	6	9.60E-06	**Klrd1,** *Il6, Il1b, H2-DMb1,* **Cd86,** *H2-DMa, H2-K1, Fas*
13	Toll-like receptor signaling pathway	11.46770397	9	1.00E-05	**Il12b,** *Il6, Il1b,* **Cxcl10, Spp1, Ccl4,** *Irf7,* **Jun, Ccl3,** *Nfkbia, Irf5,* **Cd86,** *Ikbkg,* **Cd14,** *Akt3, Nfkb1, Tlr2*
14	Intestinal immune network for IgA production	11.37908538	6	1.10E-05	**Icos,** *Il6, H2-DMb1,* **Itgb7, Tnfrsf13c,** *Aicda,* **Cd86,** *Tnfsf13b,* **Tnfrsf13b,** *Itga4, H2-DMa*
20	Genes involved in Cytokine Signaling in Immune system	11.02381646	15	1.60E-05	*Il7, Il6, Il1b,* **Il7r,** *Il1r1, Irf7,* **Il2rb,** *Icam1, Irf5,* **Lck, Blnk,** *Irf1, Peli1,* **Cdk1,** *Ikbkg, H2-K1, Nod2, Ifngr1, Nfkb2, Gbp2*
	Other				
8	Genes involved in Metabolism of lipids and lipoproteins	12.47199543	23	3.80E-06	**Plbd1,** *Pld4,* **Ppap2b, Ch25h, Cubn, Abcb4, Mgll,** *Dhcr24,* **Mboat1, Slc27a1,** *Abca1,* **Fabp4, Sphk1,** *Grhl1,* **Trib3,** *Me1, Ppm1l, Tm7sf2,* **Pla2g2e,** *Cav1,* **Ugcg,** *Sdc1, Lss, Acsl1, Elovl6, Sgms2, Ppp1cc, Alas1, Med14,* **Gpcpd1, Acsl3,** *Glipr1,* **Arsg, Gla,** *Txnrd1,* **Smpd1,** *Pmvk, Slc44a1,* **Col4a3bp,** *Lass6,* **Hexb,** *Gpd1, Acaa1a,* **Plin2**
9	G alpha q Pathway	12.44181183	6	3.90E-06	*Nfkbia, Ikbkg, Itpr1, Akt3, Nfkb1, Nfkb2*
10	Pathways in cancer	12.25324746	19	4.80E-06	**Arnt2, Pdgfa, Bcl2,** *Il6, Met,* **Ptk2,** *Ptgs2, Gli3,* **Birc5, Rad51, Cdkn2a, Ccnd1,** *Fgf11,* **Epas1, Kitl, Kit, Igf1, Tcf7, Ets1, Jun,** *Nfkbia,* **Col4a1,** *Prkcb, Tcf7l2,* **Lef1,** *Ikbkg, Tgfb2,* **Mmp2,** *Nfkb1, Akt3,* **Mmp9,** *Csf1r,* **Vegfb,** *Birc3,* **Fn1,** *Traf4,* **Bcl2l1,** *Nfkb2, Tgfbr1, Traf5,* **Cdkn1a, Cdk4,** *Fas*
15	Apoptosis	11.18045775	9	1.40E-05	**Bcl2,** *Il1b, Il1r1,* **Prkar1b,** *Nfkbia, Prkar2b, Ikbkg, Akt3, Nfkb1, Birc3,* **Bcl2l1,** *Fas*
16	Viral myocarditis	11.17375902	7	1.40E-05	*H2-DMb1,* **Dmd, Ccnd1,** *Icam1,* **Cd86,** *Cav1, Itgal, Myh9, H2-DMa, H2-K1*
17	Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha)	11.13792197	6	1.50E-05	**Pdgfa, Hspa1a,** *Ptgs2,* **Prkar1b,** *Me1,* **Jun,** *Nfkbia, Prkcb, Prkar2b, Fat1*
18	Beta2 integrin cell surface interactions	11.07548639	5	1.50E-05	*C3, Tgfbi, Icam1, F11r, Itgal,* **Plau**
19	G alpha 13 Pathway	11.07360354	6	1.60E-05	**Bcl2, Ptk2,** *Nfkbia, Ikbkg, Map3k5, Akt3, Nfkb1,* **Gna13,** *Nfkb2*

Figure 3

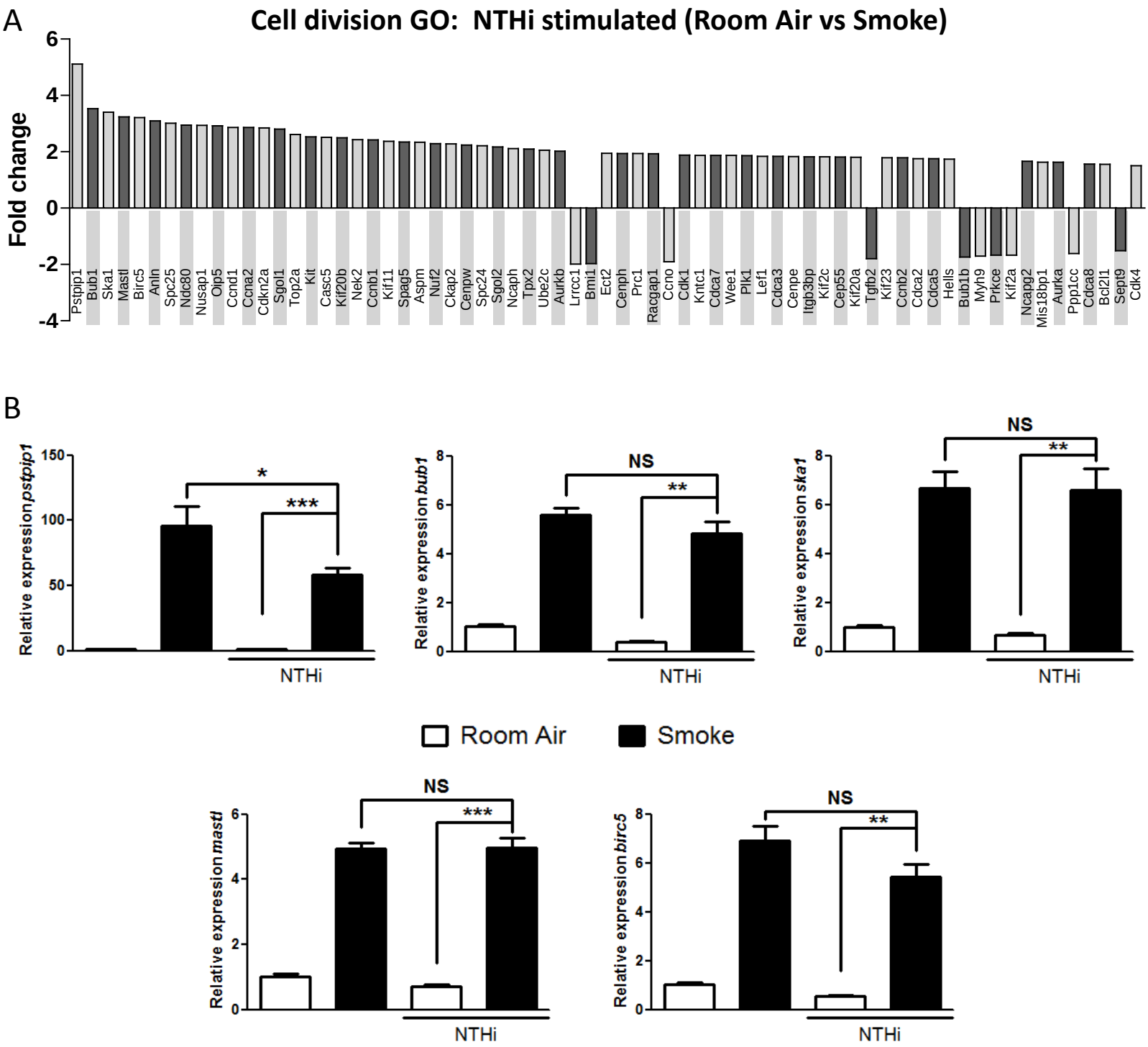

A
Cell division GO: NTHi stimulated (Room Air vs Smoke)
Fold change
6
4
2
0
-2
-4
Pstpip1
Bub1
Ska1
Mastl
Birc5
Anln
Spc25
Ndc80
Nusap1
Oip5
Ccnd1
Ccna2
Cdkn2a
Sgol1
Top2a
Kit
Casc5
Kif20b
Nek2
Ccnb1
Kif11
Spag5
Aspm
Nuf2
Ckap2
Cenpw
Spc24
Sgol2
Ncaph
Tpx2
Ube2c
Aurkb
Lrrc1
Bml1
Ect2
Cenph
Prc1
Racgap1
Ccno
Cdk1
Kntc1
Cdca7
Wee1
Plk1
Lef1
Cdca3
Cenpe
Itgb3bp
Kif2c
Cep55
Kif20a
Tgfb2
Kif23
Ccnb2
Cdca2
Cdca5
Hells
Bub1b
Myh9
Prkce
Kif2a
Ncapg2
Mis18bp1
Aurka
Ppp1cc
Cdca8
Bcl2l1
Sept9
Cdk4
B
Relative expression pstpip1
150
100
50
0
*
***
NTHi
Relative expression bub1
8
6
4
2
0
NS
**
NTHi
Relative expression ska1
8
6
4
2
0
NS
**
NTHi
Room Air
Smoke
Relative expression mastl
6
4
2
0
NS
***
NTHi
Relative expression birc5
8
6
4
2
0
NS
**
NTHi

Figure 4

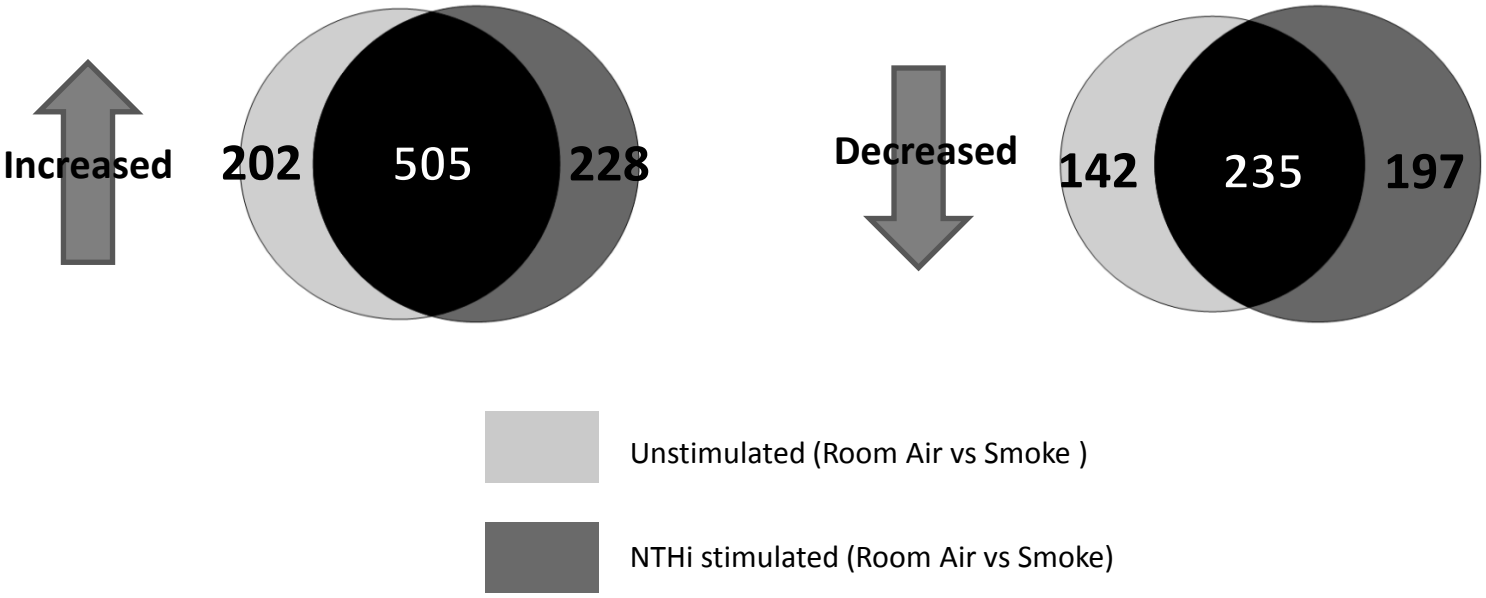

Increased
202
505
228
Decreased
142
235
197
Unstimulated (Room Air vs Smoke)
NTHi stimulated (Room Air vs Smoke)

Table 3

Symbol	Gene	Room Air vs Smoke		Room Air
		NTHi	Unstim	Unstim vs NTHi
Increased				
Gdf3	growth differentiation factor 3	14.9	-	-7.62
Il12b	interleukin 12b	11.28	4.17	14.66
Cxcl10	chemokine (C-X-C motif) ligand 10	3.79	-	98.70
Inhba	inhibin beta-A	3.49	-	3.06
Spp1	secreted phosphoprotein 1	2.76	1.82	-1.62
Ccl2	chemokine (C-C motif) ligand 2	2.51	5.1	3.31
Pglyrp1	peptidoglycan recognition protein 1	2.6	2.67	-
Kitl	kit ligand	2.45	3.12	-
Ccl17	chemokine (C-C motif) ligand 17	2.57	2.66	4.65
Ccl4	chemokine (C-C motif) ligand 4	2.17	-	30.37
Cmtm7	CKLF-like MARVEL transmembrane domain containing 7	2.15	-	-1.59
Ccl3	chemokine (C-C motif) ligand 3	2.04	-	10.45
Csf1	colony stimulating factor 1 (macrophage)	1.88	1.92	-
Lta	lymphotoxin A	1.88	-	3.20
Ltb	lymphotoxin B	1.83	2.46	1.67
Cmtm4	CKLF-like MARVEL transmembrane domain containing 4	1.68	-	-
Decreased				
Tnfsf18	tumor necrosis factor (ligand) superfamily, member 18	-18.83	-24.31	-
Il7	interleukin 7	-11.25	-	5.88
Il1f9	interleukin 1 family, member 9	-8.01	-2.75	7.45
Cxcl3	chemokine (C-X-C motif) ligand 3	-4.29	-	-
Il6	interleukin 6	-2.72	-2.34	487.17
Tnfsf4	tumor necrosis factor (ligand) superfamily, member 4	-3.82	-	4.05
Il1b	interleukin 1 beta	-3.48	-	8.34
Cxcl13	chemokine (C-X-C motif) ligand 13	-3.51	-	-
Nampt	nicotinamide phosphoribosyltransferase	-2.39	-	-
Cxcl1	chemokine (C-X-C motif) ligand 1	-2.07	-	167.42
Tnfsf13b	tumor necrosis factor (ligand) superfamily, member 13b	-1.93	-2.7	-
Cx3cl1	chemokine (C-X3-C motif) ligand 1	-1.79	-1.66	-1.88

Values represent mean fold changes (n=5 mice/group)

$p<0.05$

-, genes that showed no significant change

Table 4

Symbol	Gene	Room Air vs Smoke		Room Air
		NTHi	unstim	Unstim vs NTHi
	Cytokine Receptors			
Il18r1	interleukin 18 receptor 1	30.73	14.15	-
Il7r	interleukin 7 receptor	3.45	7.19	-
Lepr	leptin receptor	3.31	5.67	-
Tnfrsf13c	tumor necrosis factor receptor superfamily, member 13c	2.61	3.16	-
Kit	kit oncogene	2.53	3.99	-
Il2rb	interleukin 2 receptor, beta chain	2.11	2.94	-
Tnfrsf13b	tumor necrosis factor receptor superfamily, member 13b	1.69	1.93	-
Tnfrsf18	tumor necrosis factor receptor superfamily, member 18	1.68	-	-1.65
Acvr1b	activin A receptor, type 1B	-1.50	-	2.51
Fas	Fas (TNF receptor superfamily member 6)	-1.50	-	2.94
Bmpr2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	-1.52	-	-
Tgfbr1	transforming growth factor, beta receptor I	-1.55	-	-
Ifngr1	interferon gamma receptor 1	-1.58	-	-1.50
Csf1r	colony stimulating factor 1 receptor	-1.63	-1.54	-
Il1r1	interleukin 1 receptor, type I	-2.39	-1.56	-
Met	met proto-oncogene	-3.91	-1.56	-
Flt1	FMS-like tyrosine kinase 1	-4.22	-4.12	-
	Chemokine Receptors			
Cxcr6	chemokine (C-X-C motif) receptor 6	24.27	12.70	-
Cxcr5	chemokine (C-X-C motif) receptor 5	7.09	10.28	-
Ccr7	chemokine (C-C motif) receptor 7	4.91	4.36	-
Ccr6	chemokine (C-C motif) receptor 6	2.82	3.17	-

Values represent mean fold changes (n=5 mice/group)

$p<0.05$

-, genes that showed no significant change

Figure 5

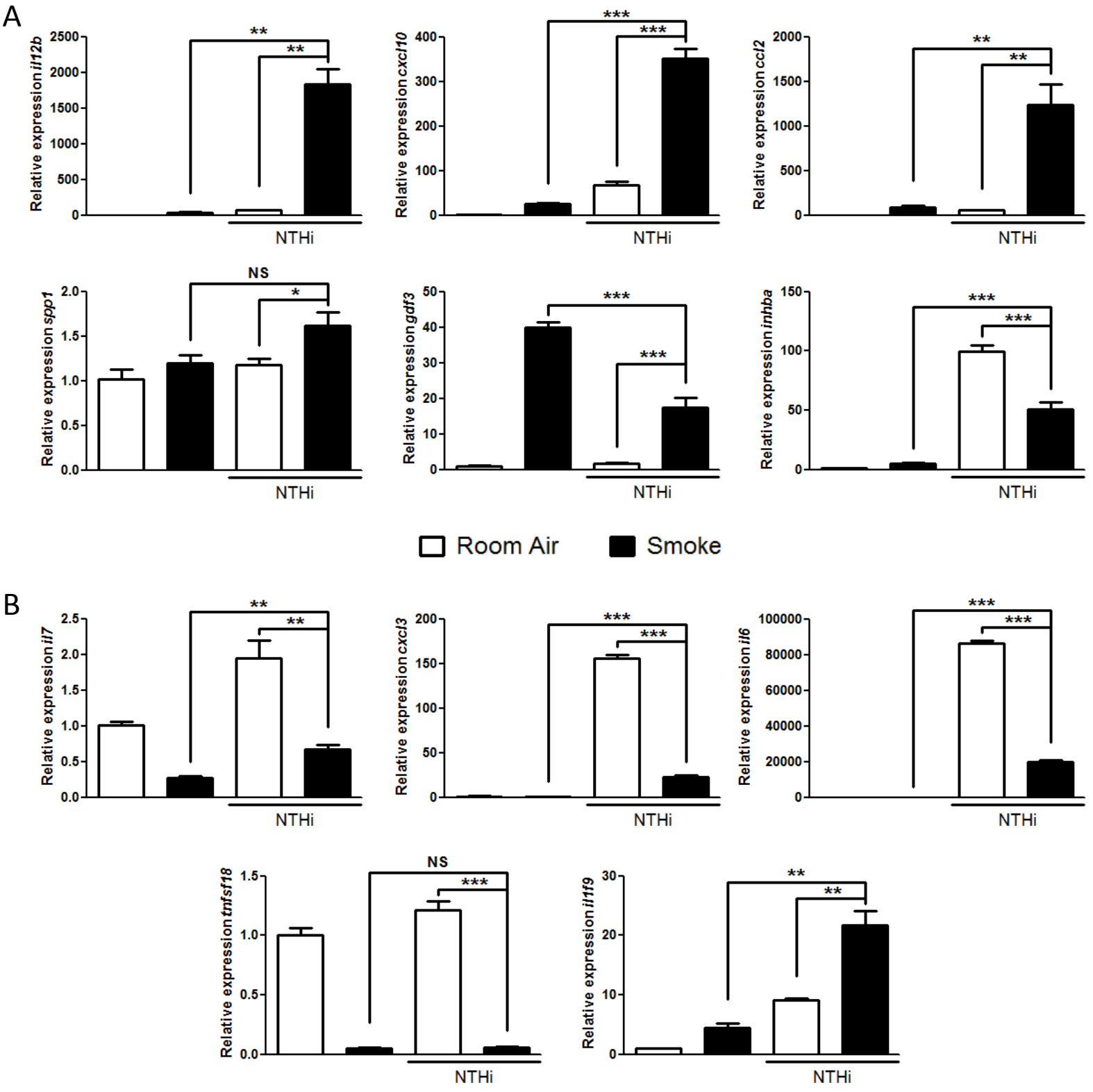
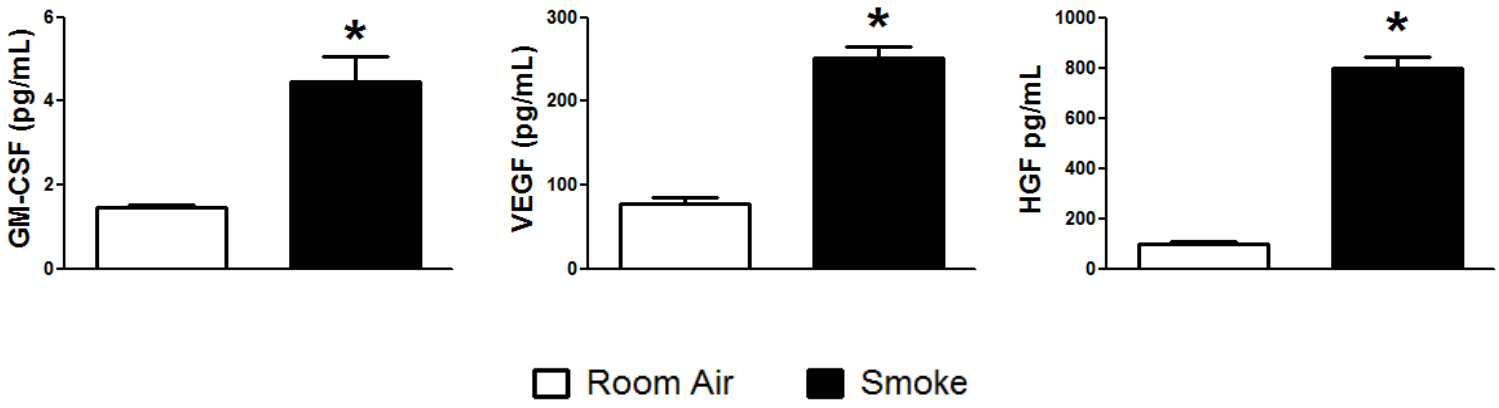

A
Relative expression il12b
2500
2000
1500
1000
500
0
**
**
NTHi
Relative expression cxcl10
400
300
200
100
0
***
***
NTHi
Relative expression ccl2
2000
1500
1000
500
0
**
**
NTHi
Relative expression spp1
2.0
1.5
1.0
0.5
0.0
NS
*
NTHi
Relative expression gdf3
50
40
30
20
10
0
***
***
NTHi
Relative expression inhba
150
100
50
0
***
***
NTHi
Room Air
Smoke
B
Relative expression il7
2.5
2.0
1.5
1.0
0.5
0.0
**
**
NTHi
Relative expression cxcl3
200
150
100
50
0
***
***
NTHi
Relative expression il6
100000
80000
60000
40000
20000
0
***
***
NTHi
Relative expression tnfsf18
1.5
1.0
0.5
0.0
NS
***
NTHi
Relative expression il1f9
30
20
10
0
**
**
NTHi

Table 5

Upstream Regulator	Gene Name	Molecule Type	Activation z-score	p-value of overlap
Csf2	Granulocyte-macrophage colony-stimulating factor	cytokine	5.627	3.27E-34
Vegf	Vascular endothelial growth factor	group	4.379	4.17E-13
Hgf	Hepatocyte growth factor	growth factor	4.127	2.82E-19
Egf	Epidermal growth factor	growth factor	2.978	5.33E-09
Agt	Angiotensin	growth factor	2.588	1.28E-08
Fgf2	Basic fibroblast growth factor	growth factor	2.552	9.80E-08
Pth	Parathyroid hormone	other	2.491	1.20E-03
Pdgf (complex)	platelet-derived growth factor	complex	2.328	1.44E-03
Igf1	Insulin-like growth factor 1	growth factor	2.273	1.73E-07
Csf1	Macrophage colony-stimulating factor	cytokine	2.183	5.22E-08
Retnlb	Resistin-like beta	other	2.034	3.87E-03

Figure 6


GM-CSF (pg/mL)
6
4
2
0
*
VEGF (pg/mL)
300
200
100
0
*
HGF pg/mL
1000
800
600
400
200
0
*
Room Air
Smoke

Supplementary Table 1

Rank	Biological functions (GO processes)	NextBio Normalized score	Common Genes	P-value	Genes (**increased**, *decreased*)
			Cell activation / differentiation		
3	cell differentiation	37.69853995	133	4.20E-17	**Adra2c, Htr2b, Mlph, Hdac9, Hbb-b1, Dok7, 5830411N06Rik,** *Ppp1r9a,* **Bex1,** *Nfib,* **Il7r, Prom1, Fcrla,** *Ttll7,* **Il12b, Ptgs1, Cd3d, Dmd, Hba-a1, Ccl2,** *Heph, Bmp2,* **Kit, Cd8a, Prickle2,** *Tgfb2,* **Cspg4, Murc,** *Fhod3, Flt1,* **Ccr7, Tnfsf8, Cadm1, Pmp22, Cdkn1c, Cdh1, Uchl1,** *Six1,* **Pitx1, Mkl2, Prm1, Satb1, Sema3e, Angpt2, Gpr183, Alas2, Kitl, Kdr, Pkdcc,** *Pbx1,* **Epas1, Adam8, Scarf1,** *Lst1,* **Nav1, Dtx1, Apbb2,** *Efna2,* **Dact2,** *Sdc1,* **Nrcam,** *Dfna5,* **Fabp4,** *Pdgfrb,* **Gata3,** *Rfx3,* **Cd24a, Mreg, Ptk2, Tcf7, Igf1,** *Lgr4,* **Gimap1, Arid5a, Vim, Bcl2, Zfp36l1,** *Epha4, Il6,* **Pde2a, Mylk2, Wee1,** *Lzts1,* **Lef1, Gimap5, Cdkn2a, Ccnb1, Basp1,** *Cebpd,* **Tmod1,** *Agrn, Rdh10,* **Stx3, Mmp2, Stmn1, Tpm1, Fst, Foxo3, Chst11, Fignl1, Foxp3,** *Fzd1,* **C230081A13Rik, Mef2c, Foxp1, Sphk1, Arhgap22, Bin1, Avil, Aspm, Csf1, Efnb2, Myo18b,** *Lmo4,* **Cd63,** *Cd74, Sipa1l1, Unc13b,* **Ccnd1, Mertk, Abcb1b,** *Hey1, Snta1,* **Celsr1, Spp1,** *Pvrl2, Six5,* **Rap2a, Lrg1, Naglu, Plaur, Mfsd7b,** *Zeb1,* **Hip1,** *F11r, Gphn,* **Anxa1, Chac1,** *H2-DMa,* **Thy1,** *Id3, Sema6d,* **Myo5a,** *Scd1,* **Bcl2l1, Rps6ka2,** *Bcl6, Runx2, Clcf1, Cdon,* **Spry2,** *Tcf7l2,* **Fn1,** *Sod2, Zcchc11,* **Dhcr7, Ctsl, Eng, Vegfb, Raph1, Grn,** *Jak2,* **Lgals1,** *Fgf10,* **Etv1,** *Psmb8,* **Coq7, Tacc3, Xrcc5,** *Irf1,* **Fem1b, Cd44, Jun,** *Id1, Klf7,* **Cd300lf,** *Smad6,* **Lyl1,** *Met, Mitf, Slc4a7,* **Brca1, Foxs1, Lck,** *Csf1r,* **Lpin1, Hipk2, Nr4a2, Pde4d,** *Htra2,* **Krt19,** *Aldh1a2, Sema4a*
7	cell activation	33.79703752	43	2.10E-15	*Tnfsf18,* **Hdac9, 5830411N06Rik, Cxcr5, 1190002H23Rik, Il7r, Nlrc3, Il12b, Cd3d, Bank1, Kit, Cd8a, Ccr7, Tnfsf8, Ms4a1, Satb1, Gpr183, Itgb3, Lat2, Il2rb, Cd86, Gata3, Cd24a, Tcf7, Gimap1, Bcl2, Zfp36l1,** *Il6,* **Lef1, Gimap5, Ikzf3, Dpp4, Foxp3, Lat, Foxp1, Mef2c,** *Cd74,* **Mertk, Anxa3,** *Prkcb,* **Fcgr4, Anxa1,** *H2-DMa, Bcl6, Clcf1, Runx2,* **Fcgr2b, Lgals1,** *Cx3cl1, Irf1, Prkce,* **Jun, Lyl1, Lck,** *Sema4a*
12	hemopoiesis	32.31425776	42	9.20E-15	**Hdac9, Hbb-b1, 5830411N06Rik, Il7r, Il12b, Cd3d, Hba-a1,** *Heph,* **Kit, Cd8a,** *Tgfb2,* **Ccr7, Tnfsf8, Satb1, Angpt2, Gpr183, Alas2, Kitl, Kdr,** *Pbx1,* **Epas1, Adam8,** *Efna2, Pdgfrb,* **Gata3, Cd24a, Tcf7, Gimap1, Bcl2, Zfp36l1, Lef1, Gimap5, Fst, Foxp3, Foxp1, Mef2c, Csf1,** *Cd74,* **Mertk, Mfsd7b, Anxa1,** *H2-DMa, Bcl6, Clcf1, Runx2, Sod2, Jak2,* **Lgals1, Tacc3, Xrcc5,** *Irf1,* **Jun, Cd300lf, Lyl1,** *Mitf,* **Lck,** *Csf1r, Sema4a*
13	regulation of cell activation	31.92065776	38	1.40E-14	*Tnfsf18,* **Ighm, Il7r, Il12b, Ccl2, Pdgfa, Ccr7, Prkcq, Slamf7, Ctla2a, Gpr183, Adam8,** *Lst1,* **Tnfrsf13c, Dtx1, Cd86, Gata3, Cd24a,** *Tnfsf13b,* **Pglyrp1, Igf1, Tnfrsf14, Bcl2,** *Il6,* **Gimap5, Cdkn2a, Ikzf3, Dpp4, Foxp3, Pdpn, Cd80,** *Bmi1,* **Lat, Mef2c,** *Lmo4, Cd74,* **Tnfrsf13b, Mertk,** *Pvrl2,* **Tec,** *Zeb1, H2-DMa,* **Thy1,** *Bcl6, Clcf1, Hsph1, Peli1,* **Fcgr2b,** *Jak2,* **Lgals1,** *Fgf10,* **Prnp,** *Irf1,* **Tnfsf14,** *Nck2, Gal,* **Lck**
			Immune response		
4	lymphocyte activation	36.49961792	36	1.40E-16	*Tnfsf18,* **Hdac9, 5830411N06Rik, Cxcr5, Il7r, Nlrc3, Il12b, Cd3d, Bank1, Kit, Cd8a, Ccr7, Tnfsf8, Ms4a1, Satb1, Gpr183, Lat2, Il2rb, Cd86, Gata3, Cd24a, Tcf7, Gimap1, Bcl2, Zfp36l1,** *Il6,* **Lef1, Ikzf3, Dpp4, Foxp3, Foxp1, Mef2c,** *Cd74,* **Mertk,** *Prkcb,* **Fcgr4, Anxa1,** *H2-DMa, Bcl6, Runx2, Clcf1,* **Lgals1,** *Irf1,* **Lyl1, Lck,** *Sema4a,*
5	regulation of immune system process	36.48912892	62	1.40E-16	*Tnfsf18,* **Ighm, 1190002H23Rik, Il7r, Il12b, Orm1, Ccl2, Kit, C3ar1,** *Tgfb2,* **Ccr7, Cadm1, Kcnn4, Prkcq, Slamf7, Ctla2a, Ccl7, C1qa, Gpr183, Kitl, Adam8, Itgb3, Lat2,** *Lst1,* **Tnfrsf13c, Dtx1, Cd86, Gata3, Cfh, Cd24a,** *C3, Clec2d,* **Cd79b,** *Tnfsf13b,* **Cd59a, Pglyrp1, Igf1, Tnfrsf14,** *Fcgr1,* **Bcl2,** *Il6,* **Lef1, Gimap5, Cdkn2a,** *Samhd1,* **Foxo3, Cfp, Ikzf3, Dpp4, Foxp3, Cd80,** *Bmi1,* **Lat, Foxp1, Mef2c, Csf1,** *Cd74,* **Tnfrsf13b, Ecm1, Mertk,** *Prkcb, Pvrl2, Trim30a,* **Tec,** *Zeb1,* **Anxa1,** *H2-DMa,* **Thy1,** *Hsph1, Bcl6, Clcf1, Peli1,* **Vegfb, Fcgr2b, Lgals1,** *Cx3cl1, Fgf10,* **Ets1,** *Colec12,* **Prnp,** *C1ra, Irf1, Prkce,* **Tnfsf14,** *Nck2,* **Cd44, Jun,** *Bst2, Mitf, Gal,* **Lck,** *Csf1r,* **Dusp10,** *2010106G01Rik*
6	leukocyte activation	35.90208261	41	2.60E-16	*Tnfsf18,* **Hdac9, 5830411N06Rik, Cxcr5, Il7r, Nlrc3, Il12b, Cd3d, Bank1, Kit, Cd8a, Ccr7, Tnfsf8, Ms4a1, Satb1, Gpr183, Lat2, Il2rb, Cd86, Gata3, Cd24a, Tcf7, Gimap1, Bcl2, Zfp36l1,** *Il6,* **Lef1, Gimap5, Ikzf3, Dpp4, Foxp3, Lat, Foxp1, Mef2c,** *Cd74,* **Mertk, Anxa3,** *Prkcb,* **Fcgr4, Anxa1,** *H2-DMa, Bcl6, Clcf1, Runx2,* **Fcgr2b, Lgals1,** *Irf1, Prkce,* **Jun, Lyl1, Lck,** *Sema4a*
8	regulation of lymphocyte activation	33.7123945	35	2.30E-15	*Tnfsf18,* **Ighm, Il7r, Il12b, Ccl2, Ccr7, Prkcq, Slamf7, Ctla2a, Gpr183, Adam8,** *Lst1,* **Tnfrsf13c, Dtx1, Cd86, Gata3, Cd24a,** *Tnfsf13b,* **Pglyrp1, Igf1, Tnfrsf14, Bcl2,** *Il6,* **Gimap5, Cdkn2a, Ikzf3, Dpp4, Foxp3, Cd80,** *Bmi1,* **Lat, Mef2c,** *Cd74,* **Tnfrsf13b, Mertk,** *Zeb1, H2-DMa,* **Thy1,** *Bcl6, Hsph1, Clcf1, Peli1,* **Fcgr2b, Lgals1,** *Fgf10,* **Prnp,** *Irf1,* **Tnfsf14,** *Nck2, Gal,* **Lck**
14	hemopoietic or lymphoid organ development	31.58784106	44	1.90E-14	**Hdac9, Hbb-b1, 5830411N06Rik, Cxcr5, Il7r, Il12b, Cd3d, Hba-a1,** *Heph,* **Kit, Cd8a,** *Tgfb2,* **Ccr7, Tnfsf8,** *Six1,* **Satb1, Angpt2, Gpr183, Alas2, Kitl, Kdr,** *Pbx1,* **Epas1, Adam8,** *Efna2, Pdgfrb,* **Gata3, Cd24a, Tcf7, Gimap1, Bcl2, Zfp36l1, Ltb, Lef1, Gimap5, Fst, Foxp3, Foxp1, Mef2c, Csf1,** *Lmo4, Cd74, Bcl2l11,* **Mertk, Mfsd7b, Anxa1,** *H2-DMa, Slc40a1, Bcl6, Clcf1, Runx2, Sod2, Jak2,* **Lgals1,** *Fgf10,* **Tacc3, Xrcc5,** *Irf1,* **Jun, Cd300lf, Lyl1,** *Mitf,* **Lck,** *Csf1r, Sema4a*
16	immune system development	31.11975692	45	3.10E-14	**Hdac9, Hbb-b1, 5830411N06Rik, Cxcr5, Il7r, Il12b, Cd3d, Hba-a1,** *Heph,* **Kit, Cd8a, C3ar1,** *Tgfb2,* **Ccr7, Tnfsf8,** *Six1,* **Satb1, Angpt2, Gpr183, Alas2, Kitl, Kdr,** *Pbx1,* **Epas1, Adam8,** *Efna2, Pdgfrb,* **Gata3, Cd24a, Tcf7, Gimap1, Bcl2, Zfp36l1, Ltb, Lef1, Gimap5, Fst, Foxp3, Foxp1, Mef2c, Csf1,** *Lmo4, Cd74, Bcl2l11,* **Mertk, Mfsd7b, Anxa1,** *H2-DMa, Slc40a1, Bcl6, Clcf1, Runx2, Sod2, Jak2,* **Lgals1,** *Fgf10,* **Tacc3, Xrcc5,** *Irf1,* **Jun, Cd300lf, Lyl1,** *Mitf,* **Lck,** *Csf1r, Sema4a*
19	leukocyte differentiation	30.35232975	31	6.60E-14	**Hdac9, 5830411N06Rik, Il7r, Il12b, Cd3d, Kit, Cd8a, Ccr7, Tnfsf8, Satb1, Gpr183, Adam8,** *Efna2,* **Gata3, Cd24a, Tcf7, Gimap1, Bcl2, Zfp36l1, Lef1, Gimap5, Foxp3, Foxp1, Mef2c, Csf1,** *Cd74,* **Mertk, Anxa1,** *H2-DMa, Bcl6, Runx2, Clcf1,* **Lgals1,** *Irf1,* **Jun, Cd300lf, Lyl1,** *Mitf,* **Lck,** *Csf1r, Sema4a*
			Cell cycle / mitosis		
9	cell division	33.69766916	44	2.30E-15	**Kit,** *Tgfb2,* **Pstpip1, Top2a, Aurkb, Mastl, Spc25, Bub1, Oip5, Arl8b, Wee1, Lef1, Cdkn2a, Ska1, Ccnb1, Tpx2, Itgb3bp, Casc5, Kif11,** *Bmi1,* **Kntc1, Nusap1, Sgol2, Aspm, Birc5, Mis18bp1, Ccnd1, Spag5, Cdc25c, Kif2c, Cenpw, Kif20b,** *Ccnd2,* **Zfyve26, Nuf2, Ckap2,** *10-Sep,* **Bcl2l1, Ccna2, Sgol1, Spc24, Cdk1, Prc1, Nek2,** *Prkce,* **Ccdc99, Cables1, Cdca7,** *Ccno,* **Lzts2**
20	kinetochore	30.19275585	21	7.70E-14	**Spc25, Bub1, Ccnb1, Ska1, Mlf1ip, Itgb3bp, Casc5, Cenpi, Kntc1, Sgol2, Birc5, Cenpk, Spag5, Kif2c, Cenpw, Cenpa, Nuf2, Sgol1, Spc24, Nek2, Ccdc99**

Supplementary Table 1 (Continued)

Rank	Biological functions (GO processes)	NextBio Normalized score	Common Genes	P-value	Genes (**increased**, *decreased*)
					Other
1	regulation of response to stimulus	48.99217694	138	5.30E-22	**Il22**, **Adra2c**, **Htr2b**, **Hbb-b1**, **Ighm**, **Sox7**, **Chi3l1**, **Lepr**, **1190002H23Rik**, **D10Bwg1379e**, **Il7r**, **Arap2**, **Il12b**, *Iqgap2*, **Ccl2**, *Bambi*, **P2ry1**, **Mefv**, *Bmp2*, **Rgs18**, **Kit**, **Cd8a**, **C3ar1**, *Tgfb2*, Cspg4, **Murc**, **Pdgfa**, **Arhgap15**, *Flt1*, **Hmgn3**, **Ccr7**, **Cadm1**, **Kcnn4**, **Ppap2b**, **Prkcq**, **Cdkn1c**, **Uchl1**, **Adra2a**, **Ctla2a**, **Nt5e**, **Ccl7**, **Angpt2**, **C1qa**, *Cnksr2*, **Gpr183**, **Kitl**, **Kdr**, **S100a4**, **Adam8**, **Scarf1**, **Itgb3**, **Lat2**, **Tnfrsf13c**, **Dtx1**, **Dact2**, **Dusp4**, **Fabp4**, **Cd86**, *Pdgfrb*, **Gata3**, **Cfh**, **Cd24a**, **Arap3**, *C3*, **Clec2d**, **Ptk2**, **Rasgrp3**, **Cd79b**, *Tnfsf13b*, **Cd59a**, **Pglyrp1**, *Rgs9*, **Igf1**, *Fcgr1*, **F3**, **Trib3**, **Bcl2**, **Mcf2l**, *Epha4*, *Il6*, *Tbc1d8*, **Pde2a**, **Mylk2**, **Ralgapa2**, **Cyp4f18**, *Lzts1*, **Lef1**, **Gimap5**, **Cdkn2a**, **Bcap31**, *Bmf*, **4930547N16Rik**, *Agrn*, *Nuak1*, **Lrpap1**, **Trim59**, *Samhd1*, **Evi5l**, **Ptgir**, **Ercc6**, **Fst**, **Cfp**, *Rhbdf1*, **Chst11**, **Hpse**, **Dpp4**, *Fzd1*, **Pdpn**, **Foxp3**, **Mgll**, **Lat**, **Mef2c**, **Rgs1**, **Sphk1**, *Asap2*, **Card6**, *Htra3*, **Aspm**, **Csf1**, **Efnb2**, *Hp*, *Cd74*, *Sipa1l1*, *Bcl2l11*, **Tbc1d2**, **Ecm1**, **Ccnd1**, *Hey1*, *Mfng*, Prkcb, Pvrl2, Trim30a, Rap2a, Plau, Tec, Ikbkg, Zeb1, Spata13, 1500003O03Rik, Pink1, Magi2, H2-DMa, Anxa1, Chac1, Thy1, Usp6nl, Myo5a, Smyd2, Rapgef5, Bcl2l1, Bcl6, Runx2, Clcf1, Cdon, Tcf7l2, Spry2, Trim13, Peli1, Sorbs3, Zcchc11, Bid, Eng, Kif7, Vegfb, Fcgr2b, Jak2, Tbc1d20, Fgf10, Lgals1, Cx3cl1, Tle2, Arhgap10, Ddit3, Wtip, Agap2, Colec12, Irf1, Prnp, Rasa3, C1ra, Fem1b, Prkce, Pid1, Tnfsf14, Cd44, Id1, Jun, Slc20a1, Smad6, Bst2, Map3k9, Dok2, Met, Dusp8, Slc7a1, Brca1, Csf1r, Lck, Hipk2, Dusp10, Htra2, Pde4d, Enpp1, Sh2d3c, Slc9a3r2, 2010106G01Rik, Rasal3, Iqgap3
2	cell periphery	47.28155114	198	2.90E-21	**Adra2c, Htr2b, S100a9,** *Lphn3, Tnfsf18,* **Mlph, Cd93, P2ry10, Ighm, Dok7, Cxcr6, 5830411N06Rik, Cdh5, Mfge8, Cd163l1, Rhoj, Cxcr5, Cadm2, Klrd1, Dlg3, Lrfn2,** *Ppp1r9a,* **Lepr, Clmp, Tmeff1,** *Epcam,* **Klra10, Il7r, Icos, Prom1, Ms4a4b, Eps8, Lims2, Cxcr1, Ptgs1, Cd3d, Dmd,** *Bambi, Mylip,* **Il1r2, P2ry1,** *Heph,* **Plxna2, Rgs18, Kit, Cd8a, C3ar1, Prickle2, Cspg4, Pstpip1,** *Clic6, Flt1, Dgkg,* **Folr2,** *Cd209b,* **Ccr7, Cadm1, Kcnn4, Pmp22, Cd207, Ms4a1, Prkcq, S100a6, Slc6a8, Slc27a1, Cdh1, Slamf7, Aqp9, Uchl1,** *Itga4,* **Emp1, Adra2a, Itga9, Cd8b1, Nt5e, Cd3g, Angpt2,** *Cnksr2,* **Gpr183,** *H2-DMb1,* **Kitl, Cd109, Kdr,** *Lypd6b,* **Slco3a1,** *Bcam,* **Perp, Adam8,** *H2-DMb2,* **Itgb3, Lat2, Ccr6, Tnfrsf13c,** *Efna2,* **Ltb4r1,** *Sdc1,* **Nrcam, Il2rb,** *Cd69, Ocln,* **Cd86,** *Pdgfrb,* **Cfh, Igf2r, Cd24a, Arap3,** *Clec2d,* **Mreg, Ptk2, Cd79b,** *Tnfsf13b,* **Cd59a,** *Rgs9, Lgr4,* **Itgae, Scamp5,** *Fcgr1,* **Tnfrsf14,** *Wnt6,* **Bcas3,** *Frk,* **Mcf2l,** *Epha4,* **St14,** *Il6, Syt7,* **Dysf, Pde2a, Cyp4f18, Fcrl1,** *Lzts1,* **Ltb,** *Ldlrad3,* **Gpr171, Bcap31, Gpnmb,** *Jph1,* **Basp1, Tmod1,** *Dtna,* **Lrpap1, Stx3, Mmp2,** *Gpr85,* **Slc16a6, Tln2, Mllt4,** *Slc52a3,* **Ptgir, Tpm1, Tnfrsf25, Lrba, Gem, Slc28a2, Cd97, Pcdh7, Dpp4, Pdcd1,** *Fzd1,* **Cd80, Pdpn, Gypc, C230081A13Rik, Lat,** *Syne2,* **Rgs1, Sphk1,** *Asap2,* **Cd72, Bbs9,** *Tmem149,* **Csf1, Efnb2,** *Cd74, Sipa1l1,* **Cd63, Lpar6, Birc5, Tnfrsf13b, Lasp1, Sgce, Thbd,** *Slc16a13,* **Itgb7, Cltb,** *Snta1,* **Ccnd1, Abcb1b, Anxa3, Celsr1, Cgnl1,** *Glrb, Prkcb, Pvrl2,* **Pdk1,** *Nos3, Emr1,* **Atp6v0a1, Rell1, Abcg2, Fcgr4, Melk, Tec,** *Spata13,* **Plaur,** *1500003O03Rik, F11r,* **Tst, Rab26,** *Magi2, Gphn,* **Rhou,** *H2-DMa, Slc40a1,* **Anxa1,** *10-Sep, Sema6d,* **Gng8, Abcb4, Thy1, Lrp12, Trem2,** *Itpr2, Prkar2b, Cdon, Slc6a9,* **Spry2,** *Vdr,* **Fn1, Sorbs3,** *Tle6, Abca1, Slc44a1,* **Ctsl, Eng, Mr1,** *Jak2,* **Fcgr2b, Usp2,** *Aplp1, Fgf10, Cx3cl1,* **Arhgap10,** *Phlda1,* **Slc26a11, Mgst2, Prnp, Rasa3, Vezt, Prc1,** *Acsl1, Prkce, Slc39a14,* **Ctsb,** *Dapp1,* **Sdc4, Tnfsf14, Cd44, Slc20a1,** *Agpat3, Bst2,* **Cd300lf,** *Il1r1, Met, Rp2h, Slc4a7,* **Iqgap1, Pgm5,** *Csf1r,* **Plin2, Slc7a1, Brca1, Lck,** *Abcg1,* **Nkain1,** *Fat1, Htra2,* **Adi1, Pde4d, Krt19, Enpp1, Parvb,** *Abcc1, Sema4a, Slc9a3r2, 2010106G01Rik, Smagp,* **Sdcbp, Rasal3, Iqgap3**
10	single-organism process	33.17682726	283	3.90E-15	**Dach1, Adra2c, Htr2b,** *Lphn3, Tnfsf18,* **Hdac9, Hbb-b1, Arnt2, P2ry10, Eya4, Ighm, Il18r1, Sox7, Dok7, Chi3l1, Cxcr6, 5830411N06Rik, Cdh5, Mfge8, Rhoj, Cxcr5,** *Ppp1r9a,* **Lepr, 1190002H23Rik, Tmeff1, Bex1,** *Nfib,* **Il7r, Asprv1, Prom1,** *Ttll7,* **Arap2, Eps8, Nlrc3, Il12b, Cxcr1,** *Iqgap2,* **Ptgs1, Cd3d, Dmd,** *Plcb4,* **Hba-a1, Stat4, Ccl2,** *Bambi, Mylip,* **Il1r2, P2ry1, Plxna2,** *Heph, Bmp2,* **Rgs18, Kit, Spic, Cd8a, C3ar1, Prickle2,** *Tgfb2,* **Cspg4, Murc, Pdgfa, Arhgap15, Stac,** *Flt1, Dgkg,* **Ddah1,** *Cd209b,* **Ccr7, Tnfsf8, Cadm1, Kcnn4, Ppap2b, Pmp22, Fcna, Prkcq, Serpinb6b, Cdkn1c, Slc27a1, Cdh1, Uchl1,** *Itga4,* **Adra2a,** *Six1,* **Pitx1, Itga9, Mkl2,** *6230427J02Rik,* **Prm1,** *Meox1,* **Satb1,** *Rassf6,* **Sema3e, Cd3g, Angpt2, C1qa, Olfr433, Gpr183,** *Cnksr2,* **Alas2,** *H2-DMb1, Maob,* **Adam19, Kitl, Kdr, Spsb4, Pkdcc,** *Pbx1,* **Epas1, Adam8,** *H2-DMb2,* **Scarf1, Itgb3, Lat2, Prkar1b,** *Lst1,* **Ccr6, Nav1, Dtx1, Apbb2, Top2a,** *Efna2,* **Ltb4r1, Pde8b, Dact2,** *Sall2,* **Dio2,** *Sdc1,* **Nrcam,** *Dfna5,* **Il2rb, Mmp9, Dusp4, Fabp4, Cd86, Tnni2,** *Akap2,* **Hivep2, Nedd4l,** *Pdgfrb, Apln,* **Gata3, Igf2r,** *Rfx3,* **Cd24a, Arap3, Mreg,** *C3, Clec2d,* **Bok, Ptk2, Rasgrp3,** *Dhcr24,* **Cd79b, Tcf7,** *Spin2,* **Cd59a, Pglyrp1, Ccl17,** *Rgs9,* **Igf1, Itgae,** *Lgr4,* **Ndst1, Gimap1, Arid5a, Vim,** *Fcgr1,* **F3, Bcl2, Papss2,** *Rnd3,* **Zfp36l1, Bub1, Tom1l1,** *Wnt6,* **Mcf2l,** *Epha4, Il6,* **Pde2a, Mylk2, Ralgapa2, Cyp4f18, Ltb, Wee1, Arl8b,** *Cib2, Lzts1,* **Lef1, Gimap5, Gpr171,** *Dpf3,* **Cdkn2a, Bcap31, Ccnb1,** *Jph1,* **Basp1,** *Apoc1,* **Fam83h,** *Cebpd, Tfpi,* **Ptpn14, Tmod1,** *Agrn, Rdh10, Spin4,* **Cdo1, Lgals3, Stx3, Mmp2,** *Ppm1l, Gpr85,* **Stmn1, Mllt4, Ptgir,** *Slc52a3,* **Tpm1, Pkp2,** *Mpzl3,* **Ercc6, Fst, Lrba, Foxo3, Cfp, Itgb3bp, Gem, Chst11, Derl3, Dapk2, Fignl1,** *Grhl1,* **Hpse, Slc28a2, Cd97, Pdpn, Foxp3,** *Fzd1, Bmi1, Pgap2,* **Hilpda, Cc2d2a, Lat,** *Napsa,* **Mef2c, Foxp1, Rgs1, Sphk1, Rad51, Arhgap22, Bin1, Avil, Sgol2, Ccbe1, Aspm, Csf1, Efnb2, Myo18b,** *Hp, Lmo4,* **Plxnc1, Lpar6,** *Cd74, Sipa1l1,* **Birc5,** *Bcl2l11, Tanc2, Afp, Foxf2, Foxn3,* **Ecm1, Thbd, Itgb7,** *Plcd1, Unc13b, Bcor, Olfr1153,* **Ccnd1, Mertk, Mki67, Abcb1b,** *Hey1, Mfng, Snta1, Tshz1,* **Celsr1, Spp1,** *Asb5,* **Pdk1,** *Glrb, Prkcb, Pvrl2,* **Uchl3,** *Nos3, Emr1, Six5,* **Abcg2, Rap2a, Plau, Tec, Naglu, Ctsd, Plaur, Mfsd7b,** *Ikbkg, Zeb1, Spata13,* **Pink1, Crem, Fbxo32, Rab26,** *Nfe2,* **Rhou,** *Magi2, Gphn, Prdx5,* **Anxa1, Chac1,** *H2-DMa, Slc40a1,* **Gng8, Sepp1, Thy1,** *Id3, Sema6d,* **Kif3a, Myo5a, Trem2, Rapgef5,** *Itpr2, Prkar2b,* **Bcl2l1, Rps6ka2,** *Bcl6, Runx2, Clcf1, Cdon,* **Ppp1r15a, Spry2,** *Tcf7l2, Trim13,* **Gtf2ird1,** *Sod2, Vdr, Ebf4, Zcchc11,* **Dhcr7, Hyal1,** *Ccdc88c,* **Bid, Ctsl, Eng, Vegfb, Sgol1, Raph1,** *Abca1,* **Fcgr2b, Grn, Usp2,** *Jak2,* **Lgals1, Arhgap10,** *Aplp1, Cdkn1b, Fgf10, Foxn2, Cx3cl1, Tle2, Adamts2,* **Ddit3, Etv1,** *Tnnt1,* **Cdk1,** *Agap2,* **Coq7, Tacc3,** *Peg12, Colec12,* **Prnp, Rasa3, Xrcc5, Vezt,** *Gclm, Irf1, C1ra,* **Fem1b, Nek2,** *Acsl1, Prkce,* **Sdc4, Tnfsf14, Gla, Cd44, Jun, Slc20a1,** *Id1, Klf7,* **Cd300lf,** *Smad6, Bst2, Map3k9,* **Lyl1, Cables1,** *Dok2, Il1r1, Met, Mitf, Tle4, Gal, Slc4a7,* **Brca1, Foxs1, Lck,** *Csf1r,* **Hipk2, Vapb,** *Abcg1, Zfp354a,* **Nr4a2, Rad18, Pde4d,** *Ctss, Gsr, Mmp14, Htra2,* **Krt19, Enpp1, Sh2d3c, Aph1b, Lzts2,** *Aldh1a2, Arhgap23,* **Cdkn2d, Sdcbp, Rasal3, Iqgap3,** *Sema4a*
11	cell surface	32.98017821	52	4.80E-15	*Tnfsf18,* **Cd93, Ighm, 5830411N06Rik, Mfge8, Klrd1,** *Epcam,* **Il7r, Icos, Prom1, Dmd,** *Bmp2,* **Kit, Cd8a,** *Tgfb2,* **Pdgfa,** *Cd209b,* **Ccr7, Ms4a1, Cdh1,** *Itga4,* **Cd8b1, Nt5e, Kdr, Slco3a1,** *Bcam,* **Adam8, Itgb3, Tnfrsf13c,** *Sdc1,* **Il2rb,** *Cd69, Ocln,* **Cd86, Igf2r, Cd24a,** *Clec2d,* **Cd79b, Cd59a, Itgae, Tnfrsf14,** *Fcgr1,* **F3,** *Wnt6, Epha4, Il6, Agrn,* **Ceacam2, Pdcd1, Dpp4, Cd80,** *Fzd1,* **Hilpda, Cd63,** *Cd74,* **Tnfrsf13b, Thbd,** *Glrb, Pvrl2, Emr1,* **Fcgr4, Plau, Plaur, Thy1,** *Cdon,* **Eng, Ctsl,** *Abca1,* **Fcgr2b, Grn, Lgals1,** *Cx3cl1, Fgf10,* **Ctsb, Sdc4, Cd44,** *Bst2, Il1r1, Csf1r, Abcg1, Ctss,* **Enpp1**
15	regulation of protein modification process	31.2642272	69	2.60E-14	**Il22, Adra2c, Htr2b, Hbb-b1, Ighm, Dok7, Chi3l1, 1190002H23Rik, Il12b, P2ry1,** *Bmp2,* **Kit,** *Tgfb2,* **Cspg4, Pdgfa,** *Flt1,* **Ccr7, Ppap2b, Cdkn1c, Slc27a1, Uchl1, Adra2a, Kitl, Adam8, Itgb3, Prkar1b, Dusp4, Fabp4,** *Pdgfrb,* **Gata3, Cd24a,** *C3,* **Ptk2, Igf1, Arid5a, Tnfrsf14, Trib3, Bcl2, Tom1l1,** *Epha4, Il6,* **Cdkn2a, Ccnb1,** *Agrn,* **Tpx2, Ercc6, Foxp3, Cd80,** *Fzd1, Bmi1,* **Sphk1, Csf1,** *Cd74, Bcor,* **Ccnd1,** *Ccnd2,* **Rap2a, Card14,** *Ikbkg,* **Pink1,** *1500003O03Rik,* **Thy1,** *Prkar2b,* **Ccna2,** *Cdon, Clcf1,* **Eef2k, Spry2,** *Peli1, Ccdc88c,* **Eng, Vegfb, Impact,** *Jak2, Cdkn1b, Fgf10, Agap2,* **Prnp,** *Irf1,* **Fem1b,** *Acsl1, Pid1, Prkce,* **Sdc4, Cd44, Jun,** *Smad6, Map3k9,* **Cables1, Dusp8,** *Met,* **Iqgap1,** *Ccno,* **Lck, Brca1,** *Csf1r,* **Lpin1, Vapb, Dusp10, Enpp1, Cdkn2d, Iqgap3**
17	regulation of multicellular organismal development	31.07771937	79	3.20E-14	**Adra2c, Hdac9, Chi3l1, 1190002H23Rik, Bex1,** *Nfib,* **Il7r, Prom1, Il12b, Ccl2,** *Bambi, Bmp2,* **Kit, C3ar1,** *Tgfb2,* **Cspg4, Pdgfa,** *Flt1,* **Ddah1, Ccr7, Pmp22, Cdh1,** *Six1,* **Ctla2a, Mkl2, Sema3e, Angpt2, Kitl, Kdr, Pkdcc,** *Pbx1,* **Adam8, Scarf1, Itgb3, Dtx1, Nrcam, Mmp9, Cd86,** *Pdgfrb,* **Gata3,** *Rfx3,* **Cd24a,** *C3, Clec2d,* **Ptk2, Cd59a, Pglyrp1, Igf1,** *Lgr4,* **Vim, F3, Bcl2,** *Wnt6, Epha4, Il6, Lzts1,* **Lef1, Gimap5, Cdkn2a, Ccnb1,** *Agrn,* **Fst, Foxo3, Ikzf3, Hpse, Foxp3,** *Fzd1, Bmi1,* **Foxp1, Mef2c, Sphk1, Rnh1, Avil, Aspm, Efnb2, Csf1,** *Sipa1l1, Cd74,* **Ecm1,** *Bcor,* **Ccnd1, Celsr1, Anxa3,** *Prkcb, Nos3,* **Rap2a, Plau,** *Zeb1, Nfe2, H2-DMa,* **Thy1,** *Runx2, Cdon, Bcl6, Clcf1, Tcf7l2, Vdr,* **Hyal1, Eng, Vegfb, Usp2, Grn, Lgals1,** *Cx3cl1, Cdkn1b, Fgf10,* **Ddit3, Ets1, Cdk1,** *Agap2,* **Xrcc5,** *Irf1,* **Jun, Cd44,** *Id1, Met, Mitf,* **Lck,** *Csf1r,* **Hipk2, Iqgap3,** *Sema4a*
18	regulation of phosphorus metabolic process	30.61847231	68	5.00E-14	**Il22, Adra2c, Htr2b, Hbb-b1, Ighm, Dok7, Chi3l1, Dlg3, 1190002H23Rik, Il12b, P2ry1,** *Bmp2,* **Kit,** *Tgfb2,* **Cspg4, Pdgfa,** *Flt1,* **Ccr7, Ppap2b, Cdkn1c, Slc27a1, Uchl1, Adra2a, Kitl, Adam8, Itgb3, Prkar1b, Dusp4, Fabp4,** *Pdgfrb, Apln,* **Cd24a,** *C3,* **Ptk2, Mastl, Igf1, Tnfrsf14, Trib3, Bcl2, Tom1l1,** *Epha4, Il6,* **Cdkn2a, Ccnb1,** *Agrn, Nuak1,* **Tpx2, Ercc6, Casc5, Cd80,** *Fzd1,* **Mef2c, Sphk1, Myo1d, Csf1,** *Cd74,* **Ccnd1,** *Ccnd2,* **Rap2a, Card14,** *Ikbkg, 1500003O03Rik, Magi2,* **Thy1,** *Prkar2b,* **Ccna2,** *Cdon, Clcf1,* **Eef2k, Spry2,** *Ccdc88c,* **Eng, Vegfb, Impact,** *Jak2, Cdkn1b, Fgf10, Agap2,* **Prnp,** *Irf1, Acsl1, Pid1, Prkce,* **Sdc4,** *Dapp1,* **Cd44, Jun,** *Smad6, Map3k9,* **Cables1, Dusp8,** *Met,* **Iqgap1,** *Ccno,* **Lck,** *Csf1r,* **Vapb, Dusp10, Enpp1, Sdcbp, Cdkn2d, Iqgap3**

Supplementary Table 2

Rank	Biological functions (GO processes)	NextBio Normalized score	Common Genes	P-value	Genes (**increased**, *decreased*)
			Biosynthetic process		
3	regulation of biosynthetic process	24.65176269	89	2.00E-11	**Dach1, Il22, S100a9,** *Tnfsf18,* **Hdac9, Arnt2, Eya4, Sox7, Zranb3, 1190002H23Rik, Bex1,** *Nfib,* **Nlrc3, Dmd, Stat4, Ccl2,** *Bambi,* **P2ry1,** *Bmp2,* **Kit, Spic,** *Tgfb2,* **Murc, Pdgfa, Ddah1,** *Cd209b,* **Hmgn3, Efcab6,** *Ssbp2,* **Ccr7, Tnfsf8, Ppap2b, Prkcq, Cdkn1c, Slc27a1, Cdh1, Adra2a,** *Six1,* **Pitx1, St18, Mkl2,** *Meox1,* **Satb1, Kitl, Kdr,** *Pbx1,* **Epas1, Adam8, Itgb3, Tnfrsf13c, Apbb2, Top2a,** *Tsc22d1,* **Pde8b,** *Sall2,* **Aurkb, Fabp4, Tnni2, Hivep2, E2f7,** *Pdgfrb,* **Gata3,** *Rfx3, C3,* **Tcf7, Igf1,** *Lgr4,* **Arid5a,** *Kdm4a,* **Trib3, Bcl2, Zfp36l1,** *Wnt6,* **Mcf2l, Irx2,** *Il6,* **Pde2a, Ltb, Lef1, Gimap5,** *Dpf3,* **Cdkn2a, Basp1,** *Apoc1, Cebpd, Agrn, Zfp827,* **Ptgir, Mlf1ip, Ercc6, Fst, Foxo3, Itgb3bp, Ikzf3,** *Grhl1,* **Taf1, Foxp3,** *Fzd1, Bmi1,* **Mef2c, Foxp1, Sphk1, Arhgap22,** *Lmo4,* **Cdca7l, Birc5,** *Foxf2, Foxn3,* **Ecm1, Cenpk,** *Plcd1, Bcor, Hey1, Tshz1,* **Anxa3,** *Bmyc, Prkcb,* **Igf2bp3,** *Nos3, Trim30a, Six5, Ikbkg, Zeb1,* **Ckap2, Hip1,** *1500003O03Rik,* **Crem,** *Nfe2, Prdx5,* **Anxa1,** *Id3,* **Smyd2, Ccna2,** *Bcl6, Runx2, Hsph1, Cdon,* **Ppp1r15a,** *Tcf7l2, Peli1, Zbtb10,* **Sorbs3, Gtf2ird1,** *Sod2, Vdr, Trps1, Ebf4, Zcchc11,* **Dhcr7,** *Trerf1,* **Eng,** *Abca1,* **Impact, Usp2,** *Jak2, Aplp1, Cdkn1b, Fgf10, Foxn2, Tle2, Psip1,* **Ddit3, Etv1, Ets1,** *Wtip,* **Cdk1,** *Agap2, Zscan18,* **Prnp, Xrcc5,** *Irf1,* **Eaf1,** *Pid1, Nck2,* **Jun,** *Id1, Klf7, Smad6, Mcts2, Bbx, Zfp710, Map3k9,* **Lyl1, Zfp30,** *Met, Mitf, Tle4,* **Cdca7,** *Mycbp,* **Brca1, Foxs1, Lpin1, Hipk2,** *Abcg1, Zfp354a, Ift57, Batf3,* **Nr4a2,** *Mmp14, Irf5,* **Enpp1**
4	regulation of RNA biosynthetic process	23.72886383	79	5.00E-11	**Dach1, Il22,** *Tnfsf18,* **Hdac9, Arnt2, Eya4, Sox7, 1190002H23Rik, Bex1,** *Nfib,* **Nlrc3, Dmd, Stat4,** *Bambi,* **P2ry1,** *Bmp2,* **Kit, Spic, Murc, Hmgn3, Efcab6,** *Ssbp2,* **Tnfsf8, Ppap2b, Prkcq, Cdkn1c, Cdh1,** *Six1,* **Pitx1, St18, Mkl2,** *Meox1,* **Satb1,** *Pbx1,* **Epas1, Adam8, Apbb2, Top2a,** *Tsc22d1,* **Pde8b,** *Sall2,* **Aurkb, Fabp4, Tnni2, Hivep2, E2f7, Gata3,** *Rfx3,* **Tcf7, Igf1,** *Lgr4,* **Arid5a,** *Kdm4a,* **Trib3,** *Wnt6,* **Mcf2l, Irx2,** *Il6,* **Pde2a, Lef1,** *Dpf3,* **Cdkn2a, Basp1,** *Cebpd, Agrn, Zfp827,* **Mlf1ip, Ercc6, Fst, Foxo3, Itgb3bp, Ikzf3,** *Grhl1,* **Taf1, Foxp3,** *Fzd1, Bmi1,* **Mef2c, Foxp1, Sphk1, Arhgap22,** *Lmo4,* **Cdca7l, Birc5,** *Foxf2, Foxn3,* **Ecm1, Cenpk,** *Bcor, Hey1, Tshz1,* **Anxa3,** *Bmyc, Prkcb, Trim30a, Six5, Ikbkg, Zeb1,* **Ckap2, Hip1,** *1500003O03Rik,* **Crem,** *Nfe2, Prdx5, Id3,* **Smyd2, Ccna2,** *Bcl6, Runx2, Cdon, Tcf7l2, Peli1, Zbtb10,* **Sorbs3, Gtf2ird1,** *Sod2, Vdr, Trps1, Ebf4, Zcchc11, Trerf1,* **Eng, Usp2,** *Jak2, Cdkn1b, Fgf10, Foxn2, Tle2, Psip1,* **Ddit3, Etv1, Ets1,** *Wtip, Agap2, Zscan18,* **Prnp, Xrcc5,** *Irf1,* **Eaf1,** *Pid1, Nck2,* **Jun,** *Id1, Klf7, Smad6, Mcts2, Bbx, Zfp710, Map3k9,* **Lyl1, Zfp30,** *Met, Mitf, Tle4,* **Cdca7,** *Mycbp,* **Brca1, Foxs1, Lpin1, Hipk2,** *Abcg1, Zfp354a, Ift57, Batf3,* **Nr4a2,** *Mmp14, Irf5*
5	regulation of cellular macromolecule biosynthetic process	21.60874497	81	4.10E-10	**Dach1, Il22,** *Tnfsf18,* **Hdac9, Arnt2, Eya4, Sox7, Zranb3, 1190002H23Rik, Bex1,** *Nfib,* **Nlrc3, Dmd, Stat4,** *Bambi,* **P2ry1,** *Bmp2,* **Kit, Spic, Murc, Pdgfa, Hmgn3, Efcab6,** *Ssbp2,* **Ccr7, Tnfsf8, Ppap2b, Prkcq, Cdkn1c, Cdh1,** *Six1,* **Pitx1, St18, Mkl2,** *Meox1,* **Satb1, Kitl,** *Pbx1,* **Epas1, Adam8, Apbb2, Top2a,** *Tsc22d1,* **Pde8b,** *Sall2,* **Aurkb, Fabp4, Tnni2, Hivep2, E2f7,** *Pdgfrb,* **Gata3,** *Rfx3,* **Tcf7, Igf1,** *Lgr4,* **Arid5a,** *Kdm4a,* **Trib3, Bcl2, Zfp36l1,** *Wnt6,* **Mcf2l, Irx2,** *Il6,* **Pde2a, Lef1,** *Dpf3,* **Cdkn2a, Basp1,** *Cebpd, Agrn, Zfp827,* **Mlf1ip, Ercc6, Fst, Foxo3, Itgb3bp, Ikzf3,** *Grhl1,* **Taf1, Foxp3,** *Fzd1, Bmi1,* **Mef2c, Foxp1, Sphk1, Arhgap22,** *Lmo4,* **Cdca7l, Birc5,** *Foxf2, Foxn3,* **Ecm1, Cenpk,** *Bcor, Hey1, Tshz1,* **Anxa3,** *Bmyc, Prkcb,* **Igf2bp3,** *Trim30a, Six5, Ikbkg, Zeb1,* **Ckap2, Hip1,** *1500003O03Rik,* **Crem,** *Nfe2, Prdx5, Id3,* **Smyd2, Ccna2,** *Bcl6, Runx2, Cdon,* **Ppp1r15a,** *Tcf7l2, Peli1, Zbtb10,* **Sorbs3, Gtf2ird1,** *Sod2, Vdr, Trps1, Ebf4, Zcchc11, Trerf1,* **Eng, Impact, Usp2,** *Jak2, Aplp1, Cdkn1b, Fgf10, Foxn2, Tle2, Psip1,* **Ddit3, Etv1, Ets1,** *Wtip,* **Cdk1,** *Agap2, Zscan18,* **Prnp, Xrcc5,** *Irf1,* **Eaf1,** *Pid1, Nck2,* **Jun,** *Id1, Klf7, Smad6, Mcts2, Bbx, Zfp710, Map3k9,* **Lyl1, Zfp30,** *Met, Mitf, Tle4,* **Cdca7,** *Mycbp,* **Brca1, Foxs1, Lpin1, Hipk2,** *Abcg1, Zfp354a, Ift57, Batf3,* **Nr4a2,** *Mmp14, Irf5,* **Enpp1**
10	negative regulation of biosynthetic process	15.72045375	36	1.50E-07	**Dach1, Hdac9, Sox7, Zranb3,** *Nfib, Bmp2,* **Pdgfa, Cdkn1c, Slc27a1, Adra2a,** *Six1,* **St18, Satb1, Itgb3,** *Sall2,* **Aurkb, Fabp4, Gata3,** *Rfx3, Lgr4, Kdm4a,* **Trib3,** *Wnt6, Il6,* **Pde2a, Lef1, Gimap5, Cdkn2a, Basp1,** *Apoc1, Cebpd,* **Fst, Foxo3, Foxp3,** *Fzd1, Bmi1,* **Foxp1, Mef2c, Birc5,** *Foxn3, Foxf2, Bcor, Hey1, Zeb1, Prdx5, Id3,* **Smyd2,** *Runx2, Bcl6, Zbtb10, Tcf7l2,* **Sorbs3,** *Trps1, Vdr,* **Eng, Usp2,** *Tle2, Foxn2, Cdkn1b, Aplp1,* **Xrcc5,** *Irf1, Pid1,* **Jun,** *Id1, Met, Tle4,* **Foxs1, Brca1, Lpin1, Hipk2,** *Zfp354a,* **Enpp1**
14	negative regulation of cellular macromolecule biosynthetic process	14.4906583	33	5.10E-07	**Dach1, Hdac9, Sox7, Zranb3,** *Nfib, Bmp2,* **Cdkn1c,** *Six1,* **St18, Satb1,** *Sall2,* **Aurkb, Fabp4, Gata3,** *Rfx3, Lgr4, Kdm4a, Wnt6,* **Pde2a, Lef1, Cdkn2a, Basp1,** *Cebpd,* **Fst, Foxo3, Foxp3,** *Fzd1, Bmi1,* **Foxp1, Mef2c, Birc5,** *Foxn3, Foxf2, Bcor, Hey1, Zeb1, Prdx5, Id3,* **Smyd2,** *Runx2, Bcl6, Zbtb10, Tcf7l2,* **Sorbs3,** *Trps1, Vdr,* **Eng, Usp2,** *Tle2, Foxn2, Cdkn2a, Cdkn1b,* **Xrcc5,** *Irf1, Pid1,* **Jun,** *Id1, Met, Tle4,* **Foxs1, Brca1, Lpin1, Hipk2,** *Zfp354a,* **Enpp1**
15	positive regulation of biosynthetic process	14.35813697	41	5.80E-07	**Il22, Arnt2, Sox7, 1190002H23Rik, Bex1,** *Nfib,* **Stat4, Ccl2,** *Bambi,* **P2ry1,** *Bmp2,* **Spic,** *Tgfb2,* **Murc, Pdgfa, Ddah1,** *Cd209b,* **Hmgn3, Ccr7, Tnfsf8, Prkcq, Cdkn1c, Cdh1,** *Six1,* **Pitx1, Mkl2,** *Meox1,* **Kitl, Kdr,** *Pbx1,* **Epas1, Tnfrsf13c, Top2a, Tnni2,** *Pdgfrb,* **Gata3,** *Rfx3,* **Igf1,** *Lgr4,* **Arid5a,** *Wnt6,* **Mcf2l,** *Il6,* **Ltb, Lef1, Cdkn2a,** *Cebpd, Agrn,* **Ptgir, Ercc6, Foxo3, Taf1, Foxp3,** *Fzd1,* **Foxp1, Mef2c,** *Lmo4, Foxf2,* **Cenpk,** *Plcd1, Zeb1,* **Ckap2,** *1500003O03Rik,* **Crem,** *Prdx5,* **Anxa1, Ccna2,** *Hsph1, Runx2, Cdon, Tcf7l2, Sod2, Vdr, Trerf1,* **Eng,** *Abca1, Jak2, Psip1, Fgf10,* **Ddit3, Etv1, Ets1, Cdk1,** *Agap2, Irf1,* **Eaf1,** *Pid1, Nck2,* **Jun,** *Klf7,* **Lyl1,** *Met, Mitf,* **Foxs1, Brca1, Lpin1, Hipk2,** *Abcg1,* **Nr4a2**
			Immune response		
6	antigen processing and presentation of exogenous antigen	17.3317326	7	3.00E-08	*H2-DMb1, H2-DMb2, Fcgr1, Cd74, H2-DMa,* **Fcgr2b,** *Psme1, Psme2*
8	antigen processing and presentation	16.95063302	9	4.30E-08	**Ighm,** *H2-DMb1, H2-DMb2, Fcgr1, Cd74, H2-DMa,* **Mr1, Fcgr2b,** *Psmb8, Psme1, Psme2, Psmb9*
9	immune response	16.04631532	24	1.10E-07	*Tnfsf18,* **Ighm, Il18r1, 1190002H23Rik, Il12b, Ccl2, Kit, Cd8a, C3ar1, Ccr7, Tnfsf8, Ccl7, C1qa, Gpr183, Lat2, Gata3,** *C3, Tnfsf13b,* **Pglyrp1,** *Fcgr1, Il6,* **Ltb, Lef1, Bcap31,** *Susd2, Samhd1,* **Traf3ip2, Cfp, Foxp3,** *Bmi1,* **Lat, Mef2c, Csf1,** *Cd74,* **Anxa3,** *H2-DMa, Bcl6,* **Mr1, Fcgr2b,** *Jak2, Serpinb9,* **Lgals1,** *Cx3cl1, Colec12, C1ra, Irf1, Prkce,* **Tnfsf14,** *Smad6, Bst2, Il1r1, Csf1r, Irf5,* **Enpp1,** *Sema4a*

Supplementary Table 2 (Continued)

Rank	Biological functions (GO processes)	NextBio Normalized score	Common Genes	P-value	Genes (**increased**, *decreased*)
					Other
1	regulation of nitrogen compound metabolic process	28.36746376	96	4.80E-13	**Dach1, Il22, Htr2b,** *Tnfsf18,* **Hdac9, Arnt2, Eya4, Sox7, Zranb3, 1190002H23Rik, Bex1,** *Nfib,* **Arap2, Nlrc3,** *Iqgap2,* **Dmd,** *Celf6,* **Stat4,** *Bambi,* **P2ry1,** *Bmp2,* **Rgs18, Kit, Spic, Murc, Pdgfa, Arhgap15, Ddah1, Hmgn3, Efcab6,** *Ssbp2,* **Ccr7, Tnfsf8, Ppap2b, Prkcq, Cdkn1c, Ptbp2, Cdh1, Adra2a,** *Six1,* **Pitx1, St18, Mkl2,** *Meox1,* **Satb1,** *Maob,* **Kitl,** *Pbx1,* **Epas1, Adam8, Apbb2, Top2a,** *Tsc22d1,* **Pde8b,** *Sall2,* **Aurkb, Fabp4, Tnni2, Hivep2, E2f7,** *Pdgfrb,* **Gata3,** *Rfx3,* **Arap3, Rasgrp3, Tcf7,** *Rgs9,* **Igf1,** *Lgr4,* **Arid5a,** *Kdm4a,* **Trib3,** *Wnt6,* **Mcf2l, Irx2,** *Epha4, Il6,* **Pde2a,** *Tbc1d8,* **Ahcyl1, Ralgapa2, Lef1, Gimap5,** *Dpf3,* **Cdkn2a, Ccnb1, Basp1,** *Apoc1, Cebpd,* **4930547N16Rik,** *Agrn, Zfp827,* **Evi5l, Ptgir, Mlf1ip, Tpm1, Ercc6, Fst, Foxo3, Itgb3bp, Ikzf3,** *Grhl1,* **Taf1, Foxp3,** *Fzd1, Bmi1,* **Mef2c, Foxp1, Rgs1, Sphk1, Rad51, Arhgap22,** *Asap2,* **Bin1,** *Lmo4,* **Cdca7l,** *Sipa1l1,* **Birc5, Tbc1d2,** *Foxf2, Foxn3,* **Ecm1, Cenpk,** *Bcor, Hey1, Tshz1,* **Anxa3,** *Bmyc, Prkcb, Nos3, Trim30a, Six5, Ikbkg, Zeb1,* **Ckap2, Hip1,** *1500003O03Rik,* **Crem,** *Nfe2, Prdx5,* **Mbnl2, Thy1,** *Id3, Usp6nl,* **Smyd2, Ccna2,** *Bcl6, Runx2, Clcf1, Cdon,* **Spry2,** *Tcf7l2, Peli1, Zbtb10,* **Sorbs3, Gtf2ird1,** *Sod2, Vdr, Trps1, Ebf4, Zcchc11, Trerf1,* **Eng,** *Abca1,* **Usp2, Tbc1d20,** *Jak2,* **Arhgap10,** *Aplp1, Cdkn1b, Fgf10, Foxn2, Tle2, Psip1,* **Ddit3, Etv1, Ets1,** *Wtip,* **Cdk1,** *Agap2, Zscan18,* **Prnp, Rasa3, Xrcc5,** *Irf1,* **Eaf1,** *Pid1, Nck2,* **Jun,** *Id1, Klf7, Smad6, Mcts2, Bbx, Zfp710, Map3k9,* **Lyl1, Zfp30,** *Met, Mitf, Rp2h, Tle4,* **Cdca7,** *Mycbp,* **Brca1, Foxs1, Lpin1, Hipk2, Vapb,** *Abcg1, Zfp354a, Ift57, Batf3,* **Nr4a2, Rad18,** *Mmp14, Irf5, Arhgap23,* **Rasal3**
2	regulation of hydrolase activity	24.96102365	40	1.40E-11	**Htr2b, Sox7, Dlg3, Lepr, Serpinb2, Arap2,** *Iqgap2,* **Mefv,** *Bmp2,* **Rgs18,** *Tgfb2,* **Murc, Arhgap15,** *Flt1,* **Ccr7, Serpinb6b, Cdh1, Serpinb8, Cd109,** *Pdgfrb,* **Arap3,** *C3,* **Bok, Rasgrp3, Mastl,** *Dhcr24, Slpi, Rgs9,* **Igf1, F3,** *Epha4, Il6, Tbc1d8,* **Ralgapa2, Lef1, Bcap31, Cdkn2a,** *Serpinb1b, Apoc1, Tfpi, Agrn, Nuak1,* **Evi5l, Tpm1, Casc5, Mef2c, Rgs1, Arhgap22, Myo1d,** *Asap2,* **Bin1, Serpinb1c,** *Sipa1l1,* **Birc5, Tbc1d2,** *Bcl2l11,* **Ecm1, Serpinb1a, Fetub,** *Nos3,* **Hip1,** *1500003O03Rik, Magi2, Prdx5,* **Thy1,** *Usp6nl,* **Bcl2l1,** *Bcl6,* **Spry2, Fn1, Nol3, Tbc1d20,** *Jak2, Serpinb9,* **Arhgap10,** *Cdkn1b, Fgf10, Agap2,* **Rasa3, Tnfsf14,** *Psme1,* **Cd44, Jun,** *Psme2, Smad6, Bst2, Rp2h,* **Lck, Vapb,** *Ift57, Mmp14, Htra2, Arhgap23,* **Rasal3, Cdkn2d**
7	nucleic acid binding transcription factor activity	17.2780382	33	3.10E-08	**Dach1, Arnt2, Sox7,** *Nfib,* **Stat4, Spic,** *Six1,* **Pitx1, St18,** *Meox1,* **Satb1,** *Pbx1,* **Epas1,** *Tsc22d1,* **E2f7, Hivep2, Gata3,** *Rfx3,* **Tcf7, Irx2, Lef1, Cdkn2a,** *Cebpd,* **Foxo3,** *Grhl1,* **Foxp3, Foxp1, Mef2c,** *Foxn3, Foxf2, Tshz1, Hey1, Bmyc, Six5, Zeb1,* **Crem,** *Nfe2, Id3, Runx2, Bcl6, Tcf7l2,* **Gtf2ird1,** *Vdr, Trerf1, Foxn2,* **Ddit3, Etv1, Ets1,** *Irf1,* **Jun,** *Id1, Klf7, Smad6, Tle4, Mitf,* **Foxs1,** *Batf3, Zfp354a,* **Nr4a2,** *Mmp14, Irf5*
11	muscle cell differentiation	14.83487096	15	3.60E-07	**Dok7, Dmd,** *Bmp2,* **Murc,** *Fhod3,* **Uchl1,** *Six1,* **Pitx1, Mkl2, Epas1,** *Sdc1, Pdgfrb,* **Igf1, Mylk2, Lef1, Tmod1,** *Agrn,* **Tpm1, Foxp1, Mef2c, Bin1, Myo18b,** *Unc13b, Snta1, Hey1, Gphn, Cdon, Tcf7l2,* **Lgals1,** *Fgf10, Smad6, Met,* **Krt19**
12	negative regulation of multicellular organismal process	14.79989081	18	3.70E-07	**Adra2c, 1190002H23Rik, Il12b,** *Mylip,* **Mefv,** *Tgfb2,* **Pdgfa, Adra2a,** *Six1, Apln,* **Gata3, Cd24a, Cd59a, Pglyrp1, Bcl2,** *Il6,* **Cyp4f18, Lef1, Gimap5,** *Apoc1,* **Lrpap1, Mmp2, Foxp3, Mgll, Mef2c, Thbd, Ecm1,** *Bcor, Trim30a, Nos3,* **Lgmn, Plau,** *Nfe2, Bcl6, Chsy1,* **Fcgr2b,** *Jak2, Procr, Cx3cl1, Tnnt1,* **Prnp,** *Smad6, Bst2,* **Dusp10, Enpp1**
13	pattern specification process	14.60545866	21	4.50E-07	*Bmp2, Flt1, Six1, Meox1,* **Sema3e,** *Pbx1, Rfx3, Wnt6,* **Lef1, Fst, Foxo3, Foxp3,** *Bmi1,* **Foxp1, Mef2c,** *Foxn3, Foxf2, Bcor, Mfng, Hey1, Tshz1,* **Celsr1,** *Zeb1,* **Kif3a,** *Cdon,* **Eng,** *Foxn2, Fgf10,* **Ddit3,** *Peg12, Smad6,* **Foxs1, Hipk2,** *Aldh1a2*
16	Golgi apparatus	14.35129823	37	5.90E-07	**Fam198b, Serpinb2, Mlana, Ptgs1,** *Flt1,* **Cdh1, Pkdcc, St3gal6, Perp, Pgcp, St6gal1, Galnt9, Igf2r,** *Dhcr24,* **Cd79b, Scamp5, Ndst1,** *Rnd3,* **Tom1l1,** *Epha4,* **Pde2a,** *Lzts1,* **Bcap31,** *St3gal5,* **Mtap6, Lrba, Galnt3, Chst11,** *Rhbdf1, Grhl1,* **Dpp4,** *Pgap2, B4galt4, Asap2, Hp, Cd74, Chst15, Stx5a,* **St6galnac4, Cltb,** *Unc13b,* **Abcb1b,** *Mfng, 1190002N15Rik, Nos3,* **Atp6v0a1, Galnt2, Hip1,** *1500003O03Rik,* **Rhou,** *Galnt10, Tpst1,* **Chac1, Abcb4,** *Sema6d,* **Myo5a, Ugcg,** *Chsy1,* **Acsl3,** *Abca1, Naprt1, Aplp1,* **Slc26a11, Prnp,** *St3gal2, Slc39a14, Prkce,* **Gla, Glipr2, Cd44,** *Bst2, Gal,* **Lck, Gga2, Vapb,** *Abcg1, Ift57,* **Pde4d, Adi1, Dusp10,** *Acacb, 2010106G01Rik, Abcc1*
17	endocrine system development	12.89401386	8	2.50E-06	*Bmp2, Six1,* **Pitx1,** *Pbx1, Pdgfrb,* **Gata3,** *Rfx3, Il6, Tcf7l2, Fgf10*
18	negative regulation of response to stimulus	12.49403671	27	3.70E-06	**Htr2b, Il7r, Il12b, Ccl2,** *Bambi,* **Mefv,** *Bmp2,* **Rgs18,** *Tgfb2,* **Prkcq, Ctla2a, Nt5e, Angpt2, Dact2, Dusp4, Gata3, Arap3,** *Clec2d,* **Cd59a,** *Rgs9,* **Igf1, Bcl2,** *Epha4, Lzts1,* **Lef1, 4930547N16Rik, Lrpap1, Trim59, Fst, Chst11, Foxp3,** *Fzd1,* **Rgs1,** *Htra3, Hp, Cd74,* **Ecm1, Ccnd1,** *Hey1, Prkcb, Trim30a, 1500003O03Rik, Magi2,* **Anxa1, Chac1, Thy1, Bcl2l1,** *Runx2, Bcl6,* **Spry2,** *Tcf7l2,* **Eng,** *Kif7,* **Fcgr2b,** *Tle2,* **Ddit3,** *Wtip, Agap2,* **Rasa3, Prnp,** *Irf1, Pid1,* **Cd44,** *Smad6,* **Dusp8, Hipk2, Dusp10, Enpp1, Rasal3,** *Slc9a3r2*
19	embryo development	12.42265698	30	4.00E-06	**Htr2b, Eya4, Sox7, Sox7, Hba-a1,** *Bmp2,* **Kit, Kit, Spic, Kit,** *Tgfb2, Flt1, Bmp2,* **Ppap2b,** *Flt1,* **Hba-a1, Cdh1,** *Itga4, Six1,* **Pitx1, Mkl2,** *6230427J02Rik, Meox1,* **Spic,** *Flt1,* **Kit, Cdh1, Kitl, Kitl, Kit, Pkdcc, Kit,** *Pbx1,* **Epas1, Cdh1, Cdh1, Kitl, Kitl, Kitl, Kitl, Top2a,** *Itga4, Sall2,* **Kitl, Kitl, Kitl, Dusp4,** *Pdgfrb,* **Cdh1, Cdh1, Gata3, Kitl, Tcf7, Cdh1, Kit, Ndst1, Bub1,** *Wnt6, Itga4,* **Lef1, Epas1, Ccnb1,** *Itga4, Rdh10, Rdh10,* **Tpm1, Foxo3, Chst11, Foxp3, Tpm1,** *Bmi1,* **Mef2c, Foxp1, Dusp4, Myo18b,** *Lmo4,* **Birc5,** *Bcl2l11,* **Ccnb1,** *Lmo4, Tanc2,* **Ccnb1,** *Foxf2, Foxn3,* **Thbd,** *Plcd1, Tshz1,* **Celsr1,** *Nos3, Nos3,* **Abcg2,** *Foxn3,* **Naglu, Mfsd7b,** *Zeb1,* **Abcg2, Foxp1,** *Bmi1, Rdh10,* **Kif3a, Bcl2l1,** *Runx2, Cdon,* **Spry2,** *Tcf7l2,* **Gtf2ird1,** *Runx2, Runx2, Pbx1,* **Hyal1, Eng,** *Runx2,* **Grn, Ndst1, Mfsd7b,** *Runx2, Runx2, Runx2, Fgf10, Foxn2, Nos3,* **Bcl2l1, Bcl2l1,** *Runx2,* **Coq7,** *Peg12, Foxn3,* **Vezt,** *Runx2, Cdon,* **Nek2,** *Runx2,* **Bcl2l1, Coq7,** *Smad6,* **Brca1, Foxs1, Hipk2, Bcl2l1, Krt19,** *Aldh1a2*
20	hair follicle maturation	12.37504306	6	4.20E-06	*Tgfb2, H2-DMb1, H2-DMb2,* **Mreg,** *Fcgr1, Cd74, H2-DMa,* **Myo5a, Fcgr2b**

CHAPTER FIVE

Discussion

COPD places a large burden on society that persists because of the addictive nature of cigarettes and the lack of pharmacological interventions that target the pathogenic aspects of cigarette smoke exposure (214–216). A better understanding of the fundamental cellular and molecular processes elicited by cigarette smoke that drive disease pathogenesis will be crucial to the effective management of COPD. Another critical component for the effective management of COPD is better treatment of disease exacerbations by understanding the mechanisms underlying these episodes. The purpose of this thesis was to understand the mechanisms of cigarette smoke-induced inflammation and the inflammatory mechanisms engaged by bacteria in a smoke-exposed lung. The implicit hypothesis was that an *in vivo* model could identify mediators of cigarette-smoke induced inflammation and that the inflammatory environment of the smoke-exposed lung will alter the inflammatory profile elicited in response to bacterial infection.

Collectively the data presented in this thesis identifies mediators of cigarette smoke-induced cellular inflammation and characterizes the altered phenotype of a lung that is primed to produce exaggerated amounts of those mediators in response to bacteria. This work began with the investigation of a biomarker of the inflammatory process elicited by cigarette smoke and how this biomarker fit into the current understanding of the IL-1 signaling pathway engaged by cigarette smoke. These findings led to the investigation of IL-1 signaling in the context of a bacterial exacerbation of cigarette smoke-induced inflammation, which identified cigarette smoke as predisposing the lung to mount an excessive IL-1α response to bacterial stimuli. The key cell type in this altered response was the AM which was then transcriptomically

characterized by gene array. Collectively, these novel findings elucidate cigarettes smoke's ability to induce inflammation and alter the host response to bacteria

General discussion of the experimental approach

Considerable effort has been placed into the development of a comprehensive model of COPD, but this may prove to be an unattainable goal. COPD is a disease that manifests itself after decades of exposure to risk factors (5, 64). This considerable time period is required for the initial diagnosis of COPD, and even greater amounts of time must elapse before a patient advances to higher GOLD stages of severity. Currently, murine models measure significant airspace enlargement after 6 months of smoke exposure (88), with some groups reporting a measurable increase after 3 months of exposure (129). These changes represent mild disease, similar to GOLD 1 or possibly GOLD 2 (88), but when considering the length of time necessary for a human smoker to experience COPD symptoms, this seems to be a drastic acceleration in disease progression. It is possible that cigarette smoke exerts effects on the human lung, which could be measurable by a histological analysis of lung samples, long before noticeable lung dysfunction is experienced, though this highlights a major limitation of current model systems: There are different standards by which the presence of COPD is assessed in experimental animals versus human patients. In humans, COPD is diagnosed and staged symptomatically, by spirometric analysis of lung function leading to an FEV_1 value. In animal models, lung pathology is assessed by histology and lung function tests that do not generate an equivalent FEV_1. This disconnect implies that the clinically accepted means of diagnosing COPD in a human cannot be applied to an experimental animal and one could argue that there has never truly been a model of cigarette smoke-induced COPD. This is problematic because such differences in the timeline of disease progression and a lack of commonality between the

assessments of the disease allows for the possibility that very different biological mechanisms are engaged in model systems when compared to human patients. As technology improves to better assess the pre-symptomatic stages of COPD in smokers and better evaluate the model of disease in animals, the divide between experimental models and clinical assessment may lessen. Currently the evaluation of lung pathology in a model that incorporates cigarette smoke exposure should be viewed as the proof-of-principle that an experimental system is capable of eliciting pathology that is similar to that observed in a COPD patient, but must be considered in the context of the above listed limitations. The temporal aspect of COPD may also mask other factors which may play a crucial role in lung pathology, as many other comorbidities and pathological events could occur over such a long period of time. Over decades of cigarette smoke exposure, factors such as the frequency and severity of pulmonary infections, exposure to pollution and allergens, the triggering of autoimmunity, and unforeseen aspects of aging may each contribute to or be the necessary trigger for a smoker's lung to begin the degenerative process of COPD. The level of complexity associated with a disease that takes a minimum of 20 years under typical circumstances to manifest could mean that a comprehensive model of COPD may be impossible.

Instead of focusing on an all-encompassing model of COPD, individual aspects of the disease should be modeled, leaving researchers to use the results of these experiments to form a conceptualization of the disease as a whole. This thesis focuses on a biological process that is widely believed to be a significant contributor to the pathogenesis of COPD. The experimental system employed in these studies was not a model of COPD, but a model of cigarette smoke exposure. The conclusions drawn from these findings have implications for COPD, and could one day aid in the development of novel therapeutic strategies for the disease, but the purpose

was to further the understanding of the biology of inflammation in the context of cigarette smoke. This is a simplification of the disease by isolating one key aspect, and there are important considerations when modeling the cigarette smoke-induced inflammatory response.

The first consideration in modeling cigarette smoke-induced inflammation in mice is the method and amount of cigarette smoke exposure. As stated in Chapter one, there is no established dose or protocol that is accepted as an equivalent reproduction of the smoke exposure experienced by a human smoker. For the purposes of this thesis, an inflammatory response that involved the accumulation of neutrophils in the lungs was the desired outcome, and the SIU84 Promech smoke exposure machine is capable of eliciting a neutrophilic response in mice. Though some neutrophils can be counted in the BAL of mice after just two days of smoke exposure, time course experiments in our lab established that this neutrophilia is reliably observed after four days (217). This formed the standard protocol utilized in our lab to study cigarette smoke-induced inflammation. To keep the dose of cigarette smoke consistent, the TPM was measured every week and a dose of 600 μg/L was necessary to maintain the neutrophilic phenomenon (95). Though the focus of these experiments was the inflammatory cell profile in the lungs, other measurements conducted during the initial characterization of the smoke exposure system provided confidence that this was a clinically relevant dose of cigarette smoke. Carboxyhemoglobin, an indicator of the saturation of hemoglobin with carbon monoxide, and cotinine, a breakdown product of nicotine, were measured in the serum of smoke exposed mice collected immediately after smoke exposure and found to be comparable to levels that would be expected in human smokers (99). For these reasons, we feel an appropriate dose of cigarette smoke for eliciting a response similar to human smokers was chosen.

In the past, there have been concerns between the limitations of whole-body smoke exposure in comparison to a nose directed system. The whole-body method is preferable because of the number of animals that can be smoke exposed without the need for individual restrainers. A criticism of whole body smoke exposure is that cigarette smoke condensate is deposited on the fur of mice and is subsequently removed by grooming practices and ingested (218). This is a spurious argument as cigarettes are placed in the mouth of human smokers as they are inhaled, and almost the entirety of the mainstream tobacco smoke passes through the oral cavity. It is reasonable to assume that the condensate that would be deposited on the mouse fur would also be deposited in the oral cavity of humans and swallowed. Though a whole-body exposure system does not completely reproduce the means by which humans smoke, we feel confident that the same components of cigarette smoke are being delivered to the mice.

Exposure to cigarette smoke and placement in a restrainer for the duration of exposure are potentially stressful experiences for a mouse and the psychological stress response could potentially affect the biological responses studied in this thesis. Stress has been shown to amplify inflammation in lungs (219), and chronic stress suppresses microbial host defense mechanisms (220). To assess the potential impact of psychological stress in our experimental model, the hormone corticosterone was measured in the serum of mice within an hour of smoke exposure (99). Corticosterone levels were within the normal range (50-300 ng/mL), and the levels of corticosterone were not significantly different between smoke-exposed mice and their respective controls. As a further measure to lessen the effects of psychological stress in our findings, mice were acclimatized to the smoke exposure restrainer in room air for three days prior to the onset of smoke exposure. It is for these reasons that it is unlikely that psychological stress affects the results generated in this smoke exposure system.

Chapter one discussed the importance of selecting the proper model species, which provided the justification for pursuing studies in mice; however, the selection of the strain of mouse is another important consideration as strain specific effects have been observed. The differences in cigarette smoke-induced inflammation and lung pathology between mouse strains have been attributed to naturally occurring variations in the level of antioxidants and anti-proteases across strains (221). Strain specific effects seem to be related to the level of damage that cigarette smoke is able to exert on the lungs, as experiments found that mouse strains which have higher levels of DAMPs after smoke exposure mount a greater inflammatory response (118). This study found that BALB/c mice mount the greatest neutrophilic response to cigarette smoke, and the four day smoke exposure model utilized for this thesis came to the same conclusion. In chapter two, the inflammatory cell profile of an outbred stock of mice was shown, and these CD1 mice mount an even greater neutrophilic response than BALB/c mice. Unfortunately, because CD1 mice are outbred, there are no reagents specific to their genetic background and further experiments were not pursued with these mice. C57CL/6 mice do not elaborate the same degree of neutrophilic inflammation as BALB/c mice and their cellular response to cigarette smoke is more monocyte/macrophage dominant. Interestingly, this same trend is observed in the smoke and NTHi model where the exacerbated inflammation from the combined stimuli is more neutrophilic in BALB/c mice while the response is more monocyte-dominant in C57BL/6 mice. These data would suggest that the best strain of mice to utilize for the experiments for this thesis would be BALB/c mice. Unfortunately, with the exception of BRP-39 and CXCR2 KO mice, all of the genetic deficient mice needed were only available on the C57BL/6 background. Experiments were pursued in both strains of mice as the

inflammatory response was more clearly demonstrated in the BALB/c strain, but reagents were more available for C57BL/6 mice.

Another strain difference was observed in the accelerated clearance of NTHi from the cigarette some-exposed lung. In chapter 3, decreased NTHi burden was observed in smoke-exposed BALB/c mice when compared to infected room air-exposed controls, but this decrease was not observed in C57BL/6 mice. This differed from previous studies from our lab which demonstrated this phenomenon in both of these mouse strains (202, 212). This new strain specific phenotype is likely unrelated to genetic factors, though genetic drift in mouse strains are a consideration (222), there was likely not enough time elapse for this to be the cause. Enhanced bacterial clearance was found to be related to cigarette smoke inducing the production of NTHi specific antibodies in the lung (212). Antibody production can be influenced by a wide array of factors, even the composition of commensal bacteria in the gut can enhance protection from microbial infections (223, 224). In the studies completed for this thesis, NTHi specific antibodies were not assessed. Bacterial clearance was not the primary focus of this research, but rather a readout that gave clues to the role of increased inflammation in effective host defense, so the underlying cause of this strain difference was not investigated. The more interesting observation was that in cases where the exacerbated inflammatory response was inhibited, the bacterial burden increased dramatically, and this phenomenon was observed in both BALB/c and C57BL/6 backgrounds of mice.

There are limitations to every experimental approach, but by being conscious of the limitations of a specific system, meaningful results can be generated. The studies completed for this thesis were interpreted from this perspective, and all attempts were made to control for any experimental artifacts.

General discussion of cigarette smoke-induced inflammation

Analysis of the experiments completed for this thesis has yielded a unique perspective on the inflammatory response engaged during cigarette smoke exposure. This section will focus on the distinctive aspects of this inflammatory response in comparison to other inflammatory stimuli, while highlighting how the currently presented data fit within the dominant theories about cigarette smoke-induced inflammation and its role in disease.

Cigarette smoke exerts its damaging effects over a long period of time, yet cigarette smoke is capable of eliciting an inflammatory response after a few days of smoke exposure in our experimental model. Four days of smoke exposure was utilized for the majority of experiments in the second and third chapters that specifically examined the effects of cigarette smoke without additional stimuli. This differs from the approaches of other studies where inflammation is often assessed after six months of exposure. A six month time point is typically chosen to correspond with pathological assessment of lung tissue, as this length of exposure is required for measurable changes in lung pathology. The experiments in chapters two and three were concerned with the inflammatory response and not the resultant pathology, so a shorter smoke exposure period was chosen. There are some differences in the inflammatory profile of the lungs when comparing short and long term smoke exposure: The neutrophilic response peaks at four days in C57BL/6 mice before settling into an equilibrium at later time points (99). This phenomenon was present but less pronounced in BALB/c mice. There is also an accumulation of lymphocytes that organize into aggregates throughout the tissue in chronically smoke-exposed lungs (225). The role of these lymphoid aggregates in propagating inflammation or mediating pathology is currently unknown. Additionally, Houghton *et al* has demonstrated that the destruction of the lung provides additional inflammatory stimuli as components of the

ECM like elastin are capable of contributing to the inflammatory response (226). This implies a positive feedback cycle between lung destruction and inflammatory signaling that may only be present in the context of chronic smoke exposure where the destruction of the lung has already begun. This evidence suggests that the inflammatory profile of the lungs changes over the duration of smoke exposure. Findings made in a four day model are still relevant; however, it is important to test these findings in a more chronic smoke exposure setting. For the critical experiments completed in chapter two and three, such as the IL-1 family member KO experiments, the findings were confirmed in mice that were smoke exposed for longer time periods, but for brevity's sake, these data were not shown.

Cigarette smoke-induced inflammation is complicated by its insensitivity to well established anti-inflammatory therapeutics. This insensitivity to current medications is a major motivating force behind efforts to identify novel therapeutic targets, such as those identified in this thesis. Corticosteroids that suppress immune responses by binding the glucocorticoid receptor (GCR) are currently the cornerstone of anti-inflammatory treatment, but these drugs are largely ineffective in suppressing the inflammatory response elicited by cigarette smoke. A mechanism of steroid resistance has been identified as cigarette smoke leads to a hyperphosphorylation of HDAC2 that marks this protein for proteasome degradation (227). The GCR uses HDAC2 to deacetylate the histones of pro-inflammatory genes, making the genes inaccessible to transcriptional machinery and stopping their expression (228). In attempting to overcome this steroid insensitivity and suppress cigarette smoke-induced inflammation, there are two approaches: Correct the defective biology underlying steroid resistance and treat with corticosteroids, or bypass steroid strategies altogether and directly target critical components of the inflammatory response. Steroid sensitivity has been restored in animal models of cigarette

smoke exposure by increasing HDAC2 expression (227, 229), but a simpler therapeutic strategy that targets a key mediator of the inflammatory response could avoid the complications of treating with multiple therapeutic agents to achieve the same outcome. Steroid treatment was attempted by gavage at varying doses in the SIU48 smoke exposure system during the course of the experiments completed for this thesis. These findings concluded that the inflammation induced by four days of smoke exposure was not affected by even relatively high doses of the steroid dexamethasone. These findings provide additional relevance to the results of this thesis, as the data presented, and therapeutic targets identified, are generated in a model of *steroid insensitive* inflammation induced by cigarette smoke.

Attempts have been made to characterize cigarette smoke by comparing it to other inflammatory stimuli. Upon the initial findings that cigarette smoke induced inflammation through a TLR4/MyD88 dependent mechanism, a hypothesis that cigarette smoke could be considered as a sort of aerosolized TLR4 ligand was postulated. There is likely some merit to this hypothesis, as analysis of the composition of cigarette smoke has shown that it contains LPS and fungal components (230). Others, including the authours of the original paper, downplay the role of LPS contained within cigarette smoke as the ligand that drives the inflammatory response (121). They instead consider TLR4 to be a sensor of cellular damage, and hypothesize that the relevant TLR4 ligand is the DAMP HSP 70. Currently, no study has followed up on this hypothesis and it is unclear what the relevant TLR4 ligand is in the context of cigarette smoke-induced inflammation; there may even be multiple TLR4 ligands that contribute to the response.

The term sterile inflammation is often used to describe an organism's response to the products of cell damage and death, as this response can occur in the absence of any microbial stimuli. While accepting the caveat that cigarette smoke contains microbial components,

involvement of sterile inflammatory mechanisms makes sense given cigarette smoke's cytotoxic properties. The findings presented in chapter three identify the importance of the IL-1α/CXCL5 signaling axis in our model. This is very similar to the inflammatory response to cell death, as the importance of IL-1α and CXCL5 was demonstrated in a hydrocarbon oil-induced cell necrosis model (231). IL-1α has been identified as a crucial mediator of sterile inflammation in other studies as well (164, 232, 233); however, it is believed that the mechanisms of IL-1α-mediated inflammation is the release of intracellular stores of IL-1α upon necrotic cell death. This differs from the findings of chapter three which show that IL-1α is significantly increased on a transcriptional level by cigarette smoke exposure in AMs and primed to be further increased in response to subsequent stimuli. These data suggest that while IL-1α is passively released in models of sterile inflammation, in the context of cigarette smoke it is actively being produced to increase its level of expression. The exact implications of this active versus passive release of IL-1α are unclear; regardless, this is an important distinction that implies that cigarette smoke-induced inflammation does not fit completely within the context of a sterile inflammatory response.

Despite similarities to other stimuli, the inflammation elicited by cigarette smoke is unique, engaging a distinct combination of biological pathways. Attempting to classify it in the context of other stimulators of inflammation is an interesting academic exercise, but threatens to blind researchers to the subtle differences that comprise this process.

The reliance of the pulmonary environment on IL-1 signaling to mount an inflammatory response to cigarette smoke has been reproduced and published by at least four labs in three different countries (120, 157, 162, 163). The robust nature of this IL-1-dependency has been established, but it is a somewhat unexpected phenomenon when considering the nature of

cigarette smoke. As stated previously, cigarette smoke is a complex stimuli consisting of thousands of chemicals, reactive oxygen species, TLR ligands, and particulate matter which stimulate the production and release of many gene products (63, 230, 234), yet the inflammatory response elicited by cigarette smoke is dependent largely on a single receptor, IL-1R1. This finding is additionally impressive given the aforementioned inter-laboratory variability with respect to cigarette smoke and inflammatory data. Deficiency in IL-1 signaling effects more than monocyte and neutrophil recruitment as our lab had identified a role for IL-1 in the recruitment of DCs and the formation of lymphoid aggravates (206, 225). An important caveat to consider is that all of the data published to date has established IL-1 as crucial for the initiation of the inflammatory response and not the ongoing propagation of inflammatory processes. Even studies that blocked IL-1R1 ligands with antibody administration did so prophylactically (162), and currently no study has attempted to assess how blocking IL-1 signaling affects inflammatory cell recruitment in a smoke-exposed lung where the inflamed state has already been established. The results of the work generated in this thesis contribute to the growing evidence that the dependency on IL-1 to mount an array of responses to cigarette smoke is a highly reproducible phenomenon despite the complexity of cigarette smoke, and should be a focal point of future research in this field.

During the course of the experiments that examined the role of IL-1 in cigarette smoke induced inflammation, attempts were made to identify other novel components of this inflammatory response. These endeavors led to the examination of the chitinase-like protein BRP-39, which seemed promising due to its role in other forms of pulmonary inflammation and its induction in the lungs of smokers (169, 171). Through the use of BRP-39 KO mice, we concluded that BRP-39 was not involved in the cellular recruitment processes engaged in a

smoke-exposed lung, however, this seemed to be contradicted by a similar paper by Matsuura *et al* that observed less macrophages and neutrophils in the lungs of BRP-39 KO mice cigarette smoke-exposed for three and six months (235). The smoke exposure system utilized by Matsuura *et al* was found to induce inflammation in an IL-18 dependent manner (79, 159), but the BRP-39 deficiency had no effect on IL-18 expression. Realizing that these data suggested that BRP-39 may not be involved in cell recruitment, the authours investigated the role of BRP-39 as an anti-apoptotic agent, and found that in the absence of BRP-39 there is increased apoptosis of monocytes/macrophages, neutrophils and cells of the epithelium (235). The cumulative effects of the increased apoptosis was the observation of less inflammatory cells in the lung lumen, but without the protective effects of BRP-39, the formation of emphysematous lesions was accelerated. Our data complements this study, by assessing inflammation at a more acute time point, where the decreased survival of inflammatory cells had not yet had sufficient time to affect the cellular profile of the lung. The four day smoke exposure model confirms the findings of Matsuura *et al* by demonstrating more succinctly that BRP-39 is not required to recruit monocytes and neutrophils to the lungs, despite the necessity of BRP-39 in the survival of these cells. Additionally, we expanded the knowledge of BRP-39 biology by identifying IL-1 as the mechanism behind the increased BRP-39 expression in response to cigarette smoke. By considering the data generated in these two studies, an interesting conclusion can be drawn: IL-1 appears to have the capacity to activate mechanisms that protect the lung form cigarette smoke-induced apoptosis, through a BRP-39 dependent mechanism.

There is a well-established relationship between inflammation and the pathogenesis of COPD, but symptomatic COPD only develops in at most 50% of smokers (236). GOLD defines COPD as arising from an *abnormal* inflammatory response to noxious gases (5), but the

characteristics that differentiate an abnormal from a normal inflammatory response have not been defined. Genetic analysis of COPD patients and analysis of the lung microbiome have sought to identify predispositions to the development of COPD (237, 238), but these efforts have yet to reach a consensus on exact mechanisms of this predisposition. It is even possible that susceptibility to the direct apoptotic effects of cigarette smoke may be more important that the inflammatory response in triggering the onset of COPD (191), though this is an area of current debate. The data presented in this thesis has helped elucidate the inflammatory response to cigarette smoke, but much subtlety remains in determining how newly identified pathways differ between individuals who are susceptible to the development of COPD and those who are resistant.

General discussion of bacteria-exacerbated inflammation

The study presented in chapter three represents the first time that a mechanism has been described for the exacerbated inflammatory response observed during a bacterial infection of a cigarette smoke-exposed lung. This new understanding of cell recruitment is contributing to changing theories about the nature of the smoke-exposed lung.

When IL-1R1 KO mice were found to have a significantly abrogated inflammatory response to cigarette smoke, it was assumed that the relevant IL-1R1 ligand was IL-1β (157). This could largely be attributed to the focus given to IL-1β in inflammation research because of the recently discovered phenomenon of IL-1β processing, by a proteolytic complex known as the inflammasome, to become biologically active (239). Data did not support the assumed importance of IL-1β and subsequent studies proved that the relevant ligand was actually IL-1α (162, 163). These data concluded that the IL-1β and the inflammasome were not a significant component of cigarette smoke-induced inflammation. This IL-1α dependency could not be

assumed for exacerbations of cigarette smoke-induced inflammation and upon discovering an IL-1R1 dependency in the NTHi-exacerbated response; both IL-1α and IL-1β were examined. Though the data were not shown in a publication, the inflammasome was directly assessed in this model as well. Mice deficient in apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), a key component of the inflammasome, were taken through the smoke exposure and NTHi infection protocol. Supporting the findings indicating that IL-1α is the important ligand in the exacerbated response, this experiment concluded that the inflammasome was not involved in NTHi exacerbation of cigarette smoke-induced inflammation. Interestingly, IL-1α KO mice did not elaborate the same degree of abrogated neutrophilia as IL-1R1 KO mice in this model. Cytokine measurements revealed that in the absence of IL-1α, IL-1β levels were significantly increased. Though IL-1β is redundant in the inflammatory response examined in this model, there may be a compensatory role for IL-1β in the absence of IL-1α. These results can be expanded upon in future studies, but this potential compensation mechanism could complicate results of experiments that target IL-1α. Collectively, these data help shift the focus away from IL-1β and the inflammasome in the field of cigarette smoke research and reinforce the importance of IL-1α.

A novel finding that emerged from characterizing the exacerbated inflammatory response in the cigarette smoke and NTHi model was the perceived importance of CXCL5. We did not examine CXCL5 directly, but experiments would have been pursued in CXCL5 KO mice if they were commercially available, and currently CXCL5 has not been directly targeted in any model of cigarette smoke exposure with or without additional microbial stimulus. Instead, we demonstrated the importance of CXCR2 in the exacerbated neutrophilic response and measured all of the identified CXCR2 ligands. CXCL5 was the only ligand that seemed to correspond with

the levels of neutrophilia observed in each experimental group and it was the only CXCR2 ligand that was significantly reduced along with neutrophilia in IL-1R1 deficient mice. These data are of interest because CXCL5 has been observed to be increased in the epithelium of COPD patients during exacerbations (240), and CXCL5 has been shown to be capable of mediating lung pathology as CXCL5-secreting pulmonary epithelial cells were shown to drive destructive neutrophil inflammation in a model of tuberculosis infection (241). There are multiple CXCR2 ligands, and this raises the question as why one CXCR2 ligand would be more relevant than the others. A recent study of CXCL chemokines in the uroepithelium has demonstrated that different CXCL chemokines are responsible for migration at the endothelial and epithelial sites (242). The relative importance of distinct CXCL chemokines at different areas of the lung tissue have not been investigated, but CXCL5 may be such an important chemokine because of its specific expression at the epithelium. CXCL5 may be the 'gate keeper' chemokine that mediates the migration of neutrophils across the epithelium and into the lumen of the lung. This hypothesis is speculative as CXCL5 is not extensively studied in respiratory disease, but increasing evidence suggests this may be a critical chemokine in pulmonary neutrophil recruitment and should be the focus of future studies.

A question that has often arisen from the interpretation of this research is whether the exacerbated response to bacteria in a smoke-exposed lung is beneficial or detrimental to the host. As stated many times previously, the products of neutrophils and macrophages are key mediators of lung destruction that lead to the formation of emphysematous lesions. Intuitively, one would expect a significant increase in the number of these leukocytes to mediate even greater damage to the lung. Measurements of lung function performed in chapter three confirm the additive effects of additional inflammatory cells in creating significantly greater lung dysfunction. These

data correspond with the clinically observed accelerated decline of lung function that correlates with increased frequency of infectious exacerbations in COPD patients (46). These data would indicate that the exacerbated inflammatory response has a negative effect on the host, leading to greater lung pathology. However, with respect to the bacterial burden, the exacerbated neutrophilia may be necessary to resolve the infection. This is evident as the levels of NTHi are significantly greater in smoke-exposed KO mice that have attenuated inflammation. Aspects of bacterial host defense, such as the phagocytic ability of macrophages, are suppressed by cigarette smoke (204). It is reasonable to postulate that the exaggerated neutrophilic response is an attempt to compensate for other aspects of host defense that are defective in a smoke-exposed lung. The exacerbated inflammatory response of a smoke-exposed lung appears to be a necessary reaction to control the bacteria, at the cost of increased lung pathology. These data suggest that any anti-inflammatory therapy aimed at reducing lung destruction must also take into account measures to control bacteria. Current guidelines state that steroid treatment of exacerbations of COPD must be accompanied by antibiotics, for these reasons (5, 243).

These studies identified a signaling pathway that includes IL-1α and CXCL5 in an exaggerated neutrophilic response to bacteria. This response typifies the double edged sword analogy that is often used to describe immune responses as it has both positive and negative consequences for the smoke-exposed lung. These findings provide an initial frame work for understanding the mechanisms engaged by bacteria in a smoke exposed lung, and may inform future strategies for the management of bacterial infections in chronically inflamed lungs.

Discussion of the altered AM phenotype, and potential mechanisms

The findings of chapter three not only established the role of IL-1α in the NTHi exacerbation of a cigarette smoke-inflamed lung, but also identified that the source of IL-1α was an AM primed to

produce exaggerated levels of this cytokine. Cigarette smoke fundamentally changes the pulmonary environment, and altering the phenotype of lung resident cells is a part of that change. For example, the stress that cigarette smoke exerts on pulmonary epithelial cells induces a state of autophagy, affecting their key physiological functions (187). Additionally, the ability of cigarette smoke to suppress AM function has already been documented (201, 204), and skewed inflammatory mediator production in smoke-exposed macrophages has also previously been described (202). The data generated in this thesis identified the smoke-altered AM as a key mediator in bacterial exacerbated neutrophilia and chapter four sought to fully characterize how cigarette smoke changes the AM's response to bacterial stimuli.

Transcriptomic analysis of NTHi-stimulated AMs from cigarette smoke-exposed mice identified many inflammatory mediators that were differentially expressed because of the effects of cigarette smoke on the AM's response to bacteria. Unfortunately for the sake of experimental continuity, IL-1α was not one of these differentially expressed genes. Analyzing microarrays with a high degree of stringency can make the results prone to Type II errors (244, 245), and although there are various statistical methods to decrease the likely hood of false negatives, there is no way to completely correct this issue. Type I and Type II errors are typically controlled for in microarray studies by performing additional experiments to determine the specific expression of the genes of interest by real time qPCR. The expression of IL-1α was observed to be significantly increased in NTHi-stimulated AMs from smoke-exposed mice by qPCR and protein expression in the previous study. It is reasonable to conclude that IL-1α is one of the NTHi-induced inflammatory mediators that is differentially expressed due to cigarette smoke, but that this expression is not identified by the microarray because of Type II error.

The specifics as to how cigarette smoke alters the phenotype of AMs are currently unknown, but clues are provided by other studies that examined unique macrophage phenotypes. With respect to cigarette smoke altering the phagocytic ability of AMs, oxidized phospholipids generated by cigarette smoke exposure have been identified as the causative agent (246). Additionally, oxidized phospholipids have been shown to suppress TNF-α production in cultured macrophages stimulated with LPS (247). These data would indicate a role for oxidative modifications in the altered AM phenotype, but further studies would be necessary to determine how much of this process can be attributed to exposure to oxidized phospholipids. Another possibility is the engagement of mechanisms similar to those observed in the process of LPS tolerance. Macrophages that are repeatedly stimulated with LPS adopt a phenotype of unique gene expression where a subset of genes are not activated by the secondary LPS challenge (248). The LPS-tolerant phenotype of a macrophage has been shown to result from epigenetic gene regulation, as the acetylation and methylation of histones are responsible for the suppressed transcription of inflammatory genes observed after the second LPS challenge. Cigarette smoke has been demonstrated to both activate and suppress different histone deacetylases (187, 227), affecting gene transcription. This provides evidence that cigarette smoke could alter the AMs through changes to epigenetic gene regulation mechanisms. This is an attractive hypothesis because of the knowledge that TLR4 is being repeatedly engaged by the continuous stimulation of cigarette smoke (120), similar to the *in vitro* model used to study LPS tolerance, but with greater frequency. However, epigenetic changes in cigarette smoke-exposed macrophages have currently not been assessed. There may be a link between epigenetic modifications and oxidative changes as treatment with the antioxidant curcumin prevented HDAC2 loss in an in vitro cigarette smoke extract culture system (249).

The analysis of chapter four's microarray data with Ingenuity Pathways Analysis software allowed for the prediction of upstream regulators based on the pattern of gene expression observed. This allowed us to make predictions about the possible mediators of the altered AM phenotype directly from the data set. All of the identified soluble mediators from that list were growth factors and the strongest scoring gene was GM-CSF. We and others have observed GM-CSF to be increased by cigarette smoke and to play a role in signaling the inflammatory response (147, 148). GM-CSF plays a crucial role in shaping the phenotype of a macrophage as indicated in the assessment of inflammatory mediator expression in cultured macrophages (250), suggesting a key role for GM-CSF in the altered AM phenotype. Focusing on a single gene product may be misleading though, as many aspects of the cytokine environment of a smoke exposed lung may contribute to the observed AM phenotype. For example, CCL2 is a proinflammatory chemokine associated with monocyte recruitment that is induced by cigarette smoke and dramatically increased by the combined stimulus of cigarette smoke and NTHi in AMs (202). CCL2 has recently been shown to play more than a proinflammatory role as it shapes the macrophage's polarization by GM-CSF (251). With respect to the cytokines and growth factors that shape the phenotype of a smoke-exposed AM, there are likely multiple factors that contribute to varying degrees.

Much ambiguity about the function and relevance of the altered AM phenotype remain. Studies in our lab indicate that this phenotypic change could be evident after just four days of cigarette smoke exposure. This would indicate that this altered phenotype is present in all smokers regardless of a COPD diagnosis, potentially explaining why smokers experience more severe responses to infectious agents (252). Additionally, it is unclear how long the altered phenotype persists after smoking cessation, or whether dividing lung-resident macrophages

maintain the altered phenotype in their daughter cells. The identification of growth factors as a potential mechanism of this altered phenotype again raises the recurring theme that many of the changes associated with a smoke-exposed lung are the result of the damaging effects of this insult. These data suggest that the altered AM phenotype is the product of a pulmonary environment producing growth factors in an attempting to resolve inflammatory processes and initiate healing. This process is then further complicated by the continuous exposure to inflammatory stimulus. The data presented in this thesis indicated that the altered AM plays an active role in lung disease, and the characterization of this phenotype will inform future studies that will further elucidate the role of AMs in cigarette smoke-mediated disease.

Translation to human disease

Ultimately, the aspiration of any research that seeks to elucidate the biological processes involved in the pathogenesis of disease is for these results to be involved in the generation of novel therapeutic strategies. The work of this thesis provided additional detail to the role of IL-1 signaling in cigarette smoke-induced inflammation and bacterial exacerbation of that inflammatory response. Targeting IL-1 in COPD patients may prove beneficial in alleviating symptoms caused by the inflammatory response and help to suppress the generation of additional pathology.

Translating research findings from smoke-exposure models into treatments for COPD patients has been difficult. One of the first inflammatory mediators identified in the response to cigarette smoke was TNF-α (92) . TNF-α KO mice were protected from 70% of the emphysema formation in a smoke exposure model, but subsequent clinical trials with the drug infliximab, a TNF-α blocking monoclonal antibody, showed no positive benefit for COPD patients (172, 173). A consideration of the variability in responses to cigarette smoke observed between

different murine models may provide insight into this discrepancy. Chapter two demonstrated that the neutrophilic response in our smoke exposure system did not require IL-18. This was at odds with findings by Kang *et al*, who observed IL-18 to be a crucial component of neutrophil recruitment in their smoke-exposure system (159). This is an example of the inter-laboratory variability discussed in chapter one, and some could view this as a severe limitation in the ability to model an appropriate inflammatory response to cigarette smoke. However, the variability in the dose and type of smoke exposure systems is likely reflective of the variability between the smoking habits of human smokers. There is also likely additional variability in the response of humans to cigarette smoke due to the genetic diversity of this outbred population. This suggests that different inflammatory mediators may be more relevant in different individuals. Subtyping COPD patients based on their inflammatory mediator profile is similar to recent findings in allergic asthma clinical trials where therapeutics that targeted IL-5 initially were found to be ineffective (253). Subsequent trials found IL-5 therapy to be effective in cases of severe asthma (254–256), prompting the opinion that respiratory diseases should be more extensively phenotyped in order to properly assess novel treatments (257). If the variability between animal models of smoke exposure is evidence of the variability of responses in humans, the anti-TNF-α therapy may be effective and characterizing the inflammatory response in COPD patients may be necessary when pursuing future clinical trials.

The data generated regarding IL-1 and cigarette smoke-induced inflammation is beginning to be translated into the clinical setting. According to the National Institute of Health's listing of clinical trials, a phase II double-blind placebo-controlled study of MEDI8968, a monoclonal antibody that targets IL-1R1, has recently been concluded (258). The results of this trial have yet to be reported, but it focused on the rate of exacerbations experienced by

COPD patients over a 12 month period of treatment. This clinical focus on exacerbation was because of the identified importance of IL-1 signaling in viral exacerbation (162). This thesis provides additional data about the importance of IL-1 during bacterial exacerbations, and further justifies the use of anti-IL-1 therapy in the prevention of infectious exacerbations of COPD.

Future directions and final thoughts

Knowledge about the inflammatory response to cigarette smoke is continuously expanding, but many questions remain. Currently the literature has focused on cigarette smoke in isolation of other stimuli, so there is still much ambiguity about the combined stimulus of smoke and bacteria. These unknown areas include the involvement of the adaptive immune response. Th17-mediated neutrophil recruitment has been demonstrated in a chronic smoke exposure system (132), but it is unknown how this response contributes to a bacterial exacerbation. Additionally, the experiments pursued in this thesis only utilized NTHi as a model bacteria, but other species of bacteria have been identified as causative agents of COPD exacerbations (259). It is important to confirm experimental findings in multiple bacteria species to evaluate how applicable findings are to various infectious agents. The NTHi infection in our *in vivo* model was also given at a time point when there was likely no measurable destruction of the lung architecture. The highly damaged nature of a COPD patient's lung likely has profound effects on their ability to clear bacteria. A series of experiment that incorporated either very long term smoke exposure, or smoke exposure in a lung damaged by proteolytic enzyme administration, with a bacterial infection could provide insight into the bacterial host defense of a patient with severe COPD. These are only a few examples of the many future research projects with the potential to build on the findings presented in this thesis.

In conclusion, there are many subtleties to the data generated in this thesis but the key findings of these studies have:

- Determined the redundancy of the biomarker BRP-39 in the inflammatory response elicited by cigarette smoke
- Provided additional detail elucidating the crucial IL-1 signaling pathway in cigarette smoke-induced inflammation
- Investigated the role of IL-1 signaling in a model of bacterial exacerbation of cigarette smoke-induced inflammation.
- Provided the first transcriptomic characterization of the cigarette smoke-altered AM phenotype in the context of a bacterial infection

Cigarette smoking will continue to be a major health issue and continue to be a leading cause of global morbidity and mortality. The data generated in this thesis has expanded the understanding of the biological processes engaged by cigarette smoke, with the potential for this data to inform future research and the development of novel therapeutic strategies.

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