RelB regulation of cigarette smoke-induced pulmonary inflammation

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LIST OF ABBREVIATIONS

Abbreviation	*Meaning*
TNF	tumor necrosis factor
IL	interleukin
ICAM-1	intracellular adhesion molecule 1
TLR	toll-like receptor
PAMP	pathogen associated molecular patterns
LPS	lipopolysaccharide
TSLP	thymic stromal lymphoprotein
COX	cyclooxygenase
PG	prostaglandin
TGF-β	transforming growth factor β
COPD	Chronic obstructive pulmonary disease
ROS	reactive oxygen species
RNS	reactive nitrogen species
H_2O_2	Hydrogen peroxide
NOX2	NADPH oxidase 2
IBD	Inflammatory bowel disease
NO	Nitric oxide
SP	surfactant
DC	dendritic cells
APC	antigen presenting cells
MHC	major histocompatibility complex
CCL	C-C motif ligand
PRR	pathogen recognition receptor
IFN	interferon
Ig	immunoglobulin
TRPC	transient receptor potential channel
MPO	myeloperoxidase
MMP	matrix metalloproteinase
ECM	extracellular matrix
NET	neutrophil extracellular traps
Th	T helper
Treg	T regulatory
TRAF	TNF receptor associated factor
MAPK	Mitogen activated protein kinase
NF-κB	Nuclear factor κB
HSF	hepatocyte-stimulating factor
BCSF	B cell stimulatory factor
BCDF	B cell differentiation factor

Abbreviation	*Meaning*
gp	glycoprotein
NK	natural killer
CXCL	C-X-C motif ligand
Gro	growth regulated oncogene
KC	keratinocyte chemoattractant
MIP	macrophage inflammatory protein
MCP	monocte chemoattractant protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GDP	gross domestic product
WHO	World Health Organization
CSE	cigarette smoke extract
RSV	respiratory syncytial virus
CL_2	Chlorine
OVA	ovalbumin
LBP	LPS binding protein
CD	cluster differentiation
Myd88	myeloid differentiation response gene 88
IκB	Inhibitor of NF-κB
IKK	IκB kinase
NIK	NF-κB inducing kinase
BAFR	B-cell activating factor receptor
LβTR	lymphotoxin β receptor
RANK	receptor activator nuclear factor-κB
TWEAK	TNF-like weak inducer of apoptosis
BMC	bone marrow chimera
CS	Cigarette smoke
MIP	macrophage inhibitory protein
TARC	thymus and activation-regulated chemokine
Nqo1	NADPH dehydrogenase quinone 1

ABSTRACT

Inflammation is a response to injury and infection. Although protective under physiological conditions, excessive and persistent inflammation is linked to disease. As the lungs are continuously exposed to the external environment and subjected to injury by irritants, it is particularly liable to inflammation. RelB is a member of the non-canonical NF-κB pathway that may control lung inflammation and cell death caused by cigarette smoke (CS), a potent irritant and a leading cause of morbidity and mortality around the world. Our lab has previously shown that RelB protects against CS-induced apoptosis and inflammation *in vitro*, but less is known about pulmonary RelB *in vivo*. We hypothesized that RelB protects against acute CS-induced pulmonary inflammation. To investigate our hypothesis, we exposed wild-type ($RelB^{+/+}$) and RelB-deficient mice ($RelB^{-/-}$) mice to CS using an acute full-body regime. We found that CS-exposure caused a sustained decrease in airway granulocytes in $RelB^{-/-}$ mice; this decrease in granulocytes was predominated by neutrophils. Pulmonary inflammation caused by other irritants, including chlorine, ovalbumin (to mimic features of asthma) and lipopolysaccharide was unaffected by RelB. Differential cytokine analysis via multiplex assay suggests that alterations in chemotactic cytokines do not fully account for the CS-specific decrease in $RelB^{-/-}$ granulocytes. Flow cytometric analysis of the bronchoalveolar lavage and bone marrow cells also reveal that it is unlikely that the sustained decrease is caused by excessive cell death or decreased hematopoiesis from the bone marrow. Overall, our results indicate that RelB regulates acute CS-induced pulmonary inflammation through an unidentified mechanism. Understanding how RelB regulates CS-induced inflammation may potentiate the discovery of new therapeutic strategies for many of the inflammatory diseases caused by CS.

Le système immunitaire produit de l'inflammation en réponse à une blessure ou une infection. Bien que l'inflammation aiguë a un rôle protecteur, l'inflammation chronique peut entraîner des lésions persistantes dans les tissus et les organes. Par exemple, les poumons sont vulnérables aux irritants provenant de l'environnement extérieur et sont donc susceptibles de développer des lésions et de l'inflammation. RelB est un membre de la voie non-canonique du facteur transcriptionel NF-κB qui joue un rôle important dans l'inflammation et la mort cellulaire. Notre laboratoire a précédemment montré que RelB protège contre l'apoptose et l'inflammation induites in vitro par la fumée de cigarette ; un puissant irritant et une cause majeure de morbidité et de mortalité dans le monde. Cependant, le rôle protecteur de RelB dans les poumons est moins connu. Nous avons émis l'hypothèse que RelB protège contre l'inflammation pulmonaire induite par la fumée de cigarette. Pour valider notre hypothèse, nous avons exposé des souris de type sauvage ($RelB^{+/+}$) ou déficientes en RelB ($RelB^{-/-}$) à la fumée de cigarette Nous avons trouvé que l'exposition à la fumée de la cigarette entraîne une diminution du nombre de granulocytes des voies respiratoires chez les souris $RelB^{-/-}$; cette diminution des granulocytes est surtout liée à une diminution de nombre des neutrophiles. L'inflammation pulmonaire causée par d'autres irritants, y compris la chlorine, l'ovalbumine (pour imiter les caractéristiques de l'asthme), et les lipopolysaccharides n'est pas affectée par l'absence de RelB. L'analyse différentielle des cytokines par dosage multiplexe suggère que les altérations de l'expression de cytokines et chimiokines n'expliquent pas pleinement la diminution de nombre de neutrophiles chez les souris $RelB^{-/-}$ exposées à la fumée de cigarette. L'analyse par cytométrie de flux du lavage broncho-alvéolaire et des cellules de moelle osseuse révèle également qu'il est peu probable que la diminution de neutrophiles soit provoquée par une mort cellulaire excessive ou une diminution de l'hématopoïèse. Dans l'ensemble, nos résultats indiquent que RelB régule l'inflammation

pulmonaire aiguë induite par la fumée de cigarette par un mécanisme non identifié. La compréhension du mécanisme par lequel RelB régule l'inflammation induite par la fumée de cigarette pourrait mener à la découverte de nouvelles stratégies thérapeutiques ciblant des maladies inflammatoires infligées par une exposition prolongée à la fumée de cigarette.

CHAPTER 1: INTRODUCTION

1.1 Pulmonary Inflammation

1.1.1 Overview of the inflammatory response

Inflammation is a series of cellular and vascular processes which occur in response to infection or exposure to injurious agents [1]. While references of inflammation date back to the medical texts of Mesopotamia and ancient Egypt, the first clinical description was conceived by the ancient Roman Celsus and is still widely-acknowledged today by its cardinal signs [2, 3]. The definition coined by Celsus was comprised of four hallmark features of inflammation: redness (*rubor),* swelling *(tumor),* heat (*calore*) and pain (*dolore*) [2-4]. These features were later joined by a fifth cardinal sign, loss of function, proposed by German pathologist Virchow in the early 19th century [2, 4]. Our understanding of inflammation has greatly evolved since the identification of the five cardinal signs. While inflammation was viewed as an inherently pathological process at the end of the 19th century, we now recognize that inflammation is a response of the immune system to insult and injury which likely arose as a mechanism for restoring homeostasis [3, 4]. Under normal physiological circumstances, inflammation is a beneficial, life-preserving response.

Through the action of leukocytes, inflammation serves to eliminate the threat posed by an injury or infection. Although leukocytes are the main conductors of inflammation, many events must occur for these cells to reach the affected area. The first lines of defense are sentinel cells near the site of injury. Perivascular mast cells are one prominent example of sentinel cells that act as first-responders [5, 6]. Upon stimulation, mast cells release vasoactive agents such as histamine, eicosanoids and tryptases that induce dilation and permeablization of the vasculature [3, 5]. Vasodilatation induced by these mediators leads to hyperemia, responsible for the redness and

heat observed in inflammation [3]. Histamine also induces endothelial cells in the capillaries to contract and form intracellular gaps [2]. These gaps cause the extraversion of fluid and plasma proteins from the vasculature, leading to the edema [3, 5]. As well as initiating vascular changes, mast cells also release mediators which assemble leukocytes at the site of injury (or infection). For instance, mast cells release pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (interleukins are denoted as IL hereafter) which activate and attract immune cells from the bloodstream to the site of damage [1, 5]. The binding of cytokines to their cell surface receptors also activates local endothelial cells to upregulate the expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) [7]. This increase in ICAM-1 expression enables leukocytes to bind to the endothelium, transmigrate and gain access to extravascular tissue [1, 7].

Although mast cells are important first-responders, they are not the only sentinel cell present in the tissues that release important chemical mediators. Inflammation is dependent upon the coordinated efforts of many different cell types in both the vasculature and affected tissue. The architecture and cellular composition of tissue and their associated vasculature differ from organ to organ. Therefore, different patterns of leukocyte recruitment may take place in different locations [6]. For example, perivascular macrophages are an important means of host defense against bacterial infection in the skin but are entirely absent in the lung and spleen [6]. While this example describes a case in which location influences the abundance of cell types, differences in local microenvironments can also lead to phenotypic variations between cells of the same type [8]. For instance, tissue macrophages express many protein receptors on their surfaces which recognize and mediate interactions with microbial products and other inflammation-inducing ligands [8]. An important class of these surface receptors are the toll-like receptors (TLRs) which

recognize pathogen-associated molecular patterns (PAMPs) [9]. Studies on the gene expression profiles of macrophage populations reveal that resident macrophages derived from different organs may have differential expression of various TLRs [10]. While microbial lipopolysaccharide (LPS)-recognizing TLR4 is uniformly expressed among macrophages from different organs, lipoprotein and peptidoglycan recognizing TLR2 is much more highly expressed in resident macrophages of the lung relative to macrophages in the microglia and peritoneum [10]. As TLRs aid in the macrophage's ability to recognize inflammatory stimuli, variations in TLRs and other important cell surface receptors in different sentinel cell populations may lead to differential activation by various inflammatory stimuli. As a consequence, site-specific phenotypic heterogeneity may also affect patterns of leukocyte recruitment in inflammation [6].

As structural cells represent the dominant cell type in most tissues, they play an equally important role as sentinel cells in the development and progression of inflammation [6]. For example, airway epithelial cells are vital immunoregulatory cells that produce many cytokines that orchestrate the inflammatory response [11, 12]. Airway epithelial cells produce IL-17E, IL-33 and thymic stromal lymphoprotein (TSLP) which induce the production of additional cytokines by mast cells, macrophages, innate lymphoid cells and other leukocytes [13]. Airway epithelial cells also release classic pro-inflammatory cytokines such as IL-6 and IL-8 in response to stimuli such as cigarette smoke (CS) and bacteria, which also lead to the recruitment of inflammatory cells [14, 15]. In addition to releasing cytokines, epithelial cells also metabolize cell membrane phospholipids to produce pro-inflammatory lipid mediators such as prostaglandins and thromboxanes from the cyclooxygenase enzymes COX-1 and COX-2 [1, 16]. Prostaglandin E_2 (PGE_2; prostaglandins are denoted as PG) is one such pro-inflammatory lipid

mediator produced by epithelial cells via the actions of COX-1 and COX-2. A potent vasoactive agent, PGE_2 can contribute to vasodilation by inducing arterial dilation and increasing permeability of the microvasculature [17]. This in turn contributes to the onset of two of the classical signs of inflammation: redness and edema. By acting on the peripheral sensory neurons, PGE_2 also increases susceptibility to pain-yet another sign of inflammation [1, 16, 17].

While the identity of sentinel cells and the pattern in which they recruit leukocytes may contextually vary, the end result of an acute inflammatory episode is often very similar. One reason for this is that most pro-inflammatory cytokines and mediators show redundancy in their source and function. For example, PGE_2 is released by many cell types including macrophages, mast cells and epithelial cells [1]. Similarly both histamine and PGE_2 result in vasodilation. The combined effects of these different pro-inflammatory mediators and contributions from sentinel cells results in the accumulation of phagocytic cells at the site of inflammation [7]. Once at the site of inflammation, phagocytic cells such as neutrophils are able to ingest invading microbes and destroy them intracellularly [7]. In situations where the target cannot be fully engulfed, neutrophils release granules of cytotoxic components into the extracellular milieu [1, 7, 18]. After the threat has been eliminated, leukocytes are cleared by various mechanisms including local apoptotic death and systemic recirculation following reverse-migration from the inflamed tissue [19]. Alternatively-activated monocytes and macrophages then partake in the resolution phase by engulfing dead cells and apoptotic debris [1]. These cells also promote tissue remodelling and repair by producing pro-angiogenic and fibrogenic factors such as transforming growth factor-β (TGF-β) [1, 20].

It is interesting to note that the same processes which were used to establish an inflammatory response may be modified in the later stages and aid in the resolution of inflammation. During

the initial phases of inflammation, macrophages produce pro-inflammatory lipid mediators (*i.e.* thromboxanes and PGs) from the phospholipid metabolite arachidonic acid and COX enzymes. However, in the resolution phase macrophages shift to produce lipoxins-a groups of neutrophil recruitment-inhibiting anti-inflammatory mediators produced from the same substrates as prostaglandins and thromboxanes [1]. COX-2 correspondingly switches from producing pro-inflammatory molecule PGE_2 to producing cyclopentanone prostaglandins such as lipoxin A_4, which blocks the endothelium from expressing adhesion molecules and prevent macrophages from releasing pro-inflammatory cytokines [21]. Thus, inflammatory cells and mediators can have a broad range of effects with both pro- and anti-inflammatory consequences and under physiological conditions, both pro- and anti-inflammatory mechanisms are required to maintain or return to homeostasis.

Indeed, while inflammation is necessary to protect against pathogens and noxious substances, extensive inflammation can disrupt tissue homeostasis, overwhelm host defenses and damage host tissue [18]. As the proteolytic enzymes released by neutrophils work indiscriminately, this can often result in collateral damage to the host [1, 18]. If the inflammation is acute, such damage is reparable and the tissue may be restored to its functional state. If the inflammatory reaction persists and leukocytes are not effectively cleared, lasting tissue damage can occur [1, 3]. Thus, chronic inflammation acquires new characteristics compared to its acute counterpart [21]. The predominant leukocytes in chronic inflammation are macrophages and lymphocytes, while neutrophils predominate in acute inflammation [1]. Distinct histological features may also appear in cases of chronic inflammation. For example, mononuclear cells may form granulomas- a focal collection of macrophages and lymphocytes- to quarantine non-degradable foreign bodies [1].

Perhaps one of the most worrying aspects of chronic inflammation is the extent of its persistence. While an acute inflammatory response resolves within hours, chronic inflammation can persist for years, even after the initial stimulus is removed [19]. This continued inflammation is evident in HIV-infected patients that were treated with effective retroviral therapy [22]. Another notable example occurs is in chronic obstructive pulmonary disease (COPD). COPD is a persistent and debilitating disease characterized by expiratory airflow limitations manifesting as increased breathlessness and is associated with an abnormal chronic inflammatory response of the airways and lungs [23]. COPD encompasses two main conditions: emphysema that is characterized by permanent destruction of alveolar walls and chronic bronchitis, which is characterized by chronic cough and sputum production for a minimum of three months a year for 2 consecutive years [23]. Over 90% of COPD patients have smoked, making CS the most important casual risk factor in the development of the disease [23]. Those who develop COPD need not be current smokers and many COPD patients that ceased smoking still exhibit signs of persistent inflammation [24]. HIV and COPD are by no means an exhaustive list of persistent and debilitating chronic inflammatory diseases. Chronic inflammation is also known to contribute to the pathophysiology of many other common diseases, including atherosclerosis and asthma [7].

It is also noteworthy that inflammation may induce disease directly by causing destruction and impaired organ function or through an indirect mechanism. For instance, prolonged and excessive inflammation can increase susceptibility to disease via oxidative stress [25]. Oxidative stress is the overproduction of free radicals and reactive metabolites such as superoxide and hydrogen peroxide (H_2O_2) [25]. These reactive oxygen species (ROS) and reactive nitrogen species (RNS) can react with lipid, protein and nucleic acids in a cell and cause damage [25]. ROS/RNS may directly interact with DNA to form DNA abducts and breaks, leading to

mutagenesis [25]. During inflammation, phagocytes such as neutrophils and macrophages employ a respiratory burst, which is the rapid release of ROS as a means to combat invading pathogens [25]. Enzymes such as NADPH oxidase 2 (NOX2) present in the phagosomes of macrophages for example is responsible for producing superoxide which reacts with other molecules to generate more ROS [25]. Due to this link between inflammation and oxidative stress, chronic inflammation is associated with an increased rate of mutagenesis and as a consequence, an increased risk for malignant transformation [25, 26]. One reason for this is that mutations in genes associated with proliferation (*i.e.* oncogenes) foster an environment for cancer [27]. Some well-established examples include the higher incidence of colon cancer in patients with inflammatory bowel disease (IBD) and the higher risk of pancreatic cancer associated with chronic pancreatitis [28]. Similarly, COPD doubles the risk for the development of lung cancer [27].

Given that the inflammatory response is vital for survival, the therapeutic challenge for these chronic inflammatory diseases lies in effectively regulating inflammation; in other words, to limit excessive tissue damage while preserving the physiology of the organ [1, 18]. Because the physiological inflammatory mechanisms of each organ may be distinct, in order to target a particular inflammatory disease, it may be advantageous to characterize the nature of inflammation in the affected organ. The following section will describe the innate defense and inflammatory mechanisms occurring in the lung.

1.1.2 Inflammatory Mechanisms in the Lung

1.1.2.1 Physical barriers and the airway epithelium

The lungs contain the largest epithelial surface of the body and as such, are constantly exposed to the external environment [5, 11, 29, 30]. We breathe in over 10-12,000L of air every day, much of which contains microorganisms, viruses, allergens and pollutants [29, 30]. Given these large number of threats, the lungs are one of the most frequently inflamed organs [5]. In order to protect the lung from such threats, there are many elaborate defense mechanisms in place [11]. Biophysical processes such as mucocilliary clearance, sneeze and cough reflexes and vibrissae filtration are effective in removing larger particles [11, 30]. Moreover, the airway epithelium serves as a physical barrier and secretes many non-specific anti-microbial factors such as β-defensins, nitric oxide (NO), elafin and lactoferrin [11, 29]. Mucus secreted by the epithelium also contains anti-microbial peptides and oxidizing enzymes [30]. In the lower respiratory tract, alveolar surfactant proteins SP-A and SP-D play a dual role by reducing lung surface tension and binding to LPS on the outer membrane of gram-negative bacteria to target them for destruction [11, 30].

1.1.2.2 Mononuclear phagocytes

In addition to these mechanisms, the airways are also continually under immune surveillance by resident leukocytes. One of the first lines of defence against a pathogen in the lung are dendritic cells (DCs) [11]. DCs are bone marrow-derived antigen-presenting cells (APCs) which sample the alveolar lumen and conducting airways [11, 29, 31]. Most DCs in the lung are in the immature state as antigen-uptake cells, with low level expression of major histocompatibility

complexes (MHC) I and MHC II [32]. Prior to antigen uptake, DCs are maintained in the lung with chemokine CCL5 (C-C motif ligand 5 also known as RANTES) and other agonists for the receptors CCR1 and CCR5 [32]. Once an immature DC encounters an antigen, it downregulates expression of these receptors, enters the draining lymph node and acquires projections that allow contact with T lymphocytes [32]. In addition to initiating an immune response, certain subsets of pulmonary DCs can also release cytokines that skew developing lymphocytes to elicit a Th1, Th2 or Th17 response [32].

Macrophages in the lung also serve as a first-line of pulmonary defense. Different populations of macrophages can be found in the lung. Interstitial macrophages are located in the parenchyma between adjacent alveoli and work with DCs to present antigen to T lymphocytes [31]. Although macrophages and DCs are both phagocytic cells, the potency of lysosomal proteases in macrophages is higher than that of DCs [33]. Thus, most subsets of macrophages carry out the full degradation of phagocytosed material whereas material phagocytosed by DCs undergo controlled proteolysis [33]. The weaker potency of lysosomal proteases in DCs thus makes them better-suited for antigen processing while the highly potent lysosomal proteases in macrophages enables them to play other roles in the inflammatory response in addition to antigen presentation [33]. This diversity in macrophage function is aptly seen in alveolar macrophages [31]. Alveolar macrophages are long-lived, embryonically-derived cells which suppress DC-mediated activation of T lymphocytes in steady-state conditions, thereby increasing tolerance and preventing excessive inflammation [31, 34]. However, activation of various pathogen recognition receptors (PRR) on the alveolar macrophages switches their role to defense and the clearance of foreign targets [31]. This feature of alveolar macrophages makes them key regulators of lung homeostasis.

Another important class of phagocytes are monocytes, which are the myeloid precursors of macrophages and are produced in the bone marrow [29]. Monocytes circulate in the bloodstream and can be recruited to the lung microvasculature in response to chemokines [29]. These precursors can then differentiate into macrophages in the lung where they engulf foreign agents, and act as a main source of cytokines to regulate the inflammatory response [11, 35]. Interestingly, the role these differentiated macrophages play in regulating the inflammatory response in the lung is governed by environmental cues. Akin to the polarization of T helper (Th) lymphocytes to Th1 and Th2, environmental cues lead monocytes and macrophages to polarize to classically activated M1 and alternatively-activated M2 macrophages [35]. Classically-activated macrophages are stimulated by cytokines such as interferon γ (IFN-γ) which are products of Th1 lymphocytes [36]. M1 macrophages appear early on during acute inflammation and foster a pro-inflammatory response [35]. Exposure to stimuli such as IL-4 leads to alternatively-activated M2 macrophages that are involved in tissue repair [35, 36].

1.1.2.3 Granulocytes

Other important resident leukocytes of the lung are mast cells which are located near blood vessels, nerves and glands throughout the body [11, 29]. Mast cells contain dense membrane-bound intracellular granules with pre-formed mediators (histamine, serine proteases and cytokines) kept in an inactive state by the acidic environment of the granule [29, 37]. The mast cell plasma membrane expresses FcεRI, a high affinity receptor for immunoglobulin E (IgE; immunoglobulin is denoted as Ig), which may bind allergen-bound IgE to trigger the complete degranulation of the mast cell and the subsequent release of the pre-formed mediators [37]. Mast cells also have an array of other common immunoreceptors like those in the TLR family,

cytokine receptors and transient receptor potential family of ion channels (TRPC) which are sensitive to physical stimuli such as temperature and mechanical stress [37]. In addition to releasing pre-formed components, mast cells produce and release other important inflammatory mediators *de novo* such as PGD_2 and the pro-inflammatory cytokines IL-1β and IL-6 [29, 37, 38]. Mast cells can also engulf bacteria and polarize T lymphocyte subpopulations towards a Th1 or Th2 phenotype [29, 37].

Arguably the most essential granulocyte in an acute inflammatory response is the neutrophil. Neutrophils are the first-responders during an inflammatory reaction [11]. Neutrophils in circulation are recruited to the site of inflammation within 10-20 minutes by the action of cytokines and adhesion molecules produced by resident cells [11, 39]. Neutrophils are short-lived polymorphonuclear phagocytes which contain granules and secretory vesicles in their cytoplasm [39]. As they mature, neutrophils undergo three phases with respect to their granular contents: azurophilic (primary) granules, specific (secondary) granules and gelatinase (tertiary) granules [39]. Primary granules contain cell adhesion molecules and myeloperoxidase (MPO), a lysosomal enzyme which produces ROS during the respiratory burst [39]. Secondary granules contain the antimicrobial compound lactoferrin while tertiary granules contain metalloproteinase (MMP)-9 and other proteins that allow neutrophils to digest through the extracellular matrix (ECM) and transmigrate [39]. When a neutrophil encounters a foreign body, it may phagocytose and kill the encapsulated pathogen by releasing ROS and antimicrobial proteins from its granules to the phagosome [39]. Neutrophils can also release their granular contents into the extracellular milieu to overcome extracellular pathogens. Additionally, neutrophils can form neutrophil extracellular traps (NETs) by releasing their DNA associated with histones, degradative enzymes and anti-microbial proteins to immobilize the target [39].

Mast cells and neutrophils are not the only types of granulocyte recruited to the lung. Eosinophils are important effectors of the allergic response, asthmatic lung inflammation and parasitic infections [11, 29]. Under steady-state conditions, only a limited number of eosinophils are released from the bone marrow. However, the lung also contains a pool of resident eosinophils that are believed to play a homeostatic role under physiological conditions [40]. Under the command of Th2 lymphocytes, the number of eosinophils produced in the bone marrow dramatically increases, as does the number of eosinophils recruited to the lungs [40]. Eosinophils contain four cytotoxic cationic proteins in their granules: major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin [29]. Eosinophils release these cytotoxic proteins in addition to preformed pro-inflammatory lipid mediators and cytokines at the site of inflammation [40].

1.1.2.4 Lymphocytes

Lymphocytes are part of the adaptive immune system that provides an antigen-specific immune response, compared to the quick, non-specific response of the innate immune cells discussed earlier (*i.e.* macrophages, neutrophils, *etc*.) [11]. Due to their delayed recruitment and activity, lymphocytes are associated with sub-acute and chronic inflammation. Lymphocytes are recruited to the airways and lung parenchyma to provide cell-mediated or humoral immunity in the form of T lymphocytes and B lymphocytes, respectively [11]. T lymphocytes can be divided into two major subsets based on the expression of their surface molecules: $CD4^+$ (helper or Th) T lymphocytes and $CD8^+$ (cytotoxic) T lymphocytes [11]. When DCs present naïve Th lymphocytes with an antigen, the lymphocytes differentiate into distinct subsets with their own unique roles and cytokine profiles [41]. Th1 lymphocytes produce cytokines such as IFN-γ to

activate macrophages and other innate immune cells enabling them to clear intracellular pathogens [41, 42]. Th2 lymphocytes regulate inflammation due to extracellular pathogens and upregulate B lymphocyte activation and antibody production [11, 41, 42]. Th17 lymphocytes are involved in extracellular bacterial and fungal clearance [41, 42]. T regulatory (Treg) lymphocytes mediate mucosal homeostasis by dampening the inflammatory activity of other T helper lymphocytes [41, 42]. Cytotoxic T lymphocytes secrete cytotoxic mediators such as the pore-forming enzyme perforin to directly kill infected cells [11, 41]. Finally B lymphocytes synthesize antibodies which bind to soluble, particulate and cellular antigenic determinants [43]. Binding of the antibody to an antigen may lead to a number of outcomes including blocking or neutralization of the target, opsonisation of the target for phagocytosis or antibody-dependent cell-mediated cytotoxicity [43]. IgA is the most common antibody found in the lung while IgE is notable for its association with allergic lung inflammation and asthma [43].

1.1.3 Cytokines: Modulators of the Inflammatory Response

1.1.3.1 Overview of Cytokines

Cytokines are small signalling proteins that affect nearly every biological process- including inflammation and immunity [11, 44]. With the exception of red blood cells, cytokines are secreted by and can target all cell types to affect their movement, growth, differentiation, survival and death [31, 45]. Akin to hormones, cytokines may have paracrine functions that affect nearby cells, endocrine functions that affect distant cells or autocrine functions that affect the cytokine-producing cell itself [44, 46]. Cytokines bind to receptors on target cells and take part in cell signaling cascades that result in the transcription of genes with many diverse

functions (*i.e.* cell survival, cell proliferation) [45]. Cytokines are pleiotropic, where one cytokine can affect multiple biological processes [45, 46]. For example, IL-6 inhibits Treg differentiation but also inhibits epithelial cell apoptosis [47]. Many of the pleiotrophic effects partially overlap, such that cytokines are often redundant in their functions [45, 46]. Thus different cytokines released by the same cell under a particular condition may activate some of the same genes and enhance the robustness of a response [45]. Just as they may act synergistically with each other, cytokines may also antagonize each other's functions [46]. For example, interleukin-1 receptor antagonist (IL-1Ra) competes with pro-inflammatory IL-1 family members IL-1α and IL-1β for the IL-1 receptors (IL-1R) thereby producing anti-inflammatory results [48]. Although the binding of IL-1Ra to IL-1R does not induce a cellular response, this is not the convention [48]. The binding of a cytokine to its receptor typically leads to the production of more cytokines by its target cell, creating a complex network of interactions between cells [46]. In such a way, cytokines can participate in feedback loops to regulate several functions, acting as both inducers and suppressers [46].

Cytokines are the molecular signals of communication between cells of the immune response [45], dictating everything from initiating and amplifying inflammation to inducing the activation, maturation, differentiation and death of lymphocytes [45]. There are multiple ways to classify cytokines. One common method in classifying cytokines is based on the cell responsible for the secretion of these proteins. Although produced by many cells, macrophages and helper T lymphocytes are the two major sources of cytokines [46]. Cytokines that are synthesized by monocytes and macrophages are referred to as monokines while cytokines synthesized by lymphocytes are known as lymphokines [45]. Another efficient means to classify cytokines is by their biological roles. Monokines and lymphokines may be further grouped according to whether

they exhibit immunostimulatory or immunosuppressive actions. Similarly, we may group cytokines involved in growth and proliferation together.

1.1.3.2 Monokines

When M1 macrophages are exposed to an inflammatory stimulus, they secrete pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 [35]. These cytokines are pyrogenic, appear early on in the inflammatory process and incite many of the changes associated with inflammation such as vasodilation, antigen presentation, release of acute phase proteins (inflammation-associated proteins produced by the liver) and upregulation of cell adhesion molecules [35]. In addition to inflammation, these cytokines may partake in related or entirely distinct biological processes. An example of this is TNF-α. In addition to being produced by macrophages, TNF-α (also known as cachectin) is also produced by T lymphocytes, mast cells, natural killer cells and fibroblasts [49]. TNF-α typically binds to either TNF receptor 1 (TNFR1)- a widely expressed receptor- or TNF receptor 2 (TNFR2)- a receptor expressed on leukocytes and endothelial cells [46, 49]. One important way TNF-α exerts its pro-inflammatory effects is through its effects on endothelial cells [50]. TNF-α causes endothelial cells to produce adhesion molecules such as ICAM-1 along with cytokines such as IL-8 in order to recruit leukocytes at the inflammatory site [50]. TNF-α also induces endothelial cells to express COX-2 and vasoactive PGI_2 which locally results in increased blood flow [50]. Another well-established effect of TNF-α is its ability to induce apoptosis. TNFR1 contains a death domain and in the absence of intracellular proteins TNFR-associated factor 2 (TRAF2) and receptor-interacting protein, the binding of soluble TNF-α to TNFR1 leads to apoptosis instead of pro-inflammatory gene expression [49].

Another pro-inflammatory monokine, IL-1β, is produced by neutrophils, natural killer cells, B lymphocytes, dendritic cells, fibroblasts, endothelial and epithelial cells as well as macrophages [35, 46, 49]. IL-1β is often produced by one leukocyte and acts on another [46]. In effect, IL-1β has very diverse functions on many different cell types. IL-1β acts as chemoattractant for granulocytes, participates in the degranulation of mast cells and promotes the expansion and differentiation of Th lymphocytes [35]. IL-1β is one of eleven members of the IL-1 family [49]. Other notable members include IL-1α (a pro-inflammatory monokine) and IL-RA (an anti-inflammatory cytokine) [49]. IL-1β, IL-1α and IL-RA all bind to the interleukin-1 receptor (IL-R1) [49]. The binding of IL-1β to IL-R1 leads to activation of mitogen activated protein kinases (MAPK) and the transcription factor nuclear factor kappa-B (NF-κB), leading to pro-inflammatory gene expression [49].

IL-6 is another monokine expressed by T lymphocytes, B lymphocytes, fibroblasts, endothelial, and epithelial cells [49]. Due to its diverse roles, IL-6 has also been called hepatocyte-stimulating factor (HSF), B cell stimulatory factor 2 (BCSF-2) and B cell differentiation factor (BCDF) [49]. IL-6 is involved in hematopoiesis, T lymphocyte survival, B lymphocyte maturation and antibody production [35, 49]. IL-6 binds to its receptor IL-6R in conjunction with the ubiquitously-expressed signal transducing component glycoprotein 130 (gp130) [35, 49]. Membrane-bound IL-6R is only found on lymphocytes and hepatocytes. However, this does not mean that the effects of IL-6 is only limited to these two cell types [35, 47, 49]. Other cell types may be targeted by IL-6 through trans-signalling-a type of signalling in which a naturally-occurring soluble form of IL-6R binds to and activates gp130 on the surface of cells which do not express membrane-bound IL-6R [35, 47, 49]. The pro-inflammatory effects of IL-6 are mediated by trans-signaling [35, 47]. Alternatively, classical signaling occurs in the presence of

membrane-bound IL-6R and the binding of IL-6 to membrane-bound IL-6R actually leads to regenerative and anti-inflammatory effects such as the inhibition of epithelial cell apoptosis and the regulation of other pro-inflammatory cytokines [35, 47, 51].

It is important to note that monokines are not always pro-inflammatory. Macrophages can also release cytokines that attenuate inflammation. M2 macrophages can secrete immunosuppressive cytokines such as IL-10 [35]. IL-10 (also produced by B and T lymphocytes) inhibits the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in addition to suppressing antigen presentation and DC maturation [35, 46, 52]. Furthermore, IL-10 lowers the microbicidal activity of macrophages and diminishes their production of MMPs [35, 52]. IL-10 binds to its receptor complex IL-10R1 found constitutively on most hematopoietic cells and is up-regulated upon stimulation [52]. IL-10 also may bind to ubiquitously-expressed IL-10R2 [53]. Much of the bioactivity of IL-10 occurs by inhibiting the molecular pathways activated by the pro-inflammatory cytokines such as NF-κB [52].

1.1.3.3 Lymphokines

The polarization of T lymphocytes into Th1, Th2 or Th17 lymphocytes causes different sets of cytokines to be secreted. An important lymphokine secreted by Th1 lymphocytes is IFN-γ [44]. Interferons are a group of cytokines that alert the immune system to viral infection [54]. In addition to Th1 lymphocytes, IFN-γ is also secreted by macrophages and natural killer cells [54]. IFN-γ enhances antigen presentation by upregulating class I and II MHC molecules and is a potent activator of macrophages, inducing their production of nitric oxide (NO) [55-57]. In effect, the activity of IFN-γ is essential in enhancing the ability of the innate immune cells and

cytotoxic T lymphocytes in fighting intracellular pathogens during a Th1 response [42, 57]. On the other hand, cytokines secreted by Th2 lymphocytes, such as IL-4 and IL-5, orchestrate the inflammatory response to allergens [42]. IL-4 leads to the production of IgE by B lymphocytes [58]. Comparable to the role of IFN-γ in promoting the differentiation of Th1 lymphocytes, IL-4 supports the development of Th2 lymphocytes and inhibits Th1 and Th17 lineages [42]. IL-4 is also stored in mast cell and basophil cytoplasmic granules, although in smaller amounts than Th2 lymphocytes [59]. Th2 lymphocytes also produce IL-5, along with mast cells, NK (natural killer) cells, eosinophils and epithelial cells [60]. IL-5 is essential in the maturation, differentiation and survival of eosinophils [58]. IL-5 also has roles in IgA production in mucosal tissue [60]. Finally, Th17 cells produce the IL-17 family of cytokines (which includes IL-17A, IL-17B, IL-17C, IL-17D and IL-17E) and orchestrate innate immune responses such as neutrophil activation and trafficking [42]. IL-17A is actively involved in the recruitment of neutrophils and enhances the production of IL-6 and TNF-α from macrophages and DCs in the lung parenchyma [61]. Also produced in granulocytes, mast cells and NK cells, IL-17A may stimulate these other cell types to produce pro-inflammatory cytokines [61].

1.1.3.4 Chemokines and growth factors

Another significant class of cytokines are chemokines, which by definition are involved in the chemotaxis of leukocytes [46]. Chemokines possess three-to-four conserved cysteine residues and as such may be divided into families based on the positioning of these residues [49]. The C-X-C family members contain the first two conserved cysteines (represented by the two Cs) separated by an amino acid (represented by X) [49]. An example of a CXC chemokine is CXCL1 (also known as growth regulated oncogene-α [Gro-α] in humans and keratinocyte

chemoattractant [KC] in mice), a powerful chemoattractant for neutrophils [62]. In mice, KC functions as the murine homolog along with other cytokines such as macrophage inflammatory protein 2 (MIP-2) and C-X-C motif ligand 5 (CXCL5) [63]. CXCL1 binds to the G protein coupled receptor CXCR2 on neutrophils and glycosaminoglycans on endothelial and epithelial cells, as well as to the ECM [62]. The activity of CXCL1 affects the flux, duration and kinetics of neutrophils [62]. C-C family members contain two adjacent conserved cysteine residues [49]. Monocyte chemoattractant protein-1 (MCP-1), also known as chemokine ligand 2 or CCL2, governs the migration and infiltration of monocytes and macrophages [64]. Although MCP-1 is also produced by endothelial cells, fibroblasts, epithelial cells, monocytes and smooth muscle cells, MCP-1 is mainly produced by monocytes and macrophages [64]. MCP-1 is also implicated in stimulating the migration of other mononuclear cells such as T lymphocytes and NK cells [64]. Along with their function in chemotaxis, chemokines may function in other processes. For instance, CXCL1 and MCP-1 mediates angiogenesis [49, 65].

Cytokines also play a role in leukocyte production, particularly colony stimulating factors (CSFs). CSFs act on stem cells to boost the production and activation of hematopoietic cells [49]. GM-CSF (granulocyte-macrophage colony stimulating factor) is produced by T lymphocytes, macrophages, endothelial cells and fibroblasts [49, 66]. In a dose-dependent fashion, GM-CSF stimulates the production of multipotent progenitor cells [66]. At low concentrations, GM-CSF leads to the production of macrophage progenitors while at high quantities granulocyte, erythroid, eosinophils, megakaryocyte and multipotent progenitors are formed [66]. Similar to chemokines, CSFs can have other functions. For example, GM-CSF can recruit circulating neutrophils, monocytes and lymphocytes and is critical in the function and development of DCs [66]. Thus, CSFs not only establish a population of leukocytes, they also

dictate their recruitment and activity [66]. Overall, cytokine production can have important implications when one considers the myriad of insults the lung can be exposed to on a regular basis.

1.2 Cigarette Smoke

1.2.1 Cigarette Smoke and pulmonary inflammation

One exposure that can have significant consequences in the respiratory system is CS. Despite the gradual decline in the consumption of CS over the last half century, CS still remains a leading cause of morbidity and mortality in developed countries [67, 68]. In Canada, the most recent report indicates that 18.1% of Canadians (aged 12 and older) are active smokers [69]. This number is comparable to global trends, where about 20% of the world population smoke [70]. Given that CS affects multiple organ systems and results in many chronic diseases, the high prevalence of smoking places an enormous burden on global healthcare [68]. Nearly 6% of the annual global health expenditure is used to treat smoking-attributable diseases [71]. This translates to a total health expenditure of 1.8% of the annual global gross domestic product (GDP), a measure of all goods and services produced over a given time [71]. The consequences to human health and quality of life are even more dismal than the economic consequences [71]. The World Health Organization (WHO) reported that 5.4 million premature deaths were directly attributable to smoking in 2012, with 600,000 deaths due to second-hand exposure [71, 72]. Furthermore, it is estimated that adult smokers lose an average of 13-15 years of life expectancy from chronic diseases [73]. Many chronic diseases have been linked to smoking (COPD and lung cancer) or are exacerbated by it (asthma and tuberculosis) [68, 74].

CS is a complex mixture containing over six thousand chemicals- many of which have cytotoxic, mutagenic, carcinogenic or antigenic properties [68, 72, 73]. Many smoke components are generated by the combustion of tobacco and are not inherently present in the tobacco leaves themselves [73]. Combustion products of tobacco are gaseous or particulate [68, 73]. The particulate phase has hazardous and immunomodulatory effects [68, 73]. As the particles are in the range of microns, they are small enough to penetrate deeper into the lung and efficiently deposit in the alveoli where they may elicit an immune response [68]. Before the particles are able to deposit in the alveoli, however, their interaction with the respiratory epithelium initializes an inflammatory cascade [73, 75]. Upon contact with smoke, resident cells collectively release TNF-α, IL-1, Il-6, IL-8 (CXCL1 in mice) and GM-CSF and lower levels of anti-inflammatory cytokine IL-10 [68]. These cytokines cause the recruitment of immune cells to the lung, with the persistence of leukocytes in the lung contributing to tissue damage. Smoking markedly increases production of MMPs by macrophages and proteolytic enzymes by neutrophils, which can amplify the destruction [76]. This inflammation is a key contributor in the development or exacerbation of most smoke-related diseases

While injury by recruited immune cells is an indirect mechanism by which smoke harms the respiratory system, components of CS may also directly injure the lung. For instance, ROS generated by combustion of tobacco are present in the particulate phase where they generate secondary oxidative moieties and DNA abducts, causing lipid peroxidation in the cell membranes of the epithelial barrier [73, 77]. In acute CS exposure, the generation of ROS activates epithelial cells to produce pro-inflammatory mediators [77]. In chronic exposures, oxidative stress induced by ROS leads to metaplastic and dysplastic changes to the respiratory epithelium thereby disrupting the epithelial barrier and impairing mucociliary escalator function

[24, 76, 77]. As the epithelial barrier and muscocilliary escalator are major components of innate pulmonary defense, these events make the lung vulnerable to microbial infections and external threats [77]. In severe cases, ongoing destruction of lung tissue and aggregation of lymphoid cells in the respiratory tract generates autoantibodies which drive several chronic inflammatory diseases including COPD and lung cancer [76-79]. While diseases such as COPD and lung cancer may take several years to develop, preliminary signs of increased epithelial permeability is seen even in asymptomatic smokers [75].

The detrimental effects of smoke are not restricted to the structural components of the lung. CS also compromises immune homeostasis [73, 74]. CS increases the number of alveolar macrophages [77]. Despite the fact that CS increases the number of macrophages, the effector function of these cells is compromised, leading to reduced phagocytic ability [77]. The observation that CS promotes higher numbers of inflammatory cells whilst reducing their function is also relevant for neutrophils. Smoking causes a massive influx of neutrophils to the lung, and smokers can have up to 30% more circulating neutrophils compared to non-smokers [68]. At the same time, peripheral neutrophils from smokers exhibit decreased migration and chemotaxis [68]. Long term smoke exposure may also reduce the phagocytic ability of neutrophils [80]. One more example of innate defense being compromised due to smoke is seen in the respiratory epithelium. Despite secreting more pro-inflammatory mediators, smoke-exposed epithelial cells have suppressed inflammatory mediator release upon bacterial stimulation, indicating impaired innate immunity despite driving a heightened state of inflammation [15].

In line with its pro-inflammatory effects, CS also increases the total number of circulating lymphocytes, particularly T lymphocytes [68, 77]. Smokers with COPD have a marked elevation

of cytotoxic T lymphocytes [68, 77]. It has also been suggested that smoke suppresses Th1 polarizing cytokine production while skewing the response towards Th2 or Th17 immunity [73]. For example, *in vitro* exposure of DCs to cigarette smoke extract ([CSE] a surrogate for CS) results in reduced secretion of Th1 polarizing cytokine IL-12 when stimulated by LPS [73]. Similarly, animal models of respiratory syncytial virus (RSV) infection exposed to smoke have reduced levels of Th1 cytokines and an increase in pulmonary eosinophilia. As a consequence of the skewing in favor of Th2- and Th1-mediated immunity, smoke favors partial M2 activation of macrophages while inducing partial M1 deactivation [77]. B lymphocytes are equally affected by CS, and B lymphocyte follicles formed in the lungs of smokers may be involved in autoimmunity [77]. Along with autoimmunity, B lymphocytes are also involved with increased allergic sensitization, supporting the Th2 skewing characteristic of smoke [77]. In effect, smokers have elevated plasma levels of IgE despite reduced levels of all other classes of antibodies [68, 77].

Indeed CS is a potent oxidant and pro-inflammatory stimulus that increases lymphocyte recruitment and alters the homeostatic balance in the lung. While driving inflammation, CS simultaneously reduces the capacity of the immune system to protect the lung. In the long term, the smoke-induced lymphocyte aggregation causes severe damage and sets the stage for many chronic ailments.

1.2.2 Other environmental pro-inflammatory lung irritants

Although CS is a significant source of toxic exposure to humans, it is not the only environmental inhalant that causes pulmonary inflammation and damage [81]. Common exposures that also

cause pulmonary inflammation include microbial products (*i.e.* LPS), chemical irritants (*i.e.* chlorine [Cl_2]) and allergens (*i.e.* ovalbumin [OVA]). Many of these common exposures have similarities with CS in the mechanisms through which they cause damage. For example, oxidative stress is also an important aspect of Cl_2-induced pulmonary inflammation [73, 82]. Similarly, the OVA model of inflammation skews lymphocytes in favor of Th2-polarization as does chronic exposure to CS [77, 83].

1.2.2.1 LPS

LPS is present in CS [68, 84], with mainstream CS containing over 120 ng of LPS [84]. LPS is a constituent of the outer membrane of Gram-negative bacteria that triggers innate immunity [85, 86]. LPS is primarily composed of a water-soluble polysaccharide region joined to a toxic lipid A molecule [85]. As such, LPS may also be encountered during infections of the respiratory tract [86]. Experimental LPS inhalation shares many of the features of acute CS-induced pulmonary inflammation such as neutrophil influx, airway obstruction and bronchial hyperresponsiveness [84]. The similarities in outcome are seen despite a direct mechanism of action for LPS compared with smoke. LPS acts as a PAMP which is recognized by TLR4 [87]. LPS-binding protein (LBP) and cluster differentiation 14 (CD14) are involved in the LPS-TLR4 complex formation. Upon binding to LPS, TLR4 associates with toll-IL-1 receptor domain containing adaptor proteins [87]. Among the 5 adaptor proteins that TLR4 associates with, myeloid differentiation response gene 88 (MyD88) is especially important, as MyD88-dependent pathways causes the expression of pro-inflammatory mediators such as TNF-α and IL-1 [85, 87]. MyD88-indepdnent pathways alternatively leads to the production of IFNs by infected cells [87].

Macrophages and epithelial cells are the primary target cells for LPS and their stimulation leads to the production of chemotactic factors which call for the rapid influx of neutrophils [85].

1.2.2.2 Cl_2

Small quantities of Cl_2 can also be found in CS, with an average of 90 μg of Cl_2 present in the gaseous phase cigarette smoke in the form of methyl chloride, followed by 68 μg of Cl_2 in the particulate phase [88]. Low-level exposure to Cl_2 can also result from frequent swimming in chlorinated pools and through the use of common household products [82]. Contact with Cl_2 does not always occur in its gaseous form and it may be encountered as hypochlorous acid, chlorine dioxide and methyl chloride [82]. As Cl_2 is inhaled, it is hydrated by airway surface liquid to form hypochlorous acid and hypochloric acid-both of which irritate the airway epithelium [82]. These compounds further react with oxygen to generate ROS, damaging the epithelium further and causing cells to release pro-inflammatory mediators [82]. Recruited neutrophils and eosinophils exacerbate the oxidative stress by releasing more oxidants and proteolytic enzymes [82]. The intimate relationship between oxidative stress and inflammation is a feature of CS inhalation as well as Cl_2.

1.2.2.3 OVA

Exposure to environmental allergens can aggravate inflammatory conditions such as asthma. CS is an important environmental risk factors associated with asthma as it severely exacerbates the disease [89]. CS enhances Th2 driven sensitization and facilitates the development of allergic airway inflammation in mouse models [83, 89]. In humans, CS is of particular concern for asthmatic patients as approximately a quarter of asthmatics in developed countries are smokers

and active smoking is known to accelerate decline in lung function, increase severity of symptoms and impair the short-term therapeutic response to corticosteroids [90]. In mice, acute challenge with OVA replicates many features of allergic inflammation, including elevated levels of IgE, airway hyperresponsiveness, and eosinophilia, making it a suitable animal model of asthma [91].

1.3 The NF-κB family: master regulators of inflammation

1.3.1 The canonical NF-κB pathway

Many of the pro-inflammatory effects caused by CS and these other environmental exposures is due to the activation of cell signaling pathways such as NF-κB [73]. NF-κB is a family of transcription factors that regulate immunity [92]. Labelled as "the central mediator of the human immune response", NF-κB is activated by hundreds of stimuli including CS, LPS and oxidative stress. NF-κB regulates the transcription over 150 target genes [93] including cytokines and chemokines (*e.g.* IL-1β, TNF-α, CXCL1, and MCP-1), COX-2, C-reactive protein, cell adhesion molecules (ICAM-1, VCAM-1, P-selectin) and immunoreceptors (MHC class I and II, TNFR) [93]. In addition to controlling the expression of genes directly involved in inflammation and immunity, NF-κB also regulates genes involved in survival, apoptosis, growth and other signal transduction pathways [93].

NF-κB was named after its discovery as a constitutively-expressed nuclear transcription factor in mature B lymphocytes that bound to an element in the kappa immunoglobulin light-chain enhancer region [94]. The NF-κB family contains five protein members in mammals: RelA (p65), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB1)[92]. Rel proteins are characterized by their

conserved Rel-homology domain which allows them to form dimers, translocate to the nucleus and bind to κB consensus sequences on target genes [92]. In addition to the Rel-homology domain, these Rel proteins contain a transactivation domain which allows them to act as transcriptional activators [92]. This transactivation domain is absent p50 and p52, and as such, these must pair with one of the Rel proteins in order to transcribe genes [92]. Both p50 and p52 are initially formed as the precursor proteins p100 and p105 and require proteolytic processing to generate p52 and p50, respectively [94].

The NF-κB members form homo- or heterodimers to participate in two distinct cell signaling pathways (Figure 1) [92, 95]. With a few exceptions, most combinations of Rel-containing homodimers and heterodimers can be found *in vivo* [94]. The most common dimer pairing is between RelA and p50, which participate in the canonical NF-κB signaling pathway [93]. This may be because canonical subunit forming proteins RelA and p50 are ubiquitously expressed in all cell types [96]. However, other complexes containing RelA and c-Rel also participate in the canonical signaling pathway [95]. Unlike RelA and p50, the expression of c-Rel is not ubiquitous [97]. High levels of c-Rel expression are found in hematopoietic cells- primarily B and T lymphocytes- but low levels of expression are also seen in endothelial and epithelial cells [97]. Under steady-state conditions in most cell types, the NF-κB subunits are found in the cytoplasm in an inactive form [94]. This is due to the activity of inhibitor of NF-κB α (IκBα) which sequesters RelA:p50 heterodimers in the cytoplasm [96]. Upon activation of this pathway by upstream receptors, IκBα is phosphorylated by an IκB kinase (IKK), catalyzing the polyubiquitination and subsequent proteasomal degradation of IκBα [92]. This liberates the RelA:p50 dimers and allows them to translocate to the nucleus to regulate gene expression [92]. Activation of the canonical pathway is mediated by receptors like IL-1R, TNFR and TLR4 [95].

The canonical NF-kB pathway generally leads to a typical pro-inflammatory response with the upregulation of pro-inflammatory cytokines (*e.g.* TNF-α, IL-1β, IL-6, and IL-8) as well as cell adhesion molecules (*e.g.* E-selectin, ICAM-1) [98].

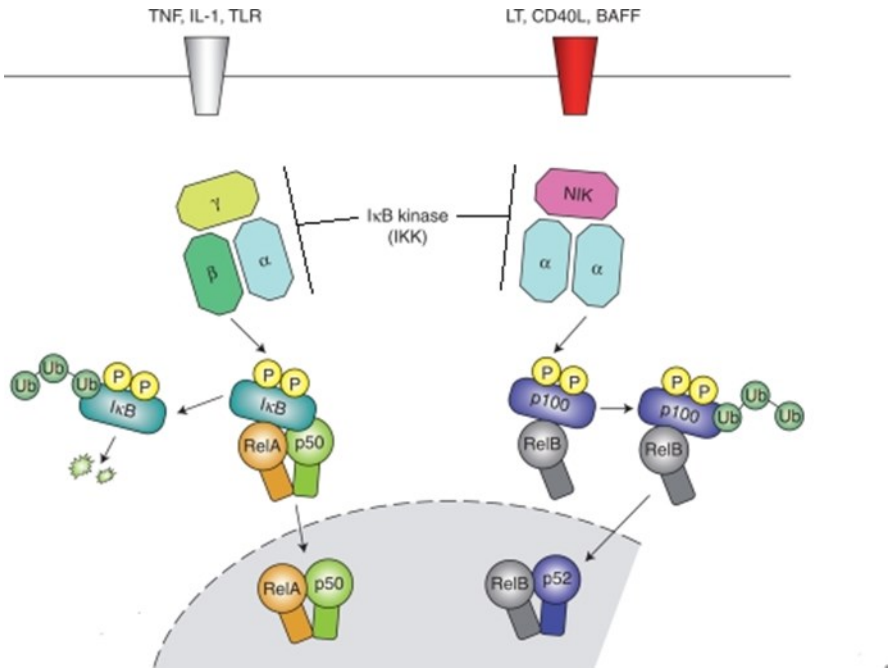

Figure 1. NF-κB family members RelA and p50 participate in the canonical (left) pathway in which upon signalling from upstream TLR and TNF and cytokine receptors, IκB-responsible for sequestering the RelA-p50 dimer in the cytoplasm-is phosphorylated and degraded by the proteasome. Members RelB and p52 participate in the non-canonical (right) pathway in which signalling from TNF family receptors such as BAFF and CD40L causes the processing of p100 to p52 and subsequent release of the Relb-p52 dimer to the nucleus. (Adapted from [95]).

1.3.2 The non-canonical NF-κB pathway

The non-canonical NF-κB pathway involves the protein RelB which typically pairs with p52 [96]. RelB is constitutively expressed in lymphocytes and DCs. However, RelB expression in most innate immune cells is low and requires stimulation to be induced/increased [99]. RelB expression is notable in the thymic medulla, the germinal centers of lymph nodes and marginal zone of spleen [96]. In the lung, RelB is expressed in many cell types including fibroblasts [100].

p52 is also found in low levels in many cell types but is expressed in higher quantities in DCs, T lymphocytes and macrophages [101]. p52 is found mostly in the form of its precursor protein p100 under steady-state conditions [99]. p100 acts as an inhibitor protein, functioning analogously to IκBα through its ability to sequester RelB in the cytoplasm [99]. The NF-κB inducing kinase (NIK) and its target IKKα phosphorylate p100, thereby processing p100 to p52 [95]. The release of the RelB:p52 permits translocation to the nucleus and DNA binding [102].

While there are many similarities between the two pathways, activation of the non-canonical pathway diverges from the canonical pathway in many regards. One major difference between the two NF-κB pathways is that fewer receptors activate the non-canonical pathway [96]. Receptor that preferentially signal to the non-canonical NF-κB pathway include those belonging to the TNFR superfamily including CD40R, B-cell activating factor receptor (BAFFR), lymphotoxin β receptor (LβTR), receptor activator nuclear factor-κB (RANK), TNFR2 and Fn14 [96, 102]. Many of these receptors play important roles in lymphoid organogenesis (*i.e.* LβTR) as well as lymphocyte development, maturation, and activation (BAFFR, CD40) [102]. The receptors for the two pathways are not entirely mutually exclusive, however. The canonical and non-canonical pathway may share some stimuli as both have receptors such as Fn14 (binding to ligand TNF-like weak inducer of apoptosis or TWEAK) and TLR4 [102] in common.

Another major difference between the canonical and non-canonical pathway is the consequences of activation of the two pathways. Although NF-κB proteins all bind to κB sites on target DNA, there is preferential binding of specific NF-κB dimers [93]. Chemokines (SDF1, BLC, ELC and SLC) involved in lymphoid organ development, splenic organogenesis and maintenance of splenic architecture are selective targets of the non-canonical pathway [103, 104]. As a consequence, one important function of the noncanonical NF-κB pathway is associated with

development [105]. For instance, RelB and p100 are required for the formation of germinal center, splenic marginal zones, and follicular DCs [96, 105]. RelB is necessary for the production of antigen specific IgG, the development of NK cells, and development of certain subsets of T lymphocytes [96].

One reason for their different roles for the two pathways is due to the differences between the kinetics of activation in the canonical versus the non-canonical pathway. While the canonical pathway is typically activated within minutes, the non-canonical pathway takes several hours to be activated [106]. This may due to the fact that NIK is rapidly degraded by upstream adaptor protein complexes containing TNF receptor-associated factor protein 3 (TRAF3) under unstimulated conditions and must be synthesized *de novo* upon activation of upstream receptors [99]. Similarly, low levels of RelB mRNA and protein are found in most cells, and RelB must first be upregulated in response to stimuli such as LPS [99]. In effect, the activation of the non-canonical pathway is delayed but long-lasting in comparison to the quick and short-lived canonical pathway [105].

Likely due to their different kinetics, the two pathways may at times work in concert to regulate each other. For example, RelA can promote the transcription of RelB by binding to the κB promoter on the RelB gene [99, 107]. On the other hand, once the non-canonical pathway is activated and RelB expression is upregulated, DNA-bound RelA-containing dimers are subsequently replaced by RelB-p52 [95]. This replacement leads to the downregulation of canonical targets, implying an antagonistic role for the non-canonical pathway [95]. This antagonistic role is most pronounced in the context of inflammation in which the non-canonical pathway (particularly RelB) has anti-inflammatory functions [96].

1.3.3 RelB: evidence for anti-inflammatory roles

There is compelling evidence that RelB is anti-inflammatory. Some of this evidence comes from animal models and in particular RelB-deficient (*RelB*$^{-/-}$) mice [95]. *RelB*$^{-/-}$ mice are sterile due to leukocyte infiltration in the reproductive organs of both *RelB*$^{-/-}$ male and female mice [108]. *RelB*$^{-/-}$ mice lack important immune structures such as the thymic medulla, Peyer's patchs and lymph nodes, further highlighting the importance of RelB in the development of lymphoid organs [96, 109, 110]. *RelB*$^{-/-}$ mice also contain a number of hematopoietic abnormalities such as splenomegaly due to extramedullary hematopoiesis and myeloid hyperplasia [110]. The development of antigen-presenting DCs, negative-selection of T lymphocytes and proliferation of lymphocytes in response to antigenic challenge are also impaired in the absence of RelB [99, 110, 111]. However, one of the most striking phenotypes of *RelB*$^{-/-}$ mice is the presence of multi-organ inflammation [96, 110, 111]. There is massive granulocytic and histiocytic cell infiltration to organs like the lung, liver, spleen, kidney, skin, epididymis and muscle [111]. In the lungs, evidence of inflammation is first apparent when the *RelB*$^{-/-}$ mice are 10 days of age. As the mice reach 3 weeks, a mix of neutrophils, eosinophils and lymphocytes is seen in the lung parenchyma, most notably near the bronchi and major blood vessels [111]. Due to the severity of the inflammation, up to 1/3 of 3-4 week old *RelB*$^{-/-}$ mice die from organ failure [108, 110, 111]. Of the mice that do survive, T-cell dependent atopic dermatitis-like skin lesions develops on the tail and around the eyes [112]. The multi-organ inflammation and increased mortality persist as the mice age, with over half the mice dying within the first couple of months [96, 108].

While the hyper-inflammatory phenotype of *RelB*$^{-/-}$ mice is indicative of an anti-inflammatory role for RelB under homeostatic conditions, there are also a few *in vitro* studies that support a

role for RelB in suppressing stimulus-induced inflammation. First, *RelB*$^{-/-}$ kidney fibroblasts over-express several cytokines (*i.e.* MCP-1, CXCL1, MIP-1α, MIP-1β, MIP-2, IL-10 and RANTES) in response to LPS [113]. Due to the ability of RelB-p52 dimers to replace DNA-bound RelA, it is suggested that in the presence of RelB, pro-inflammatory chemokine production is dampened [95, 113]. The same study used bone marrow chimeras (BMCs) to infer that non-hematopoietic cells also contribute the hyper-inflammatory phenotype in *RelB*$^{-/-}$ mice [95, 113]. These findings indicate that the importance of RelB may extend well beyond cells of the adaptive immune response, playing an important role in non-hematopoeitic cells as well. Another important lesson is that RelB may regulate inflammation induced by specific stimuli and not just under homeostatic conditions. These experiments lead to additional questions about the role of RelB in suppressing inflammation from CS. *In vitro* experiments have shown that RelB suppresses smoke-induced expression of COX-2 and PGE_2 [114] *In vivo*, RelB over-expression attenuated CS-induced COX-2 and PGE_2 in the lungs of wild-type mice [115]. Over-expression of RelB also led to decreased neutrophil infiltration as well as decreased expression of cell adhesion molecule ICAM-1 in response to smoke [115]. Pro-inflammatory cytokines and chemokine expression (including IL-6, CXCL1, TNF-α, and IFN-γ) was also dampened after smoke exposure when RelB was over-expressed [115]. While these findings support an anti-inflammatory role for RelB, the mechanism by which RelB attenuates pulmonary inflammation is largely unknown.

CHAPTER 2: HYPOTHESIS AND AIMS

HYPOTHESIS

We hypothesize that RelB protects against acute CS-induced pulmonary inflammation.

SPECIFIC AIMS:

SPECIFIC AIM 1. Determine if *RelB*$^{-/-}$ mice show heightened inflammation in response to acute CS

SPECIFIC AIM 2. Investigate how the absence of RelB leads to alterations in granulocyte numbers

CHAPTER 3: EXPERIMENTAL PROCEDURES

3.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise indicated

3.2 Animals

RelB-knockout mice (*RelB*$^{-/-}$; C57BL/6-RelbTg(H2-K1/GH1)106Bri/J) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed at the Research Institute of the McGill University Health Center (RI-MUHC, Montreal, QC). Mice were maintained on an ad libitum diet and a 12-h lighting cycle. A breeding scheme of heterozygous x heterozygous mice was used to produce *RelB*$^{-/-}$ mice and wild-type (*RelB*$^{+/+}$) littermate controls. *RelB*$^{-/-}$ mice were phenotypically-distinguishable from control mice by their smaller size, hunched posture and ruffled fur. Mouse genotypes were confirmed by TransnetYX (Cordova, TN, USA). All animal procedures were approved by the McGill University Animal Care Committee (Protocol Number: 5933) and were carried out following the guidelines set out by the Canadian Council on Animal Care.

3.3 Acute CS exposure

Age (range of 7-9 week) and gender-matched *RelB*$^{-/-}$ and RelB$^{+/+}$ mice were exposed to mainstream CS from research cigarettes (3R4F; University of Kentucky, Lexington, KY, USA) via a SCIREQ inExpose system (SCIREQ, Montreal, QC, Canada) according to the Federal Trade Commission (FTC) protocol. Briefly, mice received 1 puff/minute of 2s duration and 35-

ml volume of CS diluted with filtered air for the entire duration of the hour-long exposure. Mice were exposed to CS twice daily for three days with each exposure being an average of 4 hours apart. During these exposures, the target total particulate matter (TPM) ranged from 100-200 mg/m^3. Control mice were kept in room air for the duration of the exposure and manipulated in an identical fashion. Mice were sacrificed either 24 or 72 hours after the last exposure.

3.4 Lipopolysaccharide (LPS) Exposure

LPS from *Pseudomonas aeruginosa* (serotype 10^{22} purified by gel filtration; Sigma-Aldrich, St Louis, MO, USA) was diluted in sterile PBS and given intranasally (10 μg) to $RelB^{-/-}$ and $RelB^{+/+}$ mice. Control mice were given sterile PBS. Mice were anesthetized under isoflurane during delivery of LPS or PBS and were euthanized 24 hours following the exposure.

3.5 Chlorine (Cl_2) Exposure

Chlorine gas (Cl_2; Matheson Gas Products, Ottawa, ON, Canada) was mixed with room air using a Dynacalibrator Calibration Gas Generator (model 230-28A; VICI Metronics, WA, USA). $RelB^{-/-}$ and $RelB^{+/+}$ mice were exposed to 100 ppm Cl_2 for 5 minutes through a nose-only chamber. Control mice were exposed to room air using the nose-only chamber. Mice were sacrificed 24 hours post-exposure.

3.6 Ovalbumin (OVA) Exposure

Mice were first sensitized to 10 μg OVA in sterile PBS with a 10% solution of Imject Alum (Thermo Fisher Scientific, Rockford, IL, USA) by intraperitoneally injection (0.2 mL) or a PBS-10% alum control solution on days 0 and 7. One week after sensitization (days 14, 15, and 16), mice were challenged intranasally with a 30 μL solution of 10μg OVA in sterile PBS under the administration of light isoflurane. Mice were euthanized 48 hours after the last challenge.

3.7 Bronchoalveolar lavage (BAL)

Mice were anesthetized with an intraperitoneal injection of Avertin (2,2,2 tribromoethanol, 250mg/kg, Sigma-Aldrich, St-Louis, MO, USA) and euthanized by exsanguination via cardiac puncture. The lungs were lavaged twice with 0.5 ml PBS and the BAL collected. The BAL was centrifuged and the supernatant was collected for future cytokine analysis. The cell pellet was suspended in ACK lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) for 2 minutes to remove red blood cells. ACK buffer was inactivated by the addition of 20% fetal bovine serum (FBS)-RPMI media. The total cell counts were then determined by counting on a hemocytometer using Typhan blue (Thermo Fisher Scientific, Rockford, IL, USA). Cells were subsequently pelted, resuspended in PBS, cytospin onto a slide (at least 1000 cell/slide) and stained with Hema-Gurr Stain (Merck, Darmstadt, Germany). Images were taken at 40X on a Nikon Eclipse Ni multi-head microscope and differential cell counts were performed using the cell counter plug-in on ImageJ (National Institute of Health, Betheseda, MD, USA).

3.8 Multiplex Analysis

BAL cytokine levels were evaluated by Luminex Technology (MCYTOMAG-70K Milliplex xMAP, Millipore, Billerica, MA, USA). Eleven analytes were assessed (GM-CSF, IFN-γ, IL-1β, IL-4, IL-5, IL-6, Il-10, IL-17α, KC, MCP-1, TNF-α) in 50 uL BAL fluid using the Bio-Plex MAGPIX multiplex reader (BioRad, Hercules, CA, USA). Median Fluorescent Intensity was analyzed using a 5-paramater logistic performed with the Milliplex Analyst Software (Millipore, Billerica, MA, USA).

3.9 Bone Marrow

After sacrifice, the femur and tibia bones were isolated and placed in 10% FBS- RPMI media (Sigma-Aldrich, St-Louis, MO, USA) and cuts were made above and below the joints. Muscle tissue was removed and epiphysis cut, exposing the red bone marrow at the center of the bone. The bone was washed repeatedly with 10 mL of media (10% FBS-RPMI) by inserting a 25G needle and alternating between each side of the bone until it appeared white and translucent. The washings were then pooled, repeatedly aspirated and expulsed five times with an 18G needle connected to a syringe in order to break any cell aggregates in the solution. The cell suspension was finally aspirated and expulsed into a 15 mL tube with a 25G needle, thereby breaking any remaining clumps in the suspension. After centrifugation (1700 rpm for 5 minutes), the cell pellet was resuspended in 0.5 mL of ACK lysis buffer and incubated for 2 minutes. The ACK was neutralized with 10 mL 10%-FBS-RPMI and centrifuged again. The pellet was resuspended

in 2 mL of 10% FBS-RPMI and counted with the Beckman Coulter ACT Diff cell counter (Beckman Coulter, Brea, CA, USA) in the presence of tryphan blue (1:20).

3.10 Flow cytometry

For the bone marrow neutrophil production experiment, a solution of $1x10^6$ cells in PBS-BSA (0.05%) was blocked with Fc block for 30 minutes at 4°C. The cell suspension was then stained with 2.5µg/mL (0.25 µg per 10 cells in 100 µl volume) of rat anti-mouse APC-Cd11b, PE-Ly6C and PE-Cys7-Ly6G (BD Biosciences, San Diego, CA, USA) and subsequently washed with 0.2% PBS-BSA. All the bone marrow samples were pooled to create compensation and staining controls for the flow cytometry which included an unstained solution, three single-stained solutions with only one of the antibodies and three fluorescence minus one (FMO controls). For apoptosis, BAL cells were stained with the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) after 5 minutes of incubation in PBS-BSA. Samples were then analyzed by the BD FACSCanto II Flow Cytometer. For BAL samples 10,000 events were collected while for bone marrow 50,000 events were recorded per sample. Results were analyzed on the FlowJo software (Tree Star Inc., Ashland, OR, USA). Neutrophils were gated for $CD11b^+$Ly6G-high. Monocytes were gated for three distinct populations: $CD11b^+$ $Ly6G^-$ Ly6C-high, $CD11b^+$ $Ly6G^-$ Ly6C-intermediate, and $CD11b^+$ $Ly6G^-$ Ly6C-low.

3.11 Statistical Analysis

GraphPad Prism 7 (v7.2; La Jolla, CA, USA) was used to perform all statistical analysis. Statistical significance was assessed by two-way analysis of variance (ANOVA) followed by a

Neuman-Keuls multiple comparisons test. Evaluation of two-groups was conducted using a student's two-tailed unpaired t-test. In all cases, results are expressed as mean ± SEM and a p-value less than 0.05 was used to indicate statistical significance.

CHAPTER 4: CO-AUTHOR CONTRIBUTIONS

This thesis is based on my original work and is presented as a manuscript currently in preparation for submission. While I conducted the majority of experiments that constitute this thesis, a few experiments were overseen or were performed by the co-authors. These include:

LPS, Cl_2, and OVA challenge: In order to test whether RelB regulated other inhalational toxicants besides CS, we induced acute pulmonary or systemic inflammation in the *RelB*$^{-/-}$ mice via three other relevant environmental exposure regimes. Dr. Angela Rico de Souza performed the LPS and OVA exposures. Dr. Benoit Allard and Dr. Angela Rico de Souza performed the Cl_2 exposures.

Bone marrow extractions and flow cytometry: To test whether RelB deficiency affected the production of neutrophils, we performed flow cytometric analysis of the bone marrow of *RelB*$^{-/-}$ mice. Bone marrow extraction was performed by Dr. Hussein Traboulski. He also oversaw the flow cytometry experiments and analysis for the bone marrow neutrophil production experiments as well as the apoptosis detection in the bronchoalveolar lavage.

CHAPTER 5: RESULTS

5.1 Heightened pulmonary inflammation in naive *RelB*$^{-/-}$ mice is attenuated by acute CS exposure

It was previously demonstrated that in the absence of exogenous stimuli, *RelB*$^{-/-}$ mice have multi-organ inflammation, including in the lung [110]. To our knowledge, characterization of inflammatory cells in the airways via bronchoalveolar lavage (BAL) has not been published in mice before or after exposure to CS. Therefore, we first characterized the nature of the inflammation in the BAL of naive mice (*i.e.* not exposed to smoke). BAL collected from *RelB*$^{+/+}$ mice was mainly composed of alveolar macrophages (Fig. 1A, *closed arrows*). However, BAL from *RelB*$^{-/-}$ mice had numerous granulocytes (Fig. 1A, *arrowheads*), lymphocytes (Fig. 1A, *open arrows*) and macrophages (Fig. 1A, *closed arrows*). Quantification of these BAL cells revealed that *RelB*$^{-/-}$ mice had significantly higher numbers of total inflammatory cells in their airways (Fig. 1B). There were also significantly elevated numbers of macrophages (Fig. 1B), neutrophils (Fig. 1C), eosinophils (Fig. 1D) and lymphocytes (Fig. 1E) in the lung lavage of *RelB*$^{-/-}$ mice compared to the *RelB*$^{+/+}$ mice. These data support that RelB deficiency is associated with heightened pulmonary inflammation.

Our lab has previously published that RelB protects against CS-induced inflammation in lung fibroblasts [114]. Therefore, we also hypothesized that RelB would play a protective role against smoke-induced inflammation *in vivo*. As such we predicted that *RelB*-deficient mice exposed to an acute regimen of CS (*i.e.* 3 days) would display even higher levels of inflammation than naïve *RelB*$^{-/-}$ mice. Due to the high mortality associated with *RelB*$^{-/-}$ mice, we initially exposed the mice to a low dose of CS (200 CPD) and collected the BAL 24 hours after the last exposure. While

this level of smoke caused an insignificant increase in total cell counts of *RelB*$^{+/+}$ mice, low-dose CS caused a dramatic decrease in total cell counts in *RelB*$^{-/-}$ mice (Fig. 1B). This decrease was not characterized by a decrease in macrophages (Fig. 1C), as there was no significant change in the number of macrophages after exposure of *RelB*$^{-/-}$ mice to CS. However, there was a significant decrease in the number of neutrophils (Fig. 1D), eosinophils (Fig. 1E), but not lymphocytes (Fig.1F), in the lung lavage of *RelB*$^{-/-}$ mice exposed to smoke compared to air-exposed *RelB*$^{-/-}$ mice. These data suggest that CS negatively impacts the number of inflammatory cells in the lungs of *RelB*$^{-/-}$ mice, affecting mostly granulocytes.

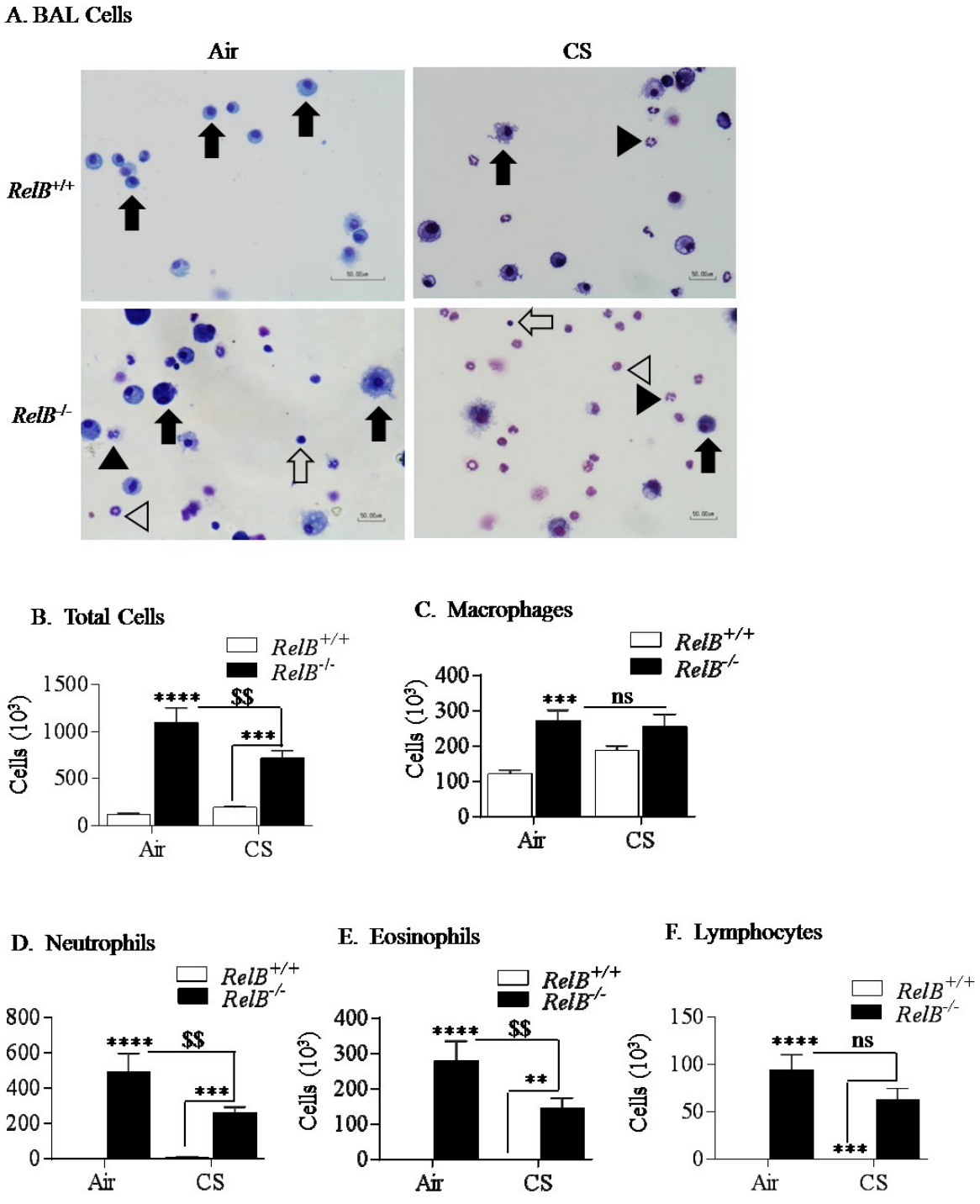

Figure 1. *RelB*$^{-/-}$ mice have decreased granulocytes in response to CS. *RelB*$^{-/-}$ (*black bars*) and *RelB*$^{+/+}$ mice (*white bars*) were exposed to cigarette smoke (CS) or room air for 3 days and BAL was performed 24 hours after the last exposure. (**A**) BAL Cells- air-exposed *RelB*$^{+/+}$ mice had a predominance of macrophages (*black arrows*) while air-exposed *RelB*$^{-/-}$ mice also had neutrophils (black *arrowheads*), eosinophils (*open arrowheads*) and lymphocytes (*open arrows*). CS-exposure caused the appearance of neutrophils in *RelB*$^{+/+}$ mice. **(B)** Total Cells- there was a

significant decrease in total BAL cells in *RelB*$^{-/-}$ mice exposed to CS compared to *RelB*$^{-/-}$ mice exposed to room air ($^{\$\$}p<0.01$). (**C**) Macrophages- the number of macrophages stayed constant after CS exposure in both *RelB*$^{-/-}$ and *RelB*$^{+/+}$ mice (**D**) Neutrophils- there was a decrease in airway neutrophilia of *RelB*$^{-/-}$ mice after exposure to CS ($^{\$\$}p<0.01$). (**E**) Eosinophils-*RelB*$^{-/-}$ also displayed a decrease in airway eosinophilia after CS exposure ($^{\$\$}p<0.01$). (**F**) Lymphocytes-CS did not alter the number of lymphocytes in the BAL in *RelB*$^{-/-}$ and *RelB*$^{+/+}$ mice. In all cases, *RelB*$^{-/-}$ mice had higher cells in their airways even after CS exposure relative to *RelB*$^{+/+}$ mice ($^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$). Results are presented as the mean ± SEM (n = 19–21 mice per group)

5.2 Decreased BAL cells in CS-exposed *RelB*$^{-/-}$ mice are not due to alterations in chemotactic cytokine levels.

Cytokines released by immune and structural cells can partake in a number of processes involved in inflammation, including the further recruitment of inflammatory cells. RelB has been implicated in the transcription of several cytokines *in vitro* [113], leading us to speculate that the decrease in BAL cells after smoke exposure was due to a corresponding decrease in chemotactic cytokines levels. To determine the effect of RelB deficiency on cytokine levels in response to CS, we quantified 11 different cytokines in the BAL fluid using a multiplex array. There were significantly higher levels of IL-6 (Fig. 2A, *panel i*), TNF-α (Fig 2B, *panel iii*) and IL-4 (Fig. 2F, *panel i*), with there being a trend (although not significant) towards an increase in KC (Fig. 2A, *panel ii*), MCP-1 (Fig. 2C), Th17 (Fig. 2D) and IL-5 (Fig. 2F, panel ii) in the BAL of naive (air-only) *RelB*$^{-/-}$ mice compared to naive *RelB*$^{+/+}$ mice. Conversely, there were significantly lower levels of the Th1 cytokine IFN-γ (Fig. 2B, panel i) and the anti-inflammatory cytokine IL-10 (Fig. 2E) in the lungs of naïve *RelB*-deficient mice relative to *RelB*$^{+/+}$ mice. These data support that there is a heightened inflammatory phenotype in the lungs of *RelB*$^{-/-}$ mice.

We also compared cytokine levels in the BAL after acute CS exposure. With the exception of IL-4 (Fig. 2F, *panel i*), which decreased in *RelB*$^{-/-}$ mice after smoke exposure, there was no significant change in cytokine levels in *RelB*$^{-/-}$ mice after exposure to CS compared to air-exposed *RelB*$^{-/-}$ mice (Fig. 2). This included neutrophil chemotactic cytokines such as IL-6 and KC (Fig. 2A), which remained the same. Cytokines involved in neutrophil production such as GM-CSF (Fig. 2A, *panel iii*) and eosinophil recruitment/activation such as IL-5 (Fig. 2F, *panel ii*) were also unaffected by CS. Thus, while our data signify that RelB plays a role in the homeostatic control of lung inflammation, with the potential exception of IL-4, decreased levels of chemotactic cytokines cannot fully explain the decrease in granulocyte (predominantly neutrophil and eosinophil) numbers in the lungs of *RelB*$^{-/-}$ mice after CS exposure.

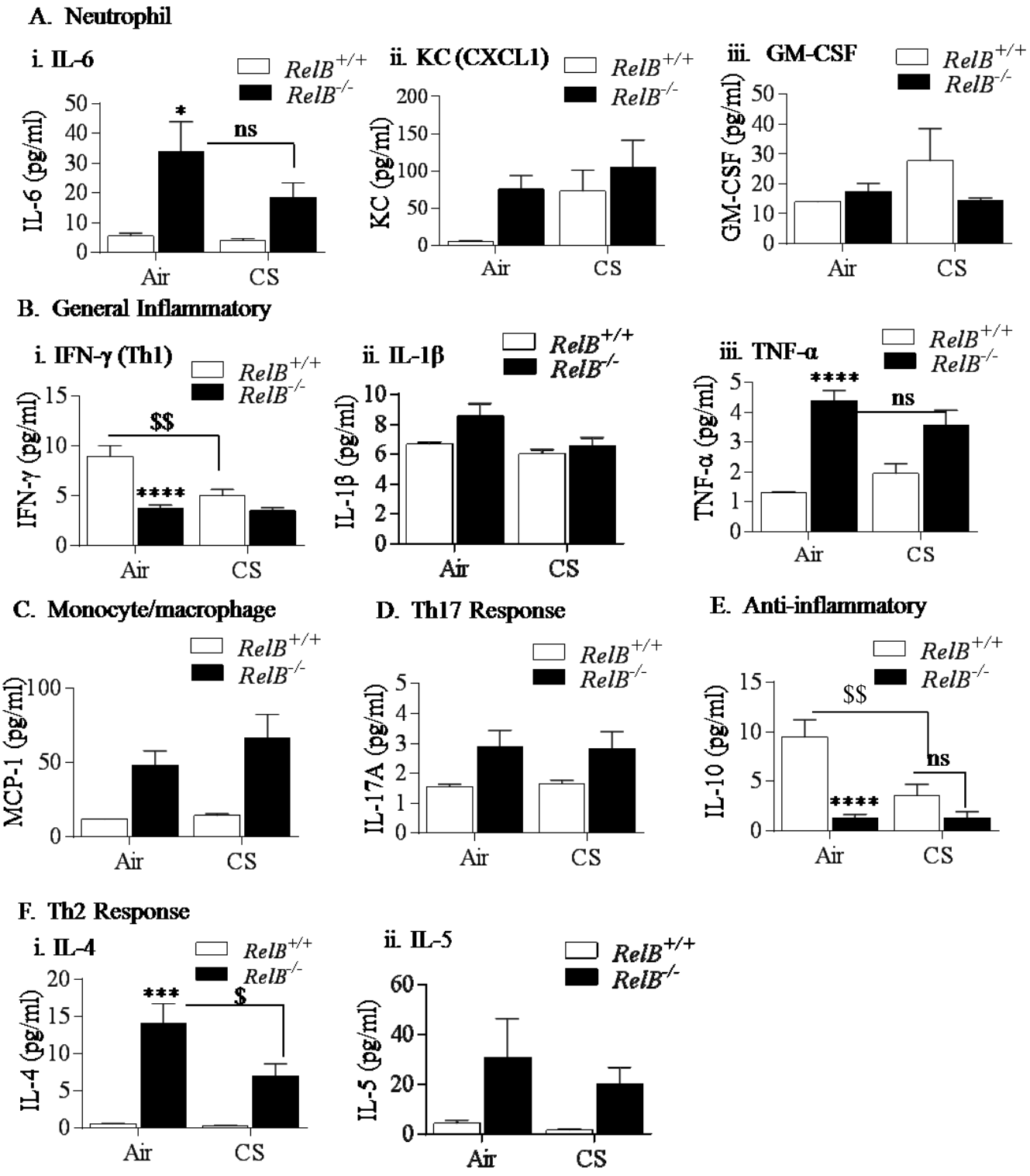

Figure 2. CS exposure does not alter the dysregulated cytokine profile of *RelB*$^{-/-}$ mice. BAL fluid from air and cigarette smoke (CS)-exposed *RelB*$^{-/-}$(*black bars*) and *RelB*$^{+/+}$ mice

(*white bars*) was analyzed by multiplex technology to assess levels of several inflammatory cytokines. In the air-exposed mice, a few cytokines were dysregulated in the *RelB*$^{-/-}$ mice compared to *RelB*$^{+/+}$ mice with IL-6, TNF-α, and IL-4 elevated and IL-10, IFN-γ decreased in the *RelB*$^{-/-}$ mice ($^{*}p<0.05$; $^{***}p<0.001$; $^{****}p<0.0001$). (**A**) CS-exposure did not significantly change the levels of cytokines involved in neutrophil activation, trafficking, or production: IL-6 **(i)**, KC **(ii)** GM-CSF **(iii)** in *RelB*$^{-/-}$ or *RelB*$^{+/+}$ mice**.** (**B**) General pro-inflammatory cytokines IFN-γ (**i**), IL-1β (**ii**), and TNF-α (**iii**) were largely unaffected by CS-exposure in *RelB*$^{-/-}$ mice although IFN-γ levels decreased in CS-exposed *RelB*$^{+/+}$ mice ($^{\$\$}p<0.01$). Similarly monocyte/macrophage trafficking cytokine MCP-1 and (**C**), Th17 cytokine were unchanged with CS-exposure. (D) Although anti-inflammatory cytokine IL-10 (**E**) did not change in CS-exposed RelB$^{-/-}$ mice, it was downregulated in the CS-exposed *RelB*$^{+/+}$ mice ($^{\$\$}p<0.01$). (F) Th2 cytokine IL-4 (**i**) was the only analyte which decreased in CS-exposed *RelB*$^{-/-}$ mice ($^{\$\$}p<0.01$) while being unaffected in the *RelB*$^{+/+}$ mice. IL-5, another Th2 cytokine was unaffected by CS-exposure (**ii**). Results are presented as the mean ± SEM (n = 5–8 mice per group).

5.3 RelB does not regulate granulocytic inflammation induced by LPS, OVA or Cl_2

Our data unexpectedly, demonstrated that acute CS exposure decreased the number of neutrophils and eosinophils in the lungs of *RelB*$^{-/-}$ mice. We therefore wondered whether other inhalational exposures would have a similar effect. To answer this question, we first used LPS as a pro-inflammatory stimulus, given that RelB has previously been shown to suppress LPS-induced inflammatory mediator production in lung fibroblasts [113]. LPS is also present in CS, making this a relevant exposure [84]. Therefore, we administered LPS intranasally to *RelB*$^{+/+}$ and *RelB*$^{-/-}$ mice and evaluated inflammatory cells in the BAL after 24 hours. There was a significant rise in inflammatory cells in the airways of *RelB*$^{+/+}$ administered 10 μg of intranasal LPS, which could be attributed to a significant increase in airway neutrophilia (Fig. 3A-B 3D). Inflammatory cells in *RelB*$^{-/-}$ mice, however, did not change in response to LPS. LPS treatment did not affect macrophage (Fig. 3C) or lymphocyte (Fig. 3F) numbers. Furthermore, neither neutrophils (Fig. 3D) or eosinophils (Fig. 3E) were affected by LPS in *RelB*$^{-/-}$ mice. This suggests that the

decrease in granulocytes (neutrophils and eosinophils) observed after exposure of *RelB*$^{-/-}$ mice to cigarette smoke is not likely due to bacterial endotoxin.

We next evaluated the response of *RelB*$^{-/-}$ mice to OVA, a commonly-used agent to induce allergic asthma in mice. We chose the OVA model because, unlike the other inhalational exposures assessed, the adaptive immune system drives OVA-induced inflammation, and OVA elicits a Th2-driven response that can cause the recruitment of eosinophils to the lung [91]. While there were more inflammatory cells in the airways of *RelB*$^{-/-}$ mice, total cell counts nor other cell types examined (macrophages, neutrophils, eosinophils or lymphocytes) changed in *RelB*$^{-/-}$ mice upon OVA exposure (Fig. 4).

Finally, we assessed whether Cl_2, another environmental lung toxicant that induces an early neutrophilic response, could alter granulocytic inflammation in the lungs of *RelB*$^{-/-}$ mice. Cl_2 is a relevant exposure, as it is present in household products and poses a risk to swimmers [82]. To our knowledge, there is no information on RelB regulating pulmonary inflammation in response to Cl_2 exposure. Although there was a perceptible decrease in total inflammatory cells in Cl_2-exposed *RelB*$^{-/-}$ mice, this decrease was not statistically-significant (Fig 5B).There was also a trend towards a decrease in airway neutrophilia in Cl_2-exposed *RelB*$^{-/-}$ mice (Fig 5D). However, there was no significant difference between Cl_2 exposed *RelB*$^{+/+}$ and *RelB*$^{-/-}$ mice. There was also no significant change in macrophages (Fig 5C), eosinophils (Fig 5E) or lymphocytes (Fig 5F) after Cl_2 exposure. We also measured BAL cytokine levels in air and Cl_2-exposed *RelB*$^{+/+}$ and *RelB*$^{-/-}$ mice (Fig. 6). In general, cytokine levels were largely unchanged by Cl_2 exposure, with the exception of IL-17A which was significantly decreased in response to Cl_2 in *RelB*$^{-/-}$ mice (Fig. 6D). IFN-γ and IL-10 significantly decreased in response to Cl_2 in *RelB*$^{+/+}$ mice (Fig. 6B and 6E, respectively). Thus, we conclude that the neutrophil-specific decrease we observed in the

RelB$^{-/-}$ was specific to CS, as there was no change in neutrophils in response to these other environmental toxicants.

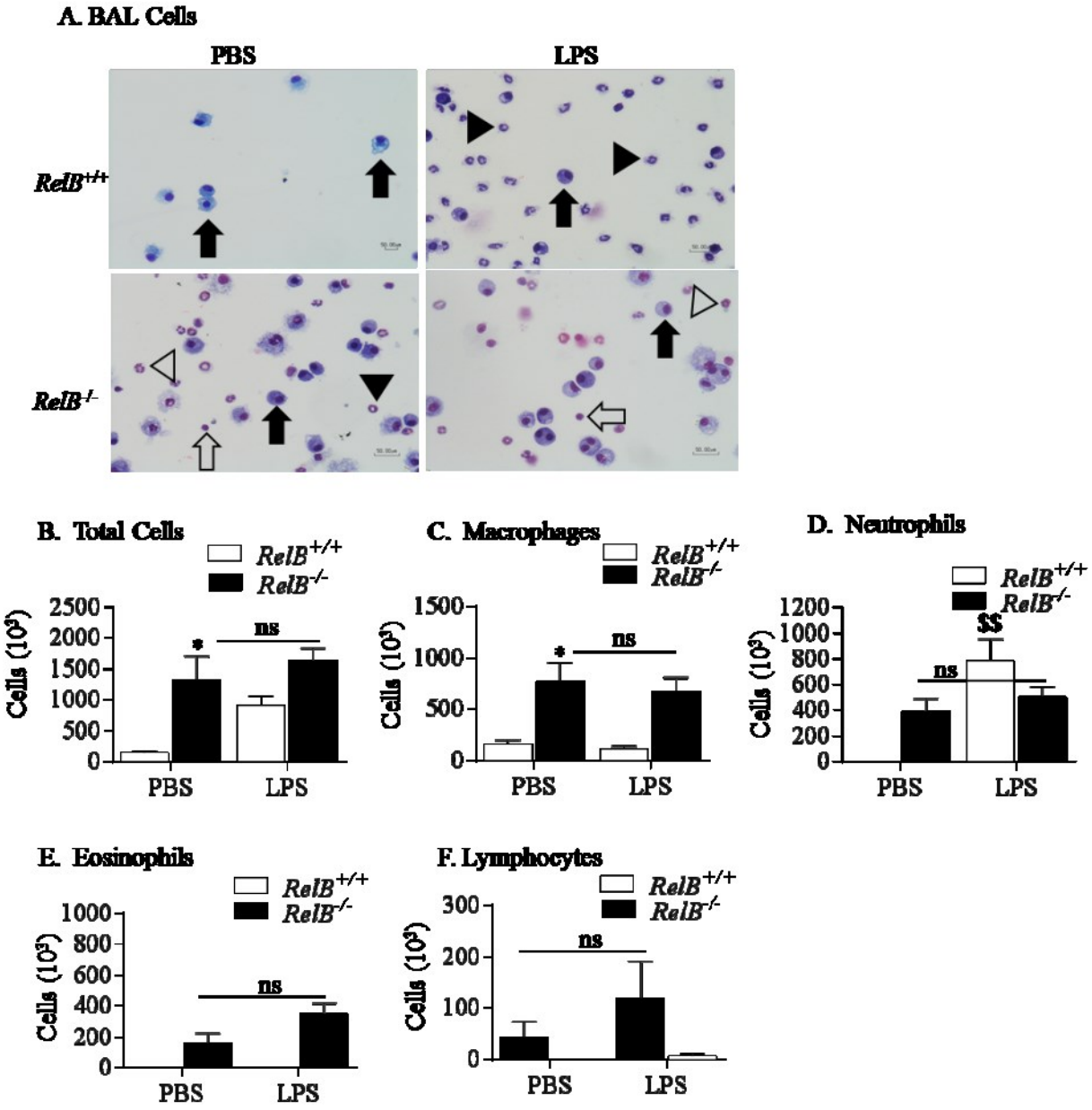

Figure 3. LPS exposure does not alter BAL cellularity in *RelB*$^{-/-}$ mice. *RelB*$^{-/-}$ and *RelB*$^{+/+}$ mice were exposed to acute model of granulocytic irritant LPS (*black bars*) or delivery control

PBS (*white bars*). (**A**) BAL Cells- Representative image of LPS or PBS-exposed *RelB*$^{+/+}$ and *RelB*$^{-/-}$ mice. Note the presence of neutrophils (black *arrowheads*) and macrophages (*black arrows*) observed in LPS-treated *RelB*$^{+/+}$ mice. *PBS and LPS*-treated *RelB*$^{-/-}$ mice additionally had eosinophils (*open arrowheads*) and lymphocytes (*open arrows*). (**B**) Total Cells-LPS exposed *RelB*$^{+/+}$ mice have higher number of cell in BAL relative to *RelB*$^{+/+}$ air controls. Cell numbers do not change in *RelB*$^{-/-}$ mice post LPS treatment ($^{*}p<0.05$). **(C)** Macrophage-counts do not change upon LPS treatment. (**D**) Neutrophils-LPS causes an increase in airway neutrophilia in *RelB*$^{+/+}$ mice but does not alter cellularity in *RelB*$^{-/-}$ mice ($^{\$\$}p<0.01$). (**E**) Eosinophils-LPS does not affect eosinophil numbers (**F**) Lymphocytes-LPS does not change lymphocyte numbers Results are presented as the mean ± SEM (n = 3–4 mice per group).

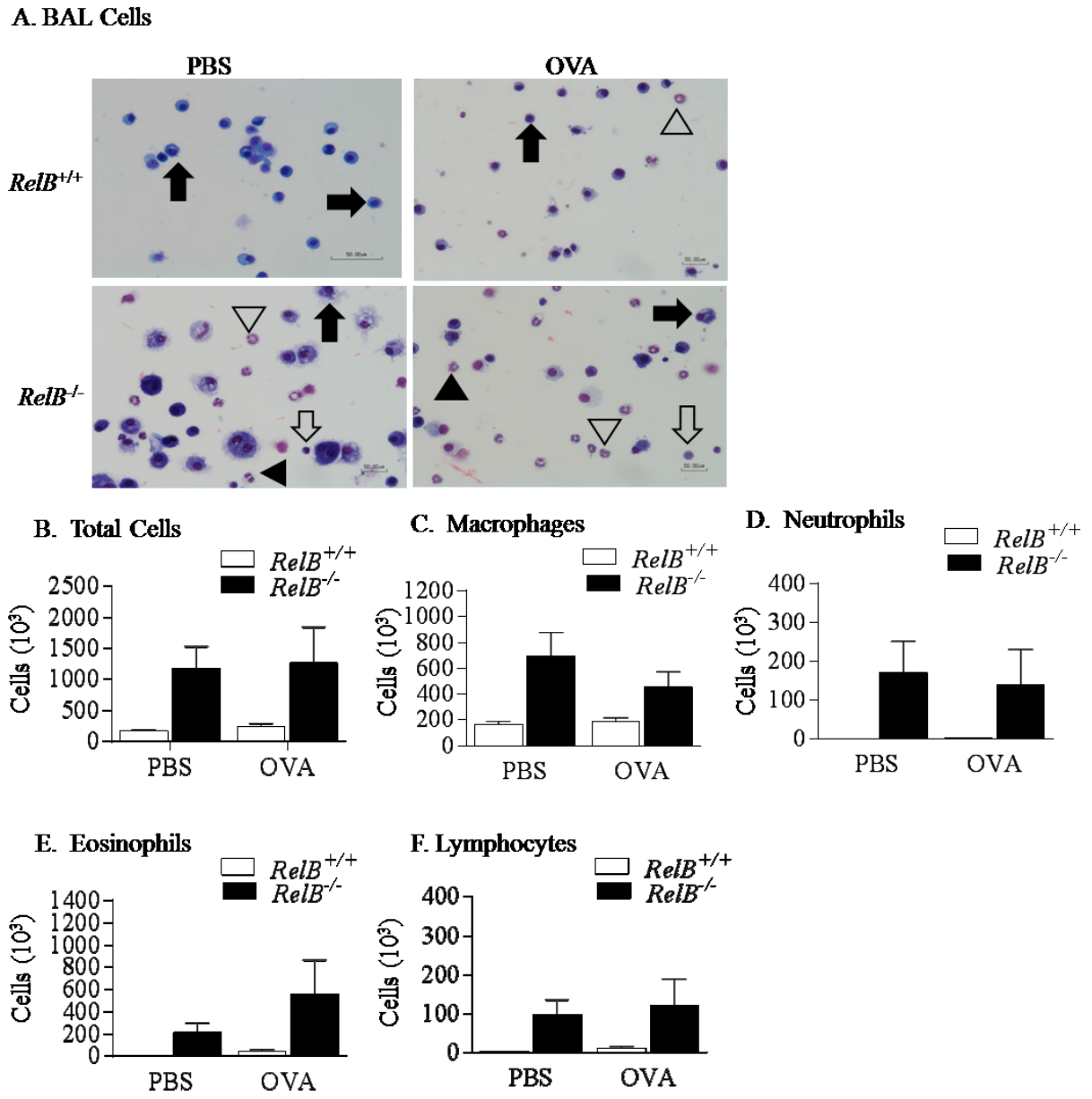

Figure 4. OVA exposure does not alter airway cellularity in *RelB*$^{-/-}$ mice. *RelB*$^{-/-}$ and *RelB*$^{+/+}$ were treated with ovalbumin (OVA; *black bars*) or PBS (*white bars)* for 3 consecutive days after sensitization and sacrificed 48 hours prior to the last exposure. (**A**) BAL Cells- Representative image of OVA or PBS-exposed *RelB*$^{+/+}$ and *RelB*$^{-/-}$ mice. Note the presence of eosinophils (*open arrowheads*) and macrophages (*black arrows*) observed in OVA-treated *RelB*$^{+/+}$ mice. *PBS and OVA*--treated *RelB*$^{-/-}$ mice additionally had neutrophils (*black arrowheads*) and lymphocytes (*open arrows*). OVA exposed mice do not have a significant change in the total BAL cells (**B**), macrophages (**C**), neutrophils **(D),** eosinophils (**E**), or lymphocytes (**F**) Results are presented as the mean ± SEM (n = 5-6 mice per group).

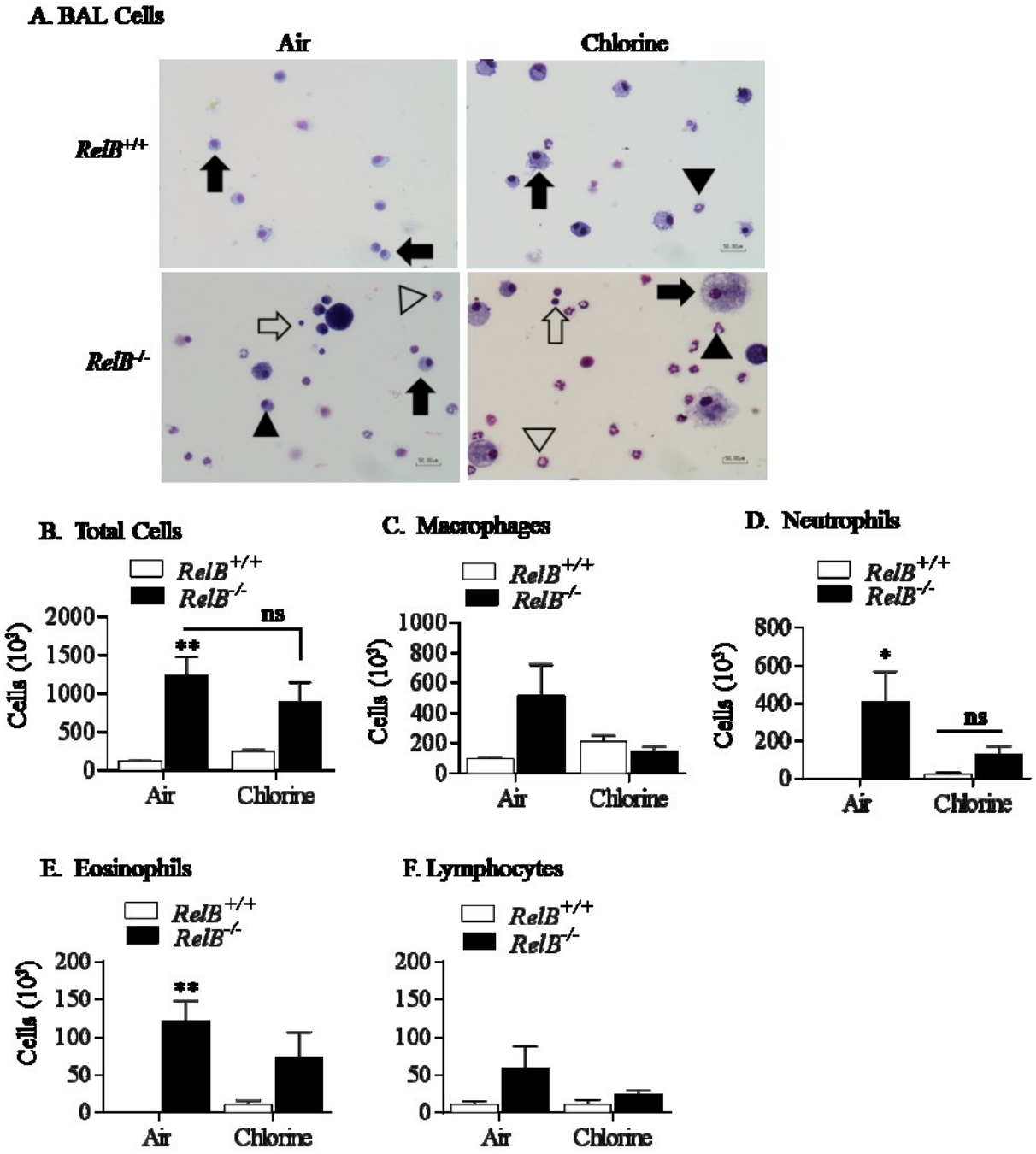

Figure 5. Cl_2 exposure does not alter airway cellularity in *RelB*$^{-/-}$ mice. *RelB*$^{-/-}$ and *RelB*$^{+/+}$ mice were exposed to Cl_2 *(black bars)* and room air (*white bars*). (**A**) BAL Cells- Representative image of Cl_2 or air-exposed *RelB*$^{+/+}$ and *RelB*$^{-/-}$ mice. Note the presence of neutrophils (black *arrowheads*) and macrophages (*black arrows*) observed in Cl_2-treated *RelB*$^{+/+}$ mice. Air and Cl_2-treated *RelB*$^{-/-}$ mice additionally had eosinophils (*open arrowheads*) and lymphocytes (*open arrows*). (**B**) Total Cells- Cl_2 exposed mice do not have a significant change in the total BAL cell numbers. The macrophage(**C**), neutrophil **(D)**, eosinophil (**E**) and lymphocyte (**F**) counts are also unaffected by Cl_2 exposure in both *RelB*$^{-/-}$ and *RelB*$^{+/+}$ mice. Note that total cell counts, neutrohilia and eosinophilia are significantly higher in the air-exposed *RelB*$^{-/-}$ mice relative to the *RelB*$^{+/+}$ mice (*p<0.05; **p<0.01) Results are presented as the mean ± SEM (n = 7-11 mice per group).

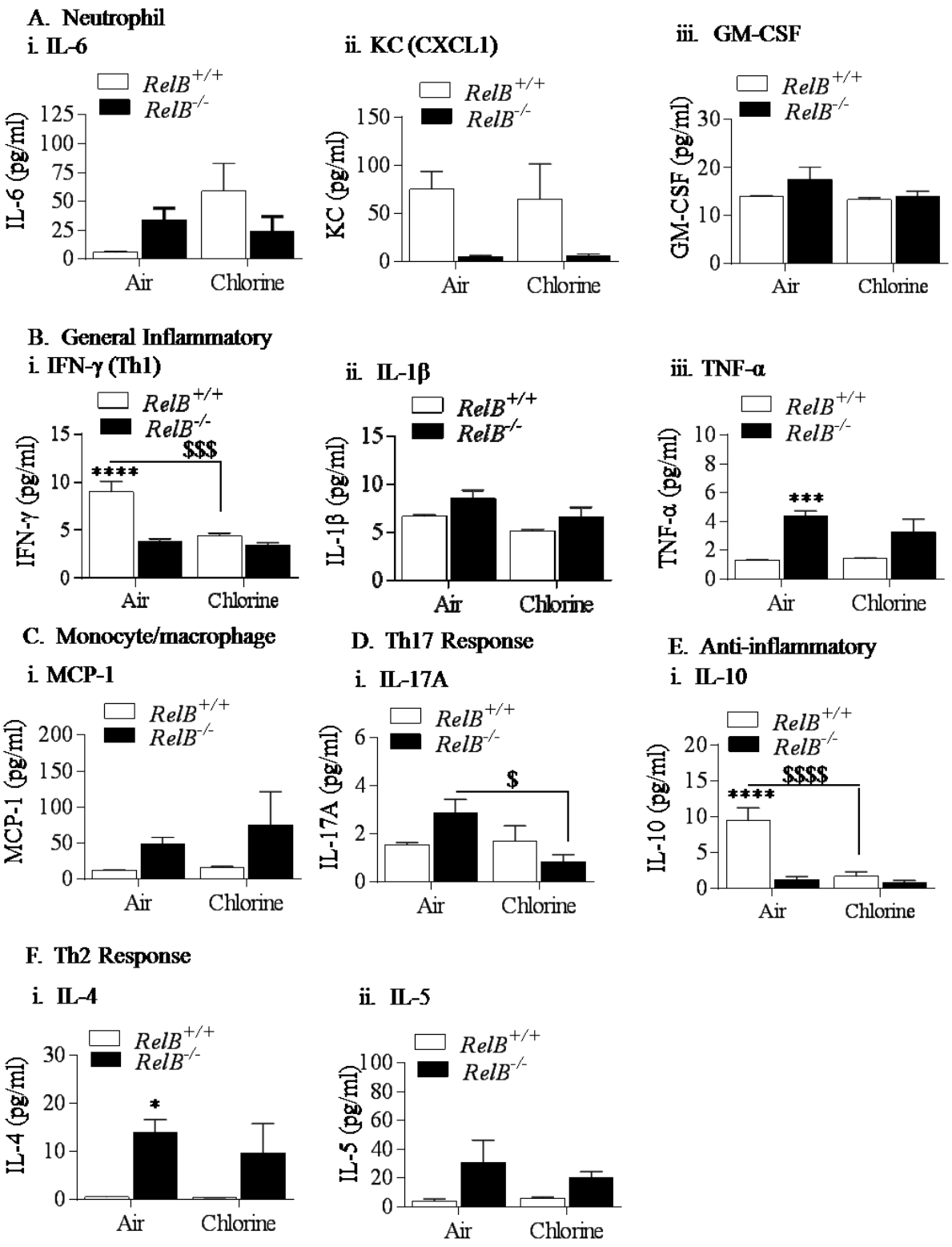
A. Neutrophil
i. IL-6
ii. KC (CXCL1)
iii. GM-CSF
B. General Inflammatory
i. IFN-γ (Th1)
ii. IL-1β
iii. TNF-α
C. Monocyte/macrophage
i. MCP-1
D. Th17 Response
i. IL-17A
E. Anti-inflammatory
i. IL-10
F. Th2 Response
i. IL-4
ii. IL-5
RelB+/+
RelB-/-
IL-6 (pg/ml)
KC (pg/ml)
GM-CSF (pg/ml)
IFN-γ (pg/ml)
IL-1β (pg/ml)
TNF-α (pg/ml)
MCP-1 (pg/ml)
IL-17A (pg/ml)
IL-10 (pg/ml)
IL-4 (pg/ml)
IL-5 (pg/ml)
Air
Chlorine
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Figure 6. Cl_2 exposure decreases IL-17A expression in airways of *RelB*$^{-/-}$ mice. BAL fluid from air and Cl_2-exposed *RelB*$^{-/-}$(*black bars*) and *RelB*$^{+/+}$ mice (*white bars*) was analyzed by multiplex technology to assess levels of several cytokine. Cl_2 exposure largely did not affect the levels of neutrophil activating and producing cytokines (**A**) IL-6 **(i)**, KC (**ii**) and GM-CSF (**iii)**. Although general pro-inflammatory cytokine (**B**) IFN-γ decreased in Cl_2-exposed *RelB*$^{+/+}$ mice ($^{\$\$\$}$p<0.001), levels in *RelB*$^{-/-}$ mice were unaffected by the exposure (**i**). Similarly IL-1β (**ii**) and TNF-α (**iii**) were unchanged as was the monocyte/macrophage chemokine (**C**) MCP-1. (**D**) Th-17 cytokine IL-17A was the only cytokine which decreased in CS-exposed *RelB*$^{-/-}$ mice ($^{\$}$p<0.05). **(E)** IL-10 was also decreased by Cl_2 exposure, however this was only pronounced in the *RelB*$^{+/+}$ mice ($^{\$\$\$\$}$p<0.0001). The Th2 cytokines (**F**) IL-4 (**i**) and IL-5 (**ii**) were unchanged by the exposure. Note several cytokines were dysregulated in the naïve *RelB*$^{-/-}$ mice relative to *RelB*$^{+/+}$ mice (****p<0.0001; ***p<0.001; *p<0.05). Results are presented as the mean ± SEM (n = 5–6 mice per group)

5.4 There is a sustained drop in BAL neutrophils even after CS cessation in *RelB*$^{-/-}$ mice

The previous experiments demonstrated that RelB regulates granulocytic inflammation in response to CS. Our data also make it unlikely that significant alterations in most of the granulocyte-activating and/or trafficking cytokines (with the potential exception of IL-4) accounts for the decrease in neutrophils and eosinophils in *RelB*$^{-/-}$ after smoke exposure. We next wondered if this decrease in granulocytes persisted even after smoke cessation. In our earlier experiments, all smoke-exposed mice were sacrificed 24 hours after the last exposure. Therefore, to assess whether the granulocytic decrease was transient, we also sacrificed the mice 72 hours after the last CS exposure. In the *RelB*$^{+/+}$ mice, there was no difference in total cell numbers either after 24 or 72 hours of smoke exposure (Fig. 7A) and there was a slight-but significant increase in neutrophils only after cessation for 72 hours (Fig. 7A). However, in the *RelB*$^{-/-}$ mice, the total number of BAL cells as well as the number of neutrophils was decreased at both the 24 and 72 hour time-points (Fig. 7B). Thus, the decrease in neutrophils persists even after cigarette smoke cessation for up to 72 hours. There was no difference in eosinophils after 72 hours of cessation compared to air-exposed mice (Fig. 7B). These results suggest that CS exposure causes an initial and sustained decrease in airway neutrophils that only occurs in the absence of RelB.

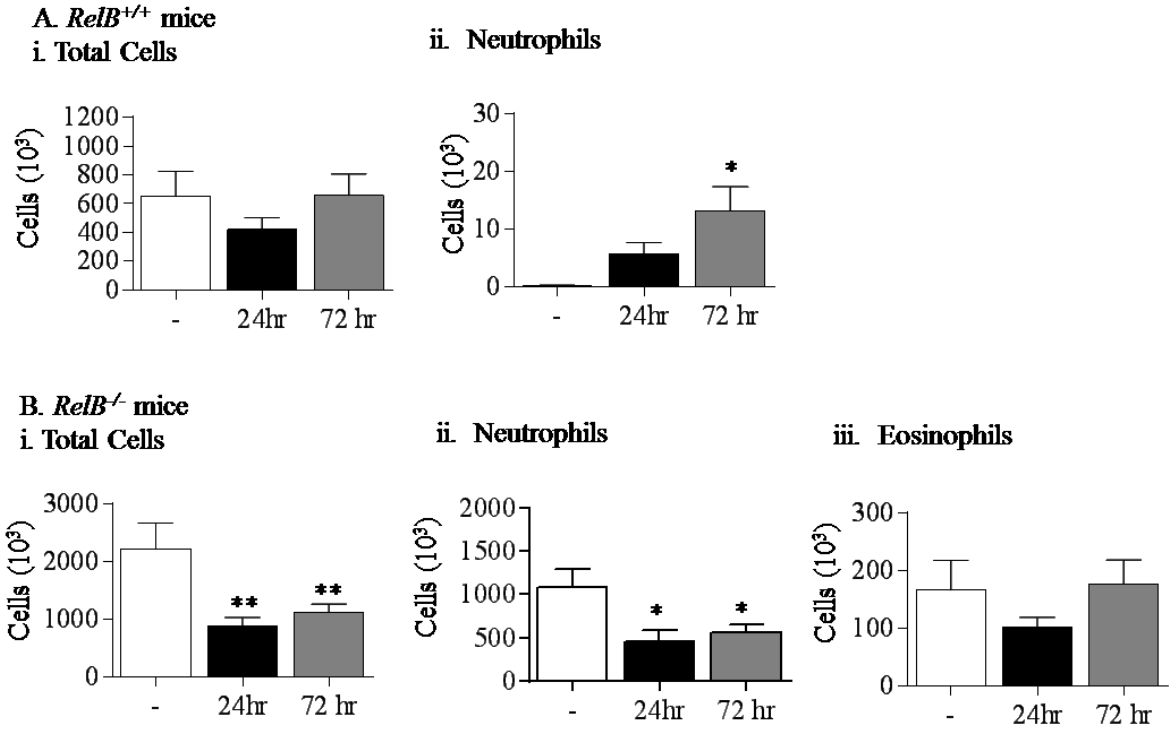

Figure 7. Decrease in CS-exposed *RelB*$^{-/-}$ neutrophils is sustained after 72 hours. To assess the nature of the CS dependent decrease in granulocytes, *RelB*$^{+/+}$ mice (A) and *RelB*$^{-/-}$ mice (B) were exposed to CS or room air (*white bars*) and sacrificed either 24 hours (*black bars*) or 72 hours (*grey bars*) post-exposure. (**A**) In *RelB*$^{+/+}$ mice, total cells (**i**) numbers did not increase upon smoke exposure in either group and neutrophil (**ii**) counts were also unaffected upon smoke exposure for both the 24 and 72 hour groups.(B) *RelB*$^{-/-}$ mice sacrificed 24 hours post CS-exposure had a decrease in total BAL cells (**i**) relative to air controls which persisted at 72 hours (**p=0.0064-0.0079). There was a decrease in neutrophils (**ii**) in *RelB*$^{-/-}$ mice 24 hours after CS (*p=0.0392) relative to air controls which persisted after 72 hours (*p=0.0426). Changes in eosinophil numbers (**iii**) were not statistically significant. Results are presented as the mean ± SEM (n = 14-26 mice per group for total cell counts, n=4-14 mice per group for neutrophil and eosinophil counts)

5.5 CS-induced decrease in airway neutrophils in *RelB*$^{-/-}$ mice is not due to altered cell death

Our observation that there is a sustained drop in airway neutrophils could be due to induction of cell death (apoptosis) from CS exposure. Given that our lab has shown that RelB protects against smoke-induced apoptosis in lung structural cells [100], this is a reasonable hypothesis. Therefore, we performed flow cytometry on the BAL of air and smoke-exposed mice using a classic apoptosis detection kit where annexin V was used as a marker for apoptosis and 7-AminoactinomycinD (7-AAD) was used as a marker for necrotic cell death. In these experiments, mice were sacrificed 24 hours after the last smoke exposure. BAL cells that highly expressed both markers were considered to be undergoing late apoptosis, while cells positive for only Annexin V or 7-AAD were classified as early apoptotic of necrotic cells, respectively (Fig. 8A). The majority of BAL cells were negative for annexin V and 7-AAD and were therefore considered healthy (Fig. 8A). The percentage of BAL cells in the early stages of apoptosis (Annexin V-positive) was significantly lower in smoke-exposed *RelB*$^{-/-}$ mice relative to air-exposed *RelB*$^{-/-}$ mice (Fig. 8B). There was no difference in the percentage of apoptotic cells in the CS-exposed *RelB*$^{+/+}$ mice compared to air-exposed mice. The percentage of late apoptotic cells and necrotic cells were similar between air- and smoke-exposed mice with very little difference between those with and without RelB (Fig. 8C and 8D). Overall, these results indicate that the initial decrease in BAL neutrophils in response to cigarette smoke in *RelB*$^{-/-}$ mice cannot be explained by increased cell death.

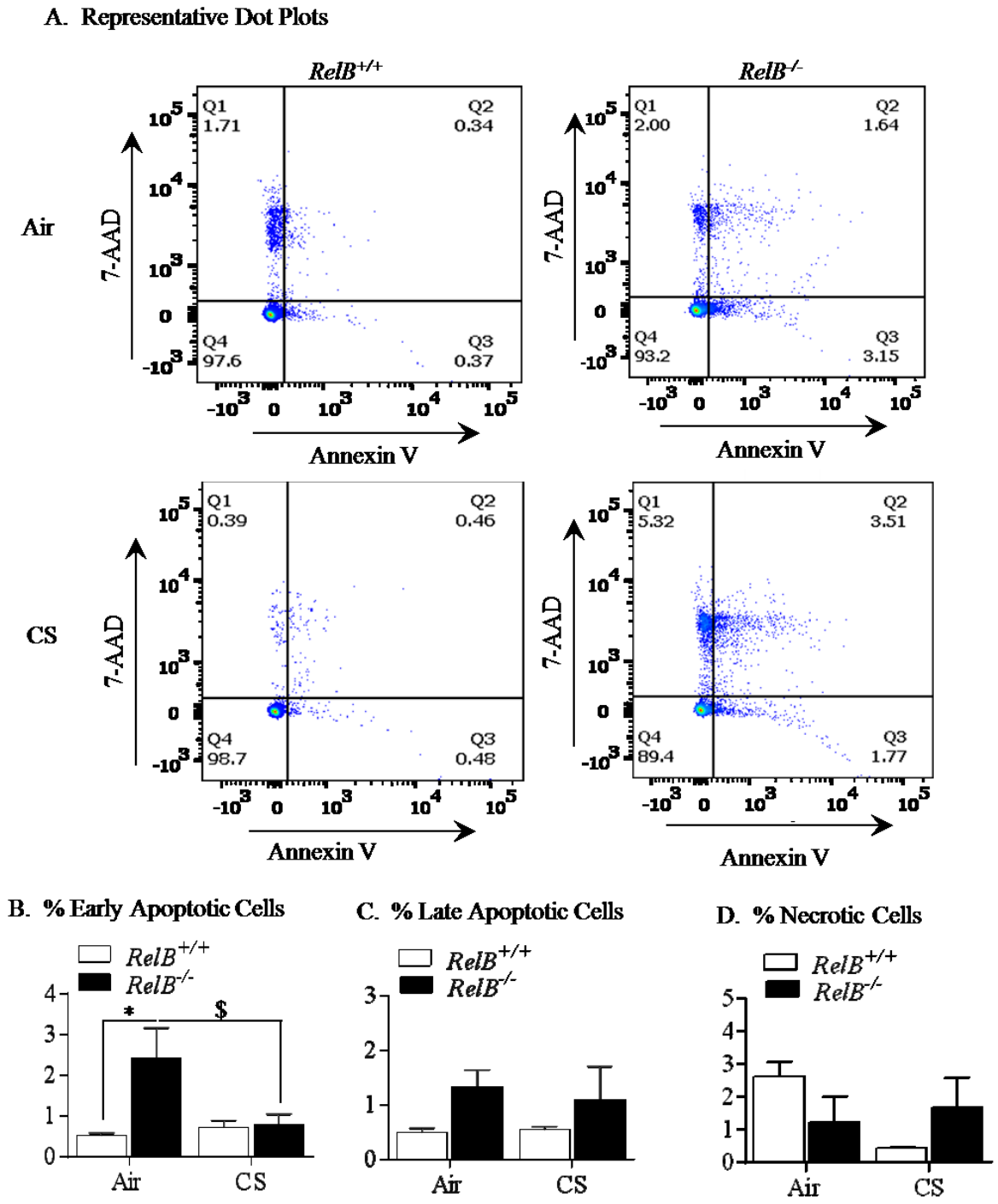

Figure 8. Decrease in CS-exposed *RelB*$^{-/-}$ granulocytes is not due to increased cell death. BAL collected from *RelB*$^{-/-}$ (*black arrows*) and *RelB*$^{+/+}$ (*white arrows*) mice exposed to CS or room air was analyzed by an apoptosis detection kit via flow cytometry. (**A**) Representative dot plot- Annexin (x-axis) marker for apoptosis and 7-AAD (y-axis) marker for necrosis were used to show levels of apoptotic and necrotic cell death in *RelB*$^{-/-}$ (right) and *RelB*$^{+/+}$ (left) mice

exposed to air (top) or CS (bottom). (**B**) % Early Apoptotic Cells- the percentage of early apoptotic cells slightly decreases in RelB$^{-/-}$ mice treated with CS ($^{\$}p<0.05$). Note that air-exposed *RelB*$^{-/-}$ mice have a higher percentage of early apoptotic cells than air exposed *RelB*$^{+/+}$ mice (*$p<0.05$). The percentage of late apoptotic cells (**C**) and the percentage of necrotic cells (**D**) do not change with CS. Results are presented as the mean ± SEM (n = 2–5 mice per group)

5.6 RelB does not alter the production of neutrophils from the bone marrow

Another possibility that could explain our observation that there are altered airway neutrophils in *RelB*$^{-/-}$ mice is if there was increased/decreased production in the bone marrow. Altered production in the bone marrow could also potentially explain our observation that neutrophils were reduced in the BAL of *RelB*$^{-/-}$ mice after smoke exposure. Therefore, we assessed the production of neutrophils from the bone marrow by flow cytometry in naive mice. However, bone marrow production of neutrophils was virtually identical between naive *RelB*$^{+/+}$ and *RelB*$^{-/-}$ mice (Fig. 9A-C). Because of the markers used in the identification of neutrophils, we were also able to quantify different population of monocytes based on Ly6C expression. These data suggest that RelB deficiency skews the relative abundances of monocytes such that *RelB*$^{-/-}$ bone marrow had significantly higher levels of Ly6C-low and Ly6C-intermediate monocytes (Fig. 9D-F). Overall, the result of this works suggests that RelB plays an important role in homeostatic control over inflammation in the respiratory system.

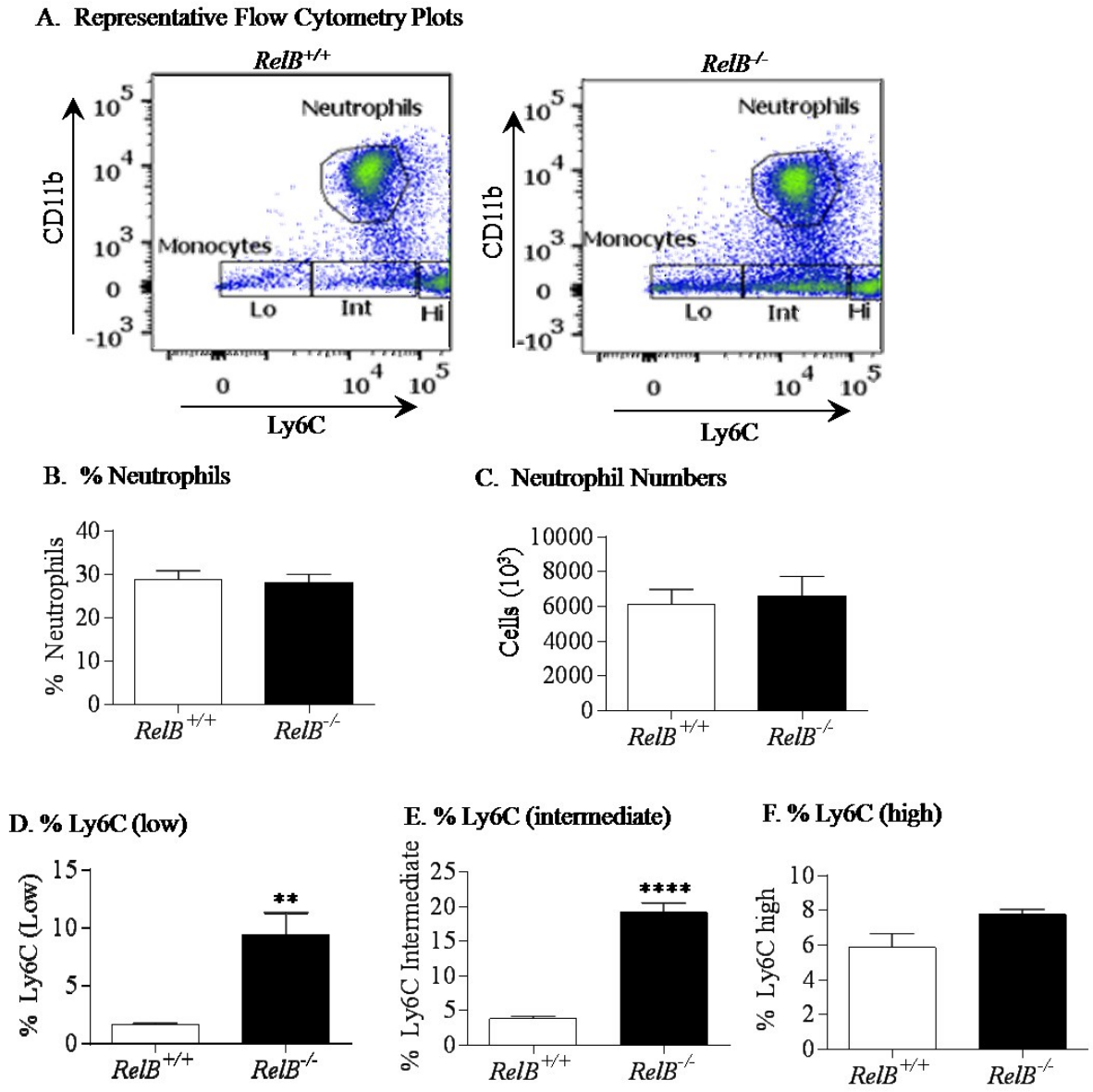

Figure 9: *RelB*$^{-/-}$ bone marrow has higher percentages of Ly6C-low and Ly6C-intermediate monocytes but the same percentage of neutrophils as *RelB*$^{+/+}$ bone marrow. Bone marrow from naïve *RelB*$^{-/-}$ mice (*black bars*) and *RelB*$^{+/+}$ mice *white bars*) was analyzed using flow cytometry markers for neutrophils and monocytes. (**A**) Representative dot plot- Ly6C(x-axis) and CD11b (y-axis) markers were used to show neutrophil and monocyte populations in naïve *RelB*$^{-/-}$ (right) and *RelB*$^{+/+}$ (left) mice . The percentage of neutrophils in the bone marrow (**B**) and number of neutrophils in the bone marrow (**C**) was not affected by RelB deficiency. The percentage of Ly6C-low monocytes (**D**) was higher in *RelB*$^{-/-}$ bone marrow than *RelB*$^{+/+}$ bone marrow ($^{**}p<0.01$). The percentage of Ly6C-intermediate monocytes (**E**) was also higher in *RelB*$^{-/-}$ bone marrow than *RelB*$^{+/+}$ bone marrow ($^{****}p<0.0001$). The percentage of Ly6C-high monocytes (**F**) was similar for both genotypes Results are presented as the mean ± SEM (n = 8 mice per group for A-C; n=4 mice per group for D-F).

CHAPTER 6: DISCUSSION

6.1 RelB may exert homeostatic control over pulmonary inflammation by maintaining the Th1/Th2 balance

Inflammation is linked to the development and exacerbation of many chronic diseases [116]. In recent years, *in vitro* experiments have exposed a regulatory role for the non-canonical NF-κB pathway- and in particular RelB- with controlling inflammation [99]. This work is one of the few studies exploring RelB-mediated attenuation of inflammation in an animal model, particularly in the context of the lung. One of our main findings is that naïve (non-stimulated) *RelB*$^{-/-}$ mice have significantly increased pulmonary inflammation and a dysregulated cytokine profile relative to *RelB*$^{+/+}$ mice (**Figure 1-2**). Thus, we propose that RelB exerts homeostatic control over pulmonary inflammation.

Such control over pulmonary inflammation may be particularly important in the context of chronic lung disease pathogenesis. For example, *RelB*$^{-/-}$ mice are prone to the development of chronic airway inflammation with increased inflammatory cells in their airways and elevated levels of many cytokines and chemotactic factors (*i.e*. macrophage inflammatory protein [MIP-1α,], RANTES, eotaxin-1 and thymus and activation-regulated chemokine [TARC]) [117]. Many of the features that characterize the airways of *RelB*$^{-/-}$ mice (i.e. high mucus-secreting cell numbers, epithelial, collagen deposition, and elevated serum IgE, IL-4, and IL-5) are also features of allergic asthma [117]. This suggests that RelB deficiency skews the pulmonary Th1/Th2 balance in favor of Th2 polarization. Our data herein also supports that there is a skewing of the Th response due to lack of RelB expression. In our experiments, the Th2 cytokines IL-4 and IL-6 were elevated in the airways of naïve *RelB*$^{-/-}$ mice while the Th1 cytokine IFN-γ was downregulated [118, 119]. Although not investigated in our study, this

observation could be due to RelB promotion of *T-bet* expression, a transcription factor involved in Th1 lymphocyte differentiation) [120-122]. Thus, deficiency in *RelB* may result in the over-expression of Th2 cytokines due to lack of T-bet expression.

It is also noteworthy that markers of Th2-driven allergic asthma (*i.e.* elevated serum IgE and airway remodelling) in the *RelB*$^{-/-}$ mice were decreased by the adoptive transfer of *RelB*-expressing DCs [117]. This suggests that RelB exerts homeostatic control of pulmonary inflammation through modulation of DC function. This is not unreasonable, given the high basal expression of RelB in DCs and its critical role in DC maturation [123]. The expression of RelB in different cell types are also likely important in the ability of RelB to fully suppress inflammation. For example, adoptive spleen transfer and bone marrow chimera experiments between *RelB*$^{-/-}$ and *RelB*$^{+/+}$ mice have provided evidence that RelB expression in non-hematopoietic cells is key in its ability to maintain immune homeostasis [113]. Finally, experiments whereby *RelB*$^{-/-}$ mice were bred with the orphan nuclear receptor *Nur77* transgene suggest that Nur77 reduces peripheral T cells and abates inflammation in *RelB*$^{-/-}$ mice [112, 124]. This last observation reveals that the hyper-inflammatory phenotype of *RelB*$^{-/-}$ mice is largely T-cell dependent [112, 124].

We propose that in our model RelB exerts its homeostatic control of pulmonary inflammation by maintaining the Th1/Th2 balance. Th polarization is driven by the interaction of naive T cells with polarizing cytokines IL-4 (Th2 differentiation) and IL-12 or IFN-γ (Th1 differentiation) [125]. As DCs are a major source for Th1-polarizing cytokines, we predict that adoptive transfer of *RelB*-expressing DCs compensates for reduced *T-bet* expression caused by *RelB*-deficiency [125, 126]. In addition to upregulating *T-bet* expression in T lymphocytes, IL-12 secreted by *RelB*-expressing DCs may also inhibit IL-4 production by Th2 lymphocytes [127, 128].

Ultimately, the net effect of these outcomes would help restore the Th1/Th2 imbalance. While earlier bone marrow chimera and adoptive transfer experiments argue that RelB expression in non-hematopoietic cells drive inflammation in *RelB*$^{-/-}$ mice, our proposition also explains their results. The polarization of T lymphocytes occurs in the periphery and not in the bone marrow or spleen [129]. It is therefore understandable why bone-marrow chimeras and adoptive transfer of spleen cells produced did not alter the level of inflammation in their host genotype. For example, after the transfer of *RelB*$^{+/+}$ bone marrow to *RelB*$^{-/-}$ mice, the *RelB*$^{+/+}$ lymphocytes are still subjected to Th2 polarizing extramedullary environment of *RelB*$^{-/-}$ mice. According to our hypothesis, this would nurture an environment for excessive inflammation.

Maintenance of the Th1/Th2 balance is one mechanism by which RelB could regulate pulmonary inflammation. However, a Th1/Th2 imbalance does not fully explain how RelB regulates pulmonary inflammation in response to inhaled toxicants, and in particular CS. We found that CS exposure decreased airway neutrophilia and eosinophilia in *RelB*$^{-/-}$ mice, which was accompanied by a significant decrease in IL-4 (**Figure 1-2**). Both the decrease in granulocytic inflammation and reduced IL-4 expression were exclusively observed in the CS model, and was not a feature of LPS, OVA, or Cl_2 exposures (**Figure 3-6**). While it is possible that IL-4 is involved in the CS-specific granulocytic decrease in the lungs of *RelB*$^{-/-}$ mice, we consider that it is unlikely that IL-4, over a 3-day exposure, would drastically change the Th1/Th2 lymphocyte ratio. It is likely that RelB is controlling another mechanism through which it mediates CS-induced pulmonary inflammation; we will explore these possible mechanisms in the next section.

Our finding that CS-exposed *RelB*$^{-/-}$ mice have reduced granulocytes was quite unexpected. One primary reason for our surprise is the body of literature proposing that RelB attenuates inflammation [99, 113, 130, 131]. Another major reason for our bewilderment is the

well-established idea that neutrophils are up-regulated with acute CS exposure [115, 132]. Moreover, active long- term smoke exposure has been demonstrated to augment eosinophilic lung inflammation and increase IL-4 production [133-135]. On the contrary, it should be noted that acute CS exposure has been shown to decrease airway eosinophilia in other mouse models [132, 136]. For example, CS was shown to attenuate eotaxin-1 (a chemotactic factor for eosinophils) and vascular cell adhesion molecule 1 (VCAM-1) without altering eosinophil survival in house dust mite-induced allergic inflammation [132, 136]. However, to our knowledge, CS exposure does not decrease neutrophilic inflammation in any other mouse model. Given the novelty of our results and fact that the CS-induced decrease in airway neutrophilia was sustained even 72 after the last CS exposure (**Figure 7**), we chose to narrow our focus specifically on neutrophils. The role of RelB in innate immunity has long-been overlooked and very little information is available directly linking RelB to the regulation of innate immune cells. RelB regulation of eosinophilic inflammation may be a promising area for future investigation.

6.2 RelB regulation of CS-specific neutrophilic inflammation

Our work presents the idea that RelB regulates CS-specific neutrophilic inflammation. It is unclear whether or not this regulation occurs due to intrinsic expression of RelB in neutrophils themselves or through the interaction of neutrophils with their surroundings. One immediate question worth investigating is whether neutrophils derived from $RelB^{+/+}$ mice express significant amounts of RelB after CSE stimulation. Thus far, RelB has only been detected in minute amounts in unstimulated murine neutrophils and has not been detected at all in unstimulated human neutrophils [137, 138]. The expression of RelB in activated neutrophils has not been explored in either model, to our knowledge [137, 138].

Another immediate inquiry following our work is how RelB regulates neutrophils. From their production in the bone marrow to their death, several processes regulate the activity and lifespan of neutrophils. Neutrophil differentiation from its myeloid precursors in the bone marrow is promoted by the granulocyte-colony stimulating factor (G-CSF) [139]. Although we did not measure G-CSF, studies have shown that other cytokines such as GM-CSF, IL-17A, and IL-6 also stimulate granulopoiesis *in vivo* [39, 140]. While GM-CSF and IL-17A were similar in unstimulated $RelB^{+/+}$ and $RelB^{-/-}$ mice in our experiments, IL-6 was elevated in the $RelB^{-/-}$ mice suggesting that perhaps granulopoiesis is elevated in these mice (**Figure 2**). Our assessment of the percentage and total cell counts of neutrophils in the bone marrows of unexposed $RelB^{+/+}$ and $RelB^{-/-}$ mice revealed that there was little difference between the two genotypes (**Figure 9**). Given the high basal neutrophilia typical of these mice in the lung as well as multiple other organs, we find these results surprising. One possible explanation for this discrepancy is that our measurements are a static reflection of the neutrophils in the bone marrow. During inflammation,

neutrophils are rapidly mobilized from the bone marrow into the blood stream [139]. The process of neutrophil mobilization from the bone marrow is governed by the CXR2-CXR4 axis.

Under stable conditions, the bone marrow constitutively expresses stromal cell-derived factor (SDF-1α also known as CXCL12) which binds to C-X-C motif receptor 4 (CXCR4) expressed on mature neutrophils [139, 141]. The binding of CXR-4 to SDF-1α retains neutrophils in the bone marrow [139, 141]. Inflammation results in the abundance of neutrophil receptor ligands such as CXCL1 (also known as KC or Gro-α) and CXCL2 (also known as MIP-2α) [139, 141]. The binding of neutrophil receptor CXCR2 to CXCL1 or CXCL2 releases neutrophils into the circulation [139, 141]. Elevated expression of G-CSF promotes CXCR2 signaling over CXCR4 thereby releasing neutrophils from the bone marrow reserve [141]. *RelB*$^{-/-}$ neutrophils may express abnormal levels of CXCR2 or CXCR4. Thus it may be that neutrophil production is in fact elevated in the RelB$^{-/-}$ mice but this is also accompanied by more rapid mobilization into the bloodstream. Evidence in favor of this theory comes from research performed on another NF-κB mouse model. As *RelA*$^{-/-}$ mice are embryonic lethal, the most severe NF-κB deficiency is that of *c-Rel*$^{-/-}$*p50*$^{-/-}$*RelA*$^{+/-}$ mice [138]. Like *RelB*$^{-/-}$ mice, *c-Rel*$^{-/-}$*p50*$^{-/-}$*RelA*$^{+/-}$ mice develop dermal inflammation associated with chronic neutrophilia [138].The chronic neutrophilia in the *c-Rel*$^{-/-}$ *p50*$^{-/-}$*RelA*$^{+/-}$ mice was attributed to elevated levels of CXCR2 which facilitated release of neutrophils into the bloodstream [138]. It is possible that RelB may regulate the CXCR2/CXR4 axis as well. There are at least seven κB sequences estimated to be on the promoter of the CXCR2 gene, strengthening the likelihood that RelB regulates this aspect of neutrophil production [142].

While increased clearance from the bone marrow is one explanation for the discrepancy between the neutrophilia and the modest percentage of neutrophils in the bone marrow, it is not the only

explanation for this incongruity. Furthermore, it does not explain how exposure to CS results in a decrease in the airway neutrophilia of *RelB*$^{-/-}$ mice. Cigarette smoke is known to enhance granulopoiesis of neutrophils as well as upregulate the expressions of CXCL1, CXCL2, and CXCR2 [143, 144]. In light of these facts, one would expect to see even higher levels of neutrophils in response to CS. It is therefore likely that increased neutrophil mobilization is not the only mechanism regulated by RelB in our model. One explanation for the apparent reduction of neutrophils in the lungs of CS-exposed *RelB*$^{-/-}$ mice is increased cell death.

We hypothesized that the most probable mechanism through which the neutrophils of *RelB*$^{-/-}$ mice are dying is via apoptosis. We have recently shown that RelB attenuates CSE-induced apoptosis in lung structural cells by controlling the expression of pro-survival anti-oxidants such as superoxide dismutase 2 (SOD-2) in concert with the aryl hydrocarbon receptor (AhR) [100]. We therefore speculated that RelB also regulates apoptotic cell death in immune cells. In fact, IL-4 (which was also decreased in response to CS in the *RelB*$^{-/-}$ mice) also delays neutrophil apoptosis [145, 146]. In this role, IL-4 incites *de novo* synthesis of actin in neutrophils which inhibits DNAse I, a key enzyme involved in the DNA fragmentation accompanied with apoptosis. This is believed to be the underlying reason for IL-4's anti-apoptotic effects [146]. When IL-4 is combined with GM-CSF, another known inhibitor of neutrophil apoptosis, these effects are compounded [146]. Thus, heightened levels of IL-4 would promote neutrophil survival, while the CS-mediated decline in IL-4 would lead to neutrophil apoptosis. However, our assessment of apoptosis and necrosis in BAL cells of CS-exposed mice did not fit with this assumption. While the loss of RelB resulted in a higher percentage of early-apoptotic cells in the naïve *RelB*$^{-/-}$ mice (**Figure 8**), CS exposure decreased the percentage of early-apoptotic cells. However, we cannot entirely rule out cell death as a potential explanation for our results. It may

be that neutrophilic cell death occurred at an earlier time point (*i.e.* after the initial CS exposure). A time course for neutrophil apoptosis would be needed to completely rule out this possibility. It is also possible that the defect lies in the macrophages. Macrophages are essential in engulfing apoptotic cells and they even have been shown to participate in the phagocytosis of very early apoptotic cells [147]. Experiments designed to quantify macrophage phagocytosis of apoptotic neutrophils could resolve this.

Non-traditional cell death mechanisms are also a possible explanation for the apparent disappearance of neutrophils in the CS-exposed *RelB*$^{-/-}$ mice. For example, reverse transendothelial migration is an emerging concept in which neutrophils from the periphery travel back to the bone marrow where they undergo apoptosis and are phagocytosed by stromal macrophages [140, 148]. Re-homing to the bone marrow is characteristic of senescent neutrophils which express higher levels of CXCR4 and thus are more resistant to CXCR2 ligands [139, 140]. It is possible that CS exposure changes RelB$^{-/-}$ neutrophil surface receptor expression to drive circulating neutrophils back to the bone marrow. This idea is solidified by the fact that both CS and RelB have been implicated in cellular senescence. CS induces cellular senescence in other cell types (i.e murine lung epithelial cells, macrophages, and fibroblasts) [149, 150]. RelB, on the other hand, inhibits p53 activity and thus suppresses cellular senescence [151]. It then follows that the loss of RelB would accelerate cellular senescence in neutrophils in response to CS, and may as a consequence result in increased reverse transendothelial migration of the neutrophils to the bone marrow [148]. Circulating neutrophils could be returning to the bone marrow or even neutrophils in the lung could be emigrating out into the circulation and returning to the bone marrow [152]. Although markers for the *in vivo* characterization of this reverse-migratory phenotype are still being explored, the current standard is the high expression of

ICAM-1 and low expression of CXCR1 [148, 153]. It would be beneficial to assess these markers in addition to CXCR2 and CXCR4. It may be that CS-exposed RelB$^{-/-}$ mice have higher levels of ICAM-1 in their lungs. Rationale for this claim comes from the fact that CS is a well-established promoter of ICAM-1 expression [143, 144]. Moreover, RelB inhibits ICAM-1 expression, suggesting that in its absence ICAM-1 will be elevated [115] .

As discussed above, high ICAM-1 expression (associated with low CXCR1) would predispose the neutrophils to reverse transendothelial migration, explaining the apparent decrease in airway neutrophilia. ICAM-1 facilitates the firm adhesion of CD18 integrins (transmembrane receptors highly-expressed on the surface of neutrophils) to the endothelium [139]. While we previously speculated that *RelB*$^{-/-}$ mice most likely have elevated levels of ICAM-1, evidence also suggests that a decrease in IL-4 may instead cause a corresponding decrease in ICAM-1 expression [154]. If ICAM-1 expression is indeed decreased in our model, it would ultimately result in the decreased adhesion and thereby decreased recruitment of neutrophils into the lungs. Future experiments to examine the expression of I-CAM1 in our model would be insightful. In particular, histological staining for ICAM-1 could reveal the role of adhesion molecules in our model. Given our claim that RelB regulates CS-specific granulocytic inflammation, we would expect to see fewer neutrophils in the CS-exposed *RelB*$^{-/-}$ histological lung sections.

In our discussion of the various mechanisms through which RelB regulates CS-induced inflammation we have mentioned various roles that IL-4 may be playing which contribute to the decreased airway neutrophilia in CS-exposed *RelB*$^{-/-}$ mice. Neutrophils express type I (IL-4Rα/CD-132) and type II IL-4 receptors (IL-4Rα/IL-13α) [141, 146]. Although we have not formally analyzed this, the expression of IL-4 and the infiltration of neutrophils appear to be positively correlated. That is, in the unstimulated *RelB*$^{-/-}$ mice, IL-4 and neutrophil infiltration are

both elevated while in the CS-exposed *RelB*$^{-/-}$ mice, both IL-4 and airway neutrophilia decrease. It is then justified to speculate whether Il-4 also regulates CS-specific airway neutrophilia in our model. If IL-4 indeed modulates airway neutrophilia, its most likely role is the promotion of adhesion molecule production, as mentioned earlier. For apoptosis and production of neutrophils from the bone marrow, there is a conflict between the function of IL-4 and our observations. IL-4 delays neutrophil apoptosis, however our indicates that in the airways of CS-exposed *RelB*$^{-/-}$ mice, there are a lower percentage of early apoptotic cells relative to the airways of unexposed *RelB*$^{-/-}$ mice [145, 146]. Similarly, others have shown that IL-4 directly binds to type II IL-4R and suppresses G-CSF-mediated neutrophil expansion in the bone marrow, blood and spleen [141]. Moreover, IL-4 inhibits CXCR2-mediated migration, inducing a bone marrow-resident non-migratory phenotype of neutrophils [141]. Given the high level of IL-4 in unstimulated *RelB*$^{-/-}$ mice, one would expect to see a larger population of neutrophils in the bone marrow reserve relative to the *RelB*$^{+/+}$ mice. In effect, these contradictory roles for IL-4 and our observations may indicate that IL-4 is not in fact a player in the mechanism. IL-4 may itself be a consequence of the decrease in granulocytes instead of being a causative factor. Although human neutrophils have been shown to secrete small quantities of IL-4, they are not considered a major source for this cytokine in mice [59, 155]. Thus, the decrease in airway IL-4 is unlikely to be a consequence of decreased airway neutrophilia. On the other hand, eosinophils have demonstrated significant production of IL-4 in parasitic infection models of the murine lung and in response to liver injury [156, 157]. Thus, the CS-specific decrease in IL-4 may simply be a reflection of the CS-specific decrease in granulocytes, particularly eosinophils. Neutralizing antibodies may be employed to confirm the contribution of IL-4 to the granulocytic decrease in the airways of CS-exposed *RelB*$^{-/-}$ mice. Administration of anti-IL-4 to the *RelB*$^{-/-}$ mice prior to CS exposure would

clarify whether its decrease is linked to the decrease in neutrophils or whether these are two independent occurrences.

6.3 RelB regulation of monocyte heterogeneity

Another interesting finding we have made is the effect of RelB on monocyte heterogeneity. We found that RelB-expressing and RelB-deficient bone marrow differ in the relative proportion of monocyte subpopulations (**Figure 9D-F**). In particular, monocytes expressing low amounts of Ly6C (referred to as Ly6C-low monocytes) and intermediate amounts of Ly6C (referred to as Ly6C-intermediate monocytes) were upregulated in the bone marrow of naive *RelB*$^{-/-}$ mice. This is interesting, as Ly6C-low monocytes patrol the endothelium and secrete the anti-inflammatory cytokine IL-10 upon bacterial infection [158]. Moreover, these cells are more likely to polarize to M2 macrophages, an anti-inflammatory macrophage subtype that is involved in tissue repair [158]. Alternatively, Ly6C-high and Ly6C-intermediate monocytes are recruited earlier in the inflammatory response and secrete ROS and pro-inflammatory cytokines such as TNF-α and IL-1β [158]. It is known that RelB is required for the Ly6C-high monocyte response following infection [159]. On the other hand, Ly6C-low monocytes function independently of RelB. In fact, Ly6C-low monocytes have a high basal expression of pro-inflammatory microRNA miRNA-146a which inhibits expression of RelB in these cells [159]. Thus, loss of RelB would disproportionately favor Ly6C-low monocytes in the naïve *RelB*$^{-/-}$ bone marrow.

How naïve *RelB*$^{-/-}$ mice have a higher proportion of anti-inflammatory monocytes yet still display heightened inflammation is not clear. For one thing, Ly6C-low monocytes secrete IL-10 [158]. Despite a higher proportion of Ly6C-low monocytes in the bone marrow of *RelB*$^{-/-}$ mice compared to *RelB*$^{+/+}$ mice, there is a relatively lower IL-10 expression in the airways of *RelB*$^{-/-}$ mice (**Figure 2E**). It may be that Ly6C-low monocytes have lost the potential to migrate to sites

of inflammation and patrol the luminal side of the endothelium in small blood vessels [158, 160]. CXCR1 on these monocytes binds to ICAM-1, allowing for the monocytes to bind to the endothelium and secrete IL-10 [158]. Alternatively Ly6C-high monocytes are recruited to the inflamed tissue, where they differentiate into Ly6C-low monocytes and aid in tissue repair and regeneration [158, 161].This inter-monocyte differentiation is dependent on Il-10 and IL-4 [158, 161]. It may be that the lack of IL-10 in our naïve *RelB*$^{-/-}$ mice inhibits the differentiation of Ly6C-high monocytes into Ly6C-low monocytes, thereby contributing to the heightened inflammation in our experiments. Then again, the higher population of Ly6C-low monocytes in the bone marrow of *RelB*$^{-/-}$ mice may simply be a reflection of decreased flux into the circulation and/or tissue. Monocyte egression from the bone marrow is similar to that of neutrophils, in that it depends on the CXCR2-CXCR4 axis [162]. Perhaps decreased expression of these cytokine receptors in one of the monocyte subpopulations contributes to the disproportionate monocyte heterogeneity we observed in *RelB*$^{-/-}$ mice. Thus, if Ly6C-low monocytes were present in the lungs (through differentiation from their Ly6C-high monocyte precursors) or in the circulation, we would expect to see higher secretion of IL-10. Instead we actually saw lower levels of IL-10 in our unstimulated *RelB*$^{-/-}$ mice. Future direction of this work should include the assessment of monocyte subpopulations in the lung after exposures, as RelB-dependent differentiation of these cells may be how RelB regulates pulmonary inflammation.

It is unknown how this disproportionate monocyte heterogeneity contributes, if at all, to the decreased neutrophils in the *RelB*$^{-/-}$ mice in response to CS. Remarkably, CS itself favors Ly6C-low monocytes by increasing their proportion in the blood and bone marrow while simultaneously decreasing the proportion of Ly6C-high monocytes [163]. These effects were seen rapidly in the blood (after 5 days of CS exposure) but required 30-day CS exposure to

observe changes in the bone marrow [163]. These data suggest that CS promotes the differentiation of Ly6C-high monocytes into Ly6C-low monocytes in acute and sub-acute models of CS. It is thought that Ly6C-low monocytes differentiate into M2 macrophages in the lung and contributing to the pathogenesis of COPD in smokers [163]. It is not known the percentage of each of the Ly6C monocyte subpopulation in the lung; however we believe that this issue is essential in determining the significance of our findings that *RelB*$^{-/-}$ bone marrow have higher levels of Ly6C-low monocytes.

We do not know how Ly6C-low monocytes might regulate neutrophils in the lung of CS-exposed *RelB*$^{-/-}$ mice. If there are few Ly6C-low monocytes in the lung, it may be that the Ly6C-low monocytes divert circulating neutrophils from the lung. Ly6C-low monocytes are believed to play a role in the maintenance of the vasculature [164]. For example, these cells recruit neutrophils to the vasculature whereby they phagocytose apoptotic endothelial cells [164]. CS upregulates endothelial cell apoptosis and thus it may be possible that, neutrophils perform clean-up tasks in the lung vasculature explaining why they are not recovered in the BAL of CS-exposed *RelB*$^{-/-}$ mice [165].

6.4 The anti-inflammatory components of CS

We present data where CS is anti-inflammatory, causing the decrease of granulocytes. This was specific to CS, as other pulmonary irritants (Cl_2, LPS, OVA) did not have this effect. There have been instances whereby CS reduces inflammation. The most notable example is in patients with ulcerative colitis (UC), a type of inflammatory bowel disease (IBD) which is characterized by chronic intestinal inflammation and ulceration [166]. CS has been linked to decreased

development of UC [166]. In patients who have already developed UC, CS results in clinical improvement in these patients, with fewer patients requiring invasive surgical treatments like colectomy [166]. It is believed that the anti-inflammatory effects of CS are largely due to nicotine, which activates the anti-inflammatory cholinergic pathway [167]. In the anti-inflammatory cholinergic pathway, the parasympathetic nervous system releases acetylcholine from the efferent vagus nerve, which binds to α7 nicotinic acetylcholine receptors on immune cells such as macrophages [168, 169]. Signalling through the α7 nicotinic acetylcholine receptor causes the inhibition of the canonical NF-κB pathway thereby attenuating inflammation [168]. This inhibition of NF-κB also occurs when nicotine binds to α7 nicotinic acetylcholine receptors on immune cells [168]. Nicotine, an agonist of the α7 nicotinic acetylcholine receptor, mimics acetylcholine and inhibits the production of pro-inflammatory mediators such as TNF-α and ICAM-1[168, 170]. The ability for nicotine to block the expression of adhesion molecules and pro-inflammatory cytokines has been demonstrated in rodent models of myocardial ischemia/reperfusion, influenza infection and LPS-induced acute lung injury [169, 171, 172]. Nicotine has also been shown to affect the adaptive immune system through its promotion of T cell anergy, for instance [170]. Interestingly, nicotine delays neutrophil apoptosis and thus prolongs neutrophil survival [173]. This last feature of nicotine may explain our finding that CS-exposed *RelB*$^{-/-}$ mice have less early apoptotic cells in the BAL. As the α7 nicotinic acetylcholine receptor is expressed on immune cells in the lung as well as the airway epithelium, it is possible that it may be part of the mechanism by which the absence of RelB incites a CS-specific decrease of neutrophils [174]. As pro-inflammatory cytokines were largely unaltered in the mice after CS-exposure, it may be that nicotine is reducing ICAM-1 levels and thus attenuating neutrophil infiltration.

Nicotine is not the only anti-inflammatory compound of CS, however. Another anti-inflammatory compound present in CS is carbon monoxide (CO) which is also produced from inflammatory cells in response to CS [132]. Like nicotine, the anti-inflammatory effects of CO also involve attenuating pro-inflammatory cytokine production such as TNF-α and IL-1β [175]. Exogenously-produced CO was also shown to inhibit neutrophil infiltration in an LPS-induced sepsis model by regulating the binding of chemokine receptor protein formyl peptide receptor I (FPR1) with its ligand N-Formyl-Met-Leu-Phe (fMLP) [176]. fMLP originates from the mitochondria and upon binding to the G-protein coupled receptor FPR1, results in de-sensitization to further stimulation by fMLP by the G protein-coupled receptor kinase 2 (GRK2)-mediated internalization of the FPR1 receptor [176, 177]. p38 mitogen-activated protein kinase (MAPK) in turn inhibits GRK2-mediated internalization [176]. In the LPS-sepsis model, CO was shown to inhibit p38/MAPK while sparing GRK2 thereby promoting the internalization of FPR1 [176]. As CO is a key component of CS, perhaps the decreased neutrophil infiltration we are seeing is attributed to this compound [178]. It would therefore be advantageous to quantify FPR1 expression and internalization via western blot and confocal microscopy respectively [176].

While CS may have some anti-inflammatory components and some beneficial effects in UC patients, the detrimental effects of CS far outweigh the positive effects. For example, CO poisoning is a major cause of morbidity and mortality and heavy smokers have carboxyhemoglobin levels up to 24%, making them susceptible to recurrent CO poisoning [178]. If nicotine and CO were indeed driving the anti-inflammatory effects of CS in our *RelB*$^{-/-}$ model, isolating these components may help in developing a targeted therapeutic approach to these

inflammatory disorders. Once we have established the positive effects of the component, we can work towards minimizing the risk associated with this compound.

6.5 Implications for disease and future directions

Taken together, our findings indicate that RelB is responsible for exerting homeostatic control over pulmonary inflammation through maintenance of the Th1/Th2 balance. RelB may also play a role in neutrophil survival and migration. Decreased expression of RelB has been shown in the lung fibroblasts of smokers with and without COPD and given this potential association, our findings may be important in the development of therapeutic strategies for this chronic lung disease [130]. Our results suggest that a deficiency in RelB would cause heightened pulmonary inflammation in COPD patients. We speculate that regular CS exposure would most likely not be beneficial in reducing neutrophil infiltration in these patients, however as the detrimental effects would outweigh the positive effects. One strategy which may turn out to be fruitful is the direct targeting of neutrophils. Neutrophils are one of the predominant immune cells in the airway walls of COPD patients and neutrophil-derived proteases are believed to be major contributors to the tissue damage and lung function decline characteristic of the disease [179] .

Before we can begin to think of potential solutions, however, it is very necessary to consider the overall implications of this CS-induced decrease in *RelB*$^{-/-}$ neutrophilia with respect to the health of the mice. The experiments we have outlined above involving the quantification of receptor expression and phagocytosis of apoptotic neutrophils are the most pressing topics that should be addressed. An equally important future direction is to consider whether these observations improve or worsen the phenotype of the *RelB*$^{-/-}$ mice. It may be that the decrease in airway

neutrophilia renders these mice incompetent of mounting a proper immune response to subsequent microbial challenge. By identifying a proper clinical endpoint, one should investigate whether the sustained granulocytic decrease in CS-exposed *RelB*$^{-/-}$ mice improves their high mortality rates. Furthermore, it is also necessary to investigate whether CS-exposed *RelB*$^{-/-}$ mice are hypo-responsive to subsequent exposures to pro-inflammatory stimuli (*i.e*. LPS). A recent paper has demonstrated that *RelB*$^{-/-}$ mice have impaired clearance of vaccinia virus supporting the idea that the *RelB*$^{-/-}$ mice may indeed be hypo-responsive to subsequent exposures of LPS after CS-treatment [122]. On the other hand, there have also been postulated roles for RelB in immune tolerance, a phenomenon in human sepsis patients in which immune cells uphold a state of unresponsiveness to challenges that would typically elicit a response [180]. In endotoxin-tolerant cells, RelB binds with heterochromatin protein 1 α (HP1α) on the promoters of pro-inflammatory genes such as IL-1β and TNF-α, thereby repressing their expression [180]. In our model, *RelB*$^{-/-}$ mice do have elevated expression of pro-inflammatory cytokines, including TNF-α, as one would expect to see in the absence of the tolerance-promoting RelB. However we also found that the inflammation in the airways of the *RelB*$^{-/-}$ mice did not behave as expected with different pro-inflammatory exposures. For example, the *RelB*$^{-/-}$ mice did not have elevated inflammation in response to LPS despite the same dose causing a robust rise in airway neutrophilia of *RelB*$^{+/+}$ mice, suggesting that *RelB*$^{-/-}$ mice are hypo-responsive to the subsequent stimulation. We thus propose that the prospective hypo-responsiveness in the *RelB*$^{-/-}$ mice occurs through a different mechanism than immune tolerance. The question of whether *RelB*$^{-/-}$ mice are hypo-responsive to subsequent infection post-CS exposure is especially relevant to the translation of our work into human disease. COPD patients suffer from an increased frequency of acute and chronic lower respiratory tract infections [181]. These infections negatively contribute

to the clinical course of COPD and therefore represent a significant comorbidity of COPD [181]. Neutrophils are the first-defense responders to infection. If the deficiency of RelB impairs the immune response after CS exposure, this may translate to the increased risk of infections. We therefore propose an experiment which assesses the long-term effects of the CS-specific neutrophilic decrease. CS may decrease the number of lung-infiltrating *RelB*$^{-/-}$ neutrophils but it may equally impact the function of these neutrophils. We therefore also propose *in vitro* experiments assaying the phagocytic ability of these neutrophils [182]. Finally as chronic and sub-acute models may be more relevant to COPD patients (who often have a history of smoking), we should assess whether the effects observed in the acute model of cigarette-smoke exposure on the *RelB*$^{-/-}$ neutrophils are reproduced in sub-acute and chronic models.

The clinical implications of our work extend far beyond COPD. Several other diseases have been associated with abnormal RelB expression. For instance, RelB over-expression has been implicated in the promotion of non-small cell lung cancer [183]. Like COPD, non-small cell lung cancer is by cigarette smoking [184]. Another example where the altered expression of RelB contributes to human disease is combined immunodeficiency, a disorder characterized by chronic lung and gastrointestinal symptoms [185]. Homozygous mutation in the *RelB* gene leads to its deficiency and this loss of RelB was associated with the development of pneumonia, reactive airway disease, repeated infections, and persistent cough [185]. Thus, we believe that further studies into the immunoregulatory functions of RelB are warranted.

CHAPTER 7: REFERENCES

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