THE ROLE OF THE INFLAMMASOME IN THE RESPIRATORY INNATE IMMUNE RESPONSE TO VIRUSES AND POLLUTANTS: INSIGHTS FOR ASTHMA PATHOGENESIS

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ABSTRACT

Rebecca N. Bauer: The role of the inflammasome in the respiratory innate immune response to viruses and pollutants: insights for asthma pathogenesis
(Under the direction of Ilona Jaspers)

The respiratory mucosal innate immune system is composed of both structural cells, such as airway epithelial cells, and immune cells, such as macrophages, that together determine the appropriate immune response to inhaled stimuli. These cells use pattern recognition receptors to distinguish between harmless and harmful stimuli by recognizing conserved microbial pathogen-associated molecular patterns (PAMPs) and endogenously derived damage-associated molecular patterns (DAMPs) from damaged tissue. Inappropriate immune responses to normally innocuous stimuli underpin the pathogenesis of a number of immune disorders, including asthma, a chronic inflammatory disease of the airway typified by difficulty breathing in response to a trigger. In this dissertation, we explored the contribution of the inflammasome signaling complex to respiratory mucosal host defense against two sources of asthma exacerbation: influenza A virus infection and inhalation of the oxidant air pollutant ozone (O_3). The inflammasome is an innate immune complex composed of a pattern recognition receptor, the protease caspase-1, and an adaptor protein (PYCARD) that when formed induces activation of caspase-1-mediated processing of the pro-inflammatory mediators IL-1β and IL-18 or cell death. Our results show that caspase-1 and the inflammasome contribute to the airway epithelial cell innate immune response to influenza and that this pathway is modified by asthma, suggesting a role for caspase-1 in virus-induced asthma exacerbation. Using a mouse model of allergic airway inflammation, we also show that caspase-1 may be involved in the development of allergic asthma, though its

function in asthma development is complex. In the context of O_3, we found increased presence of several DAMPs that may activate inflammasome signaling in the airway of healthy volunteers exposed to O_3, but no evidence to suggest that inflammasome signaling strongly contributes to the innate immune response to O_3. Finally, we identified a mechanism by which O_3 alters the interaction between epithelial cells and macrophages leading to the accumulation of DAMPs that may activate innate immune responses in the lung. In summary, our results shed light on the function of the inflammasome in the human respiratory innate immune response to viruses and pollutants, and provide insight on the contribution of inflammasome signaling to asthma pathogenesis.

To my Dad, who passed on a love of science and taught me to always do my best. To my Mom, whose ever-present memory reminds me of the necessity for scientific research.

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PREFACE

Explanation of my contribution to each chapter and acknowledgement of other contributors:

Chapter1: Parts of the "Initiation of the innate immune response by pattern recognition receptors (PRRs)" section were adapted the review article Bauer, RN; Diaz-Sanchez, D; Jaspers I. Effects of air pollutants on innate immunity: the role of toll-like receptors and nucleotide-binding oligomerization domain–like receptors. *J Allergy Clin Immunol.* 2012 Jan; 129(1):14-24 with permission from Elsevier and are marked accordingly throughout the chapter. David Diaz-Sanchez wrote the introduction, Ilona Jaspers wrote the conclusion, and I wrote the main text that was reviewed and revised by David Diaz-Sanchez and Ilona Jaspers. Only sections from the main text of this article that were authored by me are included in this dissertation. Additionally, Figures 1.3 and 1.4 are adapted from the same review article.

Chapter 2: This chapter, with minor adaptations to fit the dissertation format, was reprinted from Bauer RN, Brighton LE, Mueller L, Xiang Z, Rager JE, Fry RC; Peden D; Jaspers I. Influenza enhances caspase-1 in bronchial epithelial cells from asthmatic volunteers and is associated with pathogenesis. *J Allergy Clin Immunol.* 2012 Oct;130(4):958,967.e14 with permission from Elsevier. I wrote the article, conceived the study design, assisted with or performed all of experiments shown here, and analyzed all of the data. Luisa Brighton preformed the cell culture and immunofluorescence. Loretta Mueller helped with the immunofluorescence and provided experimental expertise. Zhidan Xiang provided the *Caspase-1* deficient and wild type mice for the study. Julia Rager and

Rebecca Fry performed the statistical analysis for the real-time PCR array and reviewed the manuscript. David Peden provided insight on the conception of the study and revisions of the manuscript. Ilona Jaspers was the senior author on the study and oversaw the conception, design, data analysis and interpretation, and manuscript preparations.

Chapter 3: The findings in this chapter are unpublished. I conceived of the hypothesis, performed all of experiments, analyzed all of the data, and wrote the chapter. Corey Jania and Stephen Tilleyprovided the mice for this study, and performed the house-dust mite sensitization protocol in their laboratory. I assisted Corey Jania with the collection of samples, preformed all subsequent sample analysis, and summarized the findings for this chapter. Luisa Brighton processed the fixed lungs and preformed the immunohistochemistry. Ellen Glista-Baker was a second scorer of the lung pathology and performed the goblet cell hyperplasia analysis. Ilona Jaspers was the senior author on the study and oversaw the conception, design, data analysis and interpretation, and manuscript preparations.

Chapter 4: The findings in this chapter are unpublished. I conceived of the hypothesis, performed all of experiments, analyzed all of the data, and wrote the chapter. The samples for this study were collected from a human exposure study performed in collaboration with Dr. Kelly Duncan and the U.S. EPA. Charles Esther performed the glutathione and purine metabolite mass spectrometry. Hye-Young Kim and Ned Porter performed the oxysterol analysis. Ilona Jaspers was the senior author on the study and oversaw the conception, design, data analysis and interpretation, and manuscript preparations.

Chapter 5: The findings in this chapter are unpublished. I conceived of the hypothesis, performed all of experiments, analyzed all of the data, and wrote the chapter. Luisa Brighton

performed the caspase-3 immunohistochemistry. Loretta Mueller helped with the design and execution of the 16HBE14o- and pulmonary macrophage co-culture model and provided technical assistance with the flow cytometry and confocal microscopy. Ilona Jaspers was the senior author on the study and oversaw the conception, design, data analysis and interpretation, and manuscript preparations.

Chapter 6: I conceived of and was the sole author of this chapter.

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16HBE	16HBE14o- (human bronchial epithelial) cell line
a_SecoB	alkynyl secosterol B
AHR	airway hyperresponsiveness
AIM2	absent in melanoma 2
ALR	AIM2-like receptor
ALI	air-liquid interface
AMP	adenosine monophosphate
AP-1	activating protein-1
ASM	airway smooth muscle
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
CLR	C-lectin type receptor
Casp1$^{-/-}$	caspase-1 deficient mice
CholEPα	cholesterol epoxide α
CholEPβ	cholesterol epoxide β
COPD	chronic obstructive pulmonary disease
Ctsg	cathepsin G
DAMP	damage associated molecular pattern
DC	dendritic cell
EC	epithelial cell
Elane	neutrophil elastase
FBS	fetal bovine serum

FLICA	measure of caspase-1 activity
FSC	forward scatter
G	alveolar tissue dampening
GM-CSF	granulocyte-macrophage colony stimulating factor
GSH	glutathione
GSSG	glutathione disulfide
H&E	hematoxylin and eosin
HA	hyaluronic acid
HBEC	human bronchial epithelial cells
HDM	house dust mite
Hr	hour
IAV	influenza virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Influenza HA	hemagglutinin
KC	keratinocyte chemoattractant
LC-MRM	liquid chromatography-multiple-reaction monitoring
LDH	lactate dehydrogenase
LGP2	laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2)
LLOD	lower limit of detection
LPS	lipopolysaccharide
Mac	pulmonary macrophage

MARCO	macrophage receptor with collagenous structure
MAVS	mitochondrial antiviral signaling protein
MDA5	melanoma differentiation associated factor 5
mDC	myeloid dendritic cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MS	mass spectrometry
MTEC	murine tracheal epithelial cells
MYD88	Myeloid differentiation primary response gene 88
ND	non-detectable
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
NLR	nucleotide-binding oligomerization domain receptors
NLRC	NLR family CARD domain-containing protein
NLRP	NACHT, LRR and PYD domains-containing protein
NLRX1	nucleotide-binding oligomerization domain, leucine rich repeat containing X1
NOD	nucleotide-binding oligomerization domain
O_3	ozone
OVA	ovalbumin
PAMP	pattern associated molecular pattern
PAS+	Periodic Acid Schiff positive
PCR	polymerase chain reaction
pDC	plasmacystoid dendritic cell

PI	propidium iodide
PMN	polymorphonuclear cell
Ppm	parts per million
PRR	pattern recognition receptor
PYCARD	PYD and CARD domain containing
qRT-PCR	quantitative real time PCR
R_{aw}	central airway resistance
RIG-I	retinoic acid-inducible gene 1
RIPK2	Receptor-interacting serine/threonine-protein kinase 2
R_L	total resistance of the lung
RLR	RIG-I-like receptor
RSV	respiratory syncytial virus
RV	rhinovirus
S. aureus	*Staphylococcus aureus*
Seco A	secosterol A
Seco B	secosterol B
SSC	side scatter
Th1	type 1 helper T lymphocyte
Th17	type 17 helper T lymphocyte
Th2	type 2 helper T lymphocyte
TLR	toll-like receptor
TSLP	thymic stromal lymphoprotein
WT	wild type

Chapter 1: Introduction[1]

1.1 Respiratory mucosal host defense against pathogens and pollutants

The respiratory mucosal surface is the first line of defense against the inhaled environment, including pathogens and pollutants. A tightly regulated immune system is necessary to effectively maintain immunologic homeostasis while permitting the appropriate response to injury (1). The immune system is composed of both an innate and adaptive component. Innate immunity is the non-specific first response to infection or injury and is composed of both structural cells, such as epithelial cells, and resident and circulating innate immune cells, such as macrophages and neutrophils (2). The crosstalk between these cell types is necessary to determine the appropriate inflammatory response to a stimulus and to inform the adaptive immune system of injury. The adaptive immunity is an antigen specific, cell- and antibody-mediated immune response to eliminate specific invading pathogens (3). The adaptive immune response is mediated by B and T lymphocytes and typically results in immunological memory of the insult (4, 5). Effective interaction between the innate and adaptive immune systems is crucial not only to clear the insult, but also to protect the body from unnecessary inflammation and self-destruction. Diseases such as asthma occur when the immune system inappropriately responds to normally harmless stimuli (6). The chronic inflammation associated with asthma causes airway remodeling and bronchoconstriction that is exacerbated by a normally innocuous stimulus, leading to difficulties breathing (7).

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[1]Sections of this introduction were adapted from Bauer, RN; Diaz-Sanchez, D; Jaspers I. Effects of air pollutants on innate immunity: the role of toll-like receptors and nucleotide-binding oligomerization domain–like receptors. J Allergy Clin Immunol. 2012 Jan; 129(1):14-24 with permission from Elsevier are indicated throughout the introduction.

The work presented in this dissertation is largely centered on the role of the innate immune system in the respiratory mucosal host response to pathogens and pollutants and how these pathways may contribute to asthma pathogenesis. A brief overview of the innate immune cells in the airway and their contribution to allergy and asthma will be covered below. Given the important interplay between innate and adaptive immunity and the crucial role that lymphocytes play in asthma development and pathogenesis, adaptive immune cells will also be briefly discussed.

1.1.1 Cells of the respiratory innate immune system

Airway epithelial cells (EC). The respiratory epithelium represents the main barrier between the inhaled environment and underlying tissue and serves as the site of oxygen exchange (8). Tight junctions and other adhesion molecules link EC together to form a mostly impermeable membrane (9). The type of EC differs depending on the anatomical location along the airway. From the nasal airways to bronchioles, the respiratory epithelium is composed primarily of pseudostratified columnar epithelial cells, but also goblet cells (mucin-producing), basal cells (precursors for differentiated epithelial cells), and club cells (nonciliated, secretory bronchial epithelial cells) (10-13). Further along the bronchioles, the EC shift to cuboidal cells and then finally to alveolar EC at the terminal alveoli, the primary site of gas exchange in the lung (14). Alveolar EC are composed of both type 1 and type 2 cells, with squamous-type 1 cells classically thought to serve a more structural function and type 2 cells that produce surfactant and regulate water and ion transport (15). The complex architecture of the lung and diverse composition of EC that line the airway allow for both efficient gas exchange and protection of the airway from the inhaled environment (14).

Beyond acting as a physical barrier, the epithelium is an active participant in host defense. EC produce an epithelial lining fluid composed of mucins and surfactants that moisten the airway and trap inhaled toxins or pathogens (16, 17). The mucins produced by EC together with movement of cilia traps foreign stimuli and moves them up the airway through a mechanism termed the "mucociliary escalator" (17, 18). The epithelial lining fluid also contains antimicrobial mediators (e.g. β-defensins and lactoferrin) and antioxidants (e.g. glutathione) that protect the airway (17). Additionally, EC play a major role in the secretion of immunoglobulin A (IgA). IgA produced by plasma cells in the lamina propria binds to the polymeric immunoglobulin receptor on the basolateral side of EC, and is transported to the luminal side before secretion as secretory IgA, which neutralizes bacteria and toxins and prevents microbe adhesion to EC (19). EC produce a number of inflammatory and chemotactic mediators that alert nearby cells of damage or infection and are thus crucial to the initiation of innate immune responses in the airway (20).

Alterations in EC function are associated with a number of airway diseases, including asthma, chronic obstructive pulmonary disease, and cystic fibrosis (8). Disruption of EC barrier function, increased goblet cell numbers, and remodeling of the airway epithelium are key characteristics of asthma, suggesting that alterations in the airway epithelium may significantly contribute the asthma pathogenesis (22). EC produce a number of DAMPs and cytokines/chemokines that attract and activate other immune cells, such as dendritic cells and eosinophils, which are major contributors to both the initiation and progression of asthma and will be described below (21).

Pulmonary macrophages (Mac). Pulmonary Mac, which are derived from monocytic precursors in the blood, compose the majority of resident immune cells in the healthy lung (23).

Mac are among the first cells to contact inhaled antigens, and thus serve a critical role in the initiation of innate immune responses (24). The primary functions of pulmonary Mac include phagocytosis of cellular/tissue debris and inhaled particulates, destruction of inhaled pathogens and infected cells by release of reactive oxidants through a mechanism termed respiratory burst, antigen presentation to T lymphocytes, and production of inflammatory and chemotactic mediators to regulate inflammation in the airway (23, 25). Adoptive transfer studies have shown that lung microenvironment plays a key role in determining Mac phenotype and function (26). Pulmonary Mac more resemble dendritic cells and have lower phagocytic and antigen presentation function compared to other tissue resident or infiltrating Mac (23, 26, 27). This subdued Mac phenotype is important to prevent inflammatory responses to the many harmless stimuli in the inhaled environment (1). Additionally, like EC, Mac phenotype and function may vary based on anatomical location within the lung, and previous studies have shown that bronchial Mac, interstitial Mac, and alveolar Mac differ in phenotype and function (28).

Recently, Mac phenotypic characterization has received increased attention and Mac have been broadly subdivided into “classically activated” and “alternatively activated” subtypes (29). Classically activated Mac are induced by proinflammatory type 1 helper T lymphocyte (Th1) cytokines, such as IFNγ and LPS, and are characterized by production of proinflammatory cytokines and microbicidal reactive oxygen and nitrogen species. Conversely, alternatively activated Mac are characterized by production of anti-inflammatory and tissue repair mediators, such IL-10 and TGFβ, and expression of scavenger receptors, such as CD206 (macrophage mannose receptor) (25). However, these categories are primarily derived from *in vitro* experiments, and distinction of Mac as either “classical” or “alternative” does not explain the phenotypic plasticity exhibited by Mac in changing tissue microenvironments (29, 30).

Moreover, these categories were primarily derived in mice or from peripheral blood/ bone marrow-derived Mac. The application of alternative and classical characterization has not been well verified in the lung or using human Mac (31, 32).

The pro- and anti-inflammatory functions of Mac may play dual roles in the pathogenesis of asthma and allergy (33). In a mouse model of adoptive transfer of Mac from unsensitized mice to allergen-sensitized mice ameliorated airway inflammation and airway hyperresponsiveness, highlighting that the anti-inflammatory functions of Mac may suppress asthma pathology (34). However, the pro-inflammatory functions of Mac can contribute to asthma pathogenesis by enhancing inflammatory cell migration to the airway. Mac from an ovalbumin mouse model of allergic asthma have been shown to produce more IL-17 that recruits neutrophils to airway, a characteristic of exacerbation and severe asthma (35). These diverging findings suggest that Mac function must be exteremely fine-tuned to mainatain a homeostatic state in the lung, and that more studies are needed to explore the anti-inflammatory versus pro-inflammatory functions of Mac in asthma pathogenesis.

Dendritic cells (DC). DC are the major antigen presenting cell in the lung, and thus serve as important linkers of the innate and adaptive immune responses. Two primary populations of DC exist in the lung, myeloid DCs (mDC) and plasmacystoid DCs (pDC), and the populations differ in recruitment and function. pDC are recruited to the lung upon viral infection, preferentially express Toll-like receptors specialized to recognize viruses (TLR7 and TLR9) and respond with massive amounts of antiviral mediators, whereas mDC are recruited continuously to the airway and serve as primary antigen presenting cells in the lung (36). DC lie on the basolateral side of EC and have long, branching extensions that extend between EC to “sample” the airway (1). DC recognize antigens and danger signals in the airway, uptake the antigen, and

process it into short fragments that are presented on major histocompatibility complex molecules to T cells (21). DC express co-stimulatory molecules such as CD40 that are required for T cell activation, and lack of co-stimulatory receptors can lead to T lymphocyte anergy, a mechanism of inactivation and tolerance (37). In addition, upon recognition of an antigen or danger signal DC produce cytokines and chemokines to attract other immune cells and promote T cell differentiation (38). As presentation of self-antigens or normally harmless allergens to T lymphocytes and differentiation of T lymphocytes to type 2 helper cells is considered an early step in the development of allergic asthma, DC have been shown to play major role in the development of asthma (39, 40).

Neutrophils. Neutrophils, along with eosinophils and basophils, are members of the granulocyte family. Neutrophils are the first immune cells to be recruited to the site of inflammation or injury in the lung (41, 42). Chemokines produced by Mac and EC, such as IL-8 and growth-regulated oncogene (GRO)-related peptides, induce recruitment of neutrophils to the lungs (42). Like Mac, neutrophils are phagocytic and play a role in clearing debris, particles, and pathogens (43). Additionally, neutrophils contain granules that harbor potent antimicrobial and cytotoxic proteins, including proteases (such as neutrophil elastase, cathepsin g, proteinase-3, and several matrix metalloproteinases), anti-microbial peptides (lactoferrin, α-defensins), and reactive species generated by enzymes such as myeloperoxidase and NADPH oxidase (44). These granules are released into phagocytic vacuoles to eliminate the ingested material or may be released into the surrounding tissue by degranulation to kill extracellular microbes, often causing tissue damage in the process. Finally, neutrophils can release Neutrophil extracellular traps (NETs), which contain antimicrobial granule proteins such as neutrophil elastase and chromatin to form fibers that trap bacteria and viruses (45). Neutrophils are extremely short-

lived. Though some neutrophils are removed by apoptosis, others may undergo necrosis leading to the release of granules that may damage the surrounding tissue (41). Neutrophils, via their high reactivity, have been shown to contribute to a number of airway diseases, including severe asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome (42, 46).

Eosinophils. Eosinophils develop and mature in the bone marrow in the presence of IL-5, IL-3, and GM-CSF and are recruited to the airway upon an inflammatory insult by Th2 cytokines such as IL-4, IL-5, and IL-13 and chemokines such as CCL5 and the eotaxins (47). Eosinophils play a major role in antimicrobial response to parasitic helminths and RNA viruses and are also a hallmark cell of allergy (47, 48). Eosinophils produce a number of proinflammatory cytokines, chemokines, and lipid mediators (such as leukotrienes) and contain granules that harbor cytotoxic cationic proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) that can damage both pathogens and tissue (47). Eosinophils are hallmark of allergic diseases and contribute to asthma by inducing mast cell and basophil degranulation, smooth muscle contraction that contributes to airway hyperresponsiveness (AHR), and mucus production (49, 50).

Basophils. Basophils account for less than 1% of granulocytes in the spleen and blood (51, 52). Like eosinophils, basophils are thought to be involved in the response to parasitic helminthes and promote allergy (52, 53). As indicated by their name, basophils contain basophilic granules in their cytoplasm that are released upon activation. Basophils are activated upon stimulation by cytokines such as IL-3 and antibodies such as IgE in allergic diseases and once activated produce Th2 cytokines (IL-4, IL-5, IL-13, and TSLP), chemokines, histamine, leukotrienes, and antimicrobial peptides (51). In the lung, the production of leukotrienes and histamine act on airway smooth muscle cells to induce bronchoconstriction, whereas Th2-family

cytokines promote Th2 lymphocyte differentiation and responses (51). Due to their responsiveness to IgE and migration to sites of ongoing allergic inflammation, basophils are considered late phase effector cells of allergic diseases, including asthma (53). Additionally, basophils may also play a role in the induction of allergic inflammation, as they have been shown to migrate to lymphoid tissue and produce mediators such as TSLP and IL-4 that promote CD4+ T cell differentiation (54, 55).

Mast cells. Mast cells are long-lived cells that reside near epithelial tissue, airway smooth muscle, blood vessels, nerves, and in mucus-producing glands (56). Like eosinophils and basophils, mast cells harbor granules that contain cytokines, histamine, proteoglycans, leukotrienes, and proteases such as chymase and β-tryptase (56). Mast cell degranulation is activated by FcεRI receptor binding of antigen-specific IgE, as well as activation of innate immune pattern recognition receptors such as the TLRs (57). The release of these mediators have important effects on the lung: mediators such as histamine and leukotrienes act on airway smooth muscle cells to induce bronchoconstriction whereas proteases play a key role in host defense by cleaving proinflammatory mediators, extracellular matrix proteins, and cell surface proteins (53, 57, 58). Since mast cells are resident in the lung, they are important mediators of the early phase of allergic responses (53).

Natural killer (NK) cells. NK cells compose approximately 10% of resident lymphocytes in the lung, though this number increases greatly with an inflammatory stimulus such as an infection (59). NK cells are important for host defense against infections and tumor cells, in part due to their cytotoxic capabilities. Cytotoxic NK cells release perforin, which pokes holes in the target cell membrane and allows entry of granzyme B to induce apoptosis or necrosis of the target cell (60). NK cells also have non-cytotoxic functions, which include the production of

inflammatory mediators, such as IFNγ, Th2 cytokines (IL-5 and IL-13), or regulatory cytokines such as IL-10 (61). Activation of NK cells is based both on interaction with receptors and cytokines. NK cell activating receptors include the natural cytotoxicity receptor NKp46, the Fc receptor CD16, and NKG2D, which recognizes ligands on "stressed" cells, such as UL16 Binding Protein 3 (ULBP3) (62). NK cells are also activated by cytokines, in particular type 1 interferons, IL-12, and IL-18 (60). In addition to antiviral and anti-tumor activity, NK cells are involved in allergy and asthma pathogenesis in the lung. NK cells can be activated by IgE and may release of a number of cytokines that contribute to the allergen sensitization, such as IL-4, IL-5 and IL-13 (63).

1.1.2 Adaptive immunity in the lung

Adaptive immunity is a specific, cell- and antibody-mediated immune response to an antigen that is mediated by T and B lymphocytes and typically results in memory (64). T lymphocytes mediate cellular immunity, whereas B lymphocytes recognize antigens and differentiate into antibody producing cells called plasma cells (64). T lymphocytes can be further broken down into CD4+ helper, CD4+ regulatory, and CD8+ cytotoxic lymphocytes, all of which express the αβ antigen receptor (65). Another type of T lymphocyte, called γδ T lymphocytes based on the antigen receptor, are also found in the airway and are considered to be more part of the innate immune response, but these cells will not be discussed here (66). Furthermore, lymphocytes may be broken down into "effector" or "memory" cells, with effector cells preforming functions such as cell-mediated cytotoxicity (CD8+ T lymphocytes), cytokine secretion and B cell activation (CD4+ helper T lymphocytes), suppression of inflammation (regulatory T lymphocytes), or antibody production (plasma cells) (64, 65). Memory cells are

conversely quiescent and may exist for years after encountering an antigen. Upon a second stimulus, memory cells quickly proliferate and mount strong immune responses (67).

In the absence of airway inflammation, the number of T and B lymphocytes in the airway is relatively low (1). Resident T lymphocytes may lie under the epithelium in the lamina propria or within the epithelial cell layer (1, 5, 68). Also within the lamina propria are plasma cells that produce mainly mucosal IgA antibodies (1, 69, 70). The adaptive immune response is closely tied to innate immune responses in the lung, thus with activation of innate immunity, an increased number of T and B lymphocytes migrate to the airway (3, 21). Innate antigen presentation cells, particularly DC, are important linkers of the two systems. DC present processed antigen to naïve T lymphocytes via a major histocompatibility (MHC) complexes (6, 38). Based on the type of MHC complex, the naïve T lymphocyte will differentiate to a CD4+ (MHC class II) or CD8+ (MHC Class I) T lymphocyte (64, 65). MHC Class I stimuli represent active intracellular infections, which require destruction of the cell by a cytotoxic T lymphocyte, whereas MHC class II mediators represent extracellular mediators that may cleared by immune cells. Based on the microenvironment, CD4+ helper T lymphocytes differentiate into subtypes such as type 1, 2, and 17 helper cells (6, 65). Type 1 helper (Th1) cells are induced by cytokines such as IFNγ and IL-12, Th2 by IL-4, and Th17 by TGFβ with IL-6 or IL-1 (6, 71, 72). Each helper cell subtype produces different sets of cytokines: Th1 produce IFNγ and IL-12, Th2 produce IL-4, IL-5, and IL-13, and Th17 produce IL-17, and these mediators then attract appropriate immune cells to clear the stimuli (65, 71). B cells differentiate inlymphoid tissue upon recognition of an antigen and, in some cases, a second stimulus from a helper T lymphocyte (64). Helper T lymphocyte co-stimulatory molecules and cytokines contribute to naive B cell class switching from IgM- or IgD- antibody production, to other mature classes,

such as IgG, IgE, or IgA isotypes (4). For example, IL-4, which is secreted by Th2 helper cells, induces a switch to the IgE isotype, which is an important mediator of allergy and asthma (6).

Increased numbers Th2 and Th17 cells, as well as Th2- or Th17-skewed cytokines produced by innate cells have been implicated in the development and pathogenesis of allergy and asthma (6). Th2 cytokines are associated with B lymphocyte isotype switching to IgE and mast cell, eosinophil, and basophil recruitment and degranulation (72). Th17 cytokines are associated with neutrophilic asthma (71).

1.1.3 Cell-cell communication underlies immunologic homeostasis

Immunologic homeostasis requires a complex interaction between cells of the innate and adaptive immune systems. Resident immune cells in the lung are constantly interacting with inhaled stimuli and must distinguish between innocuous and pathogenic agents. At baseline conditions, Mac and EC are two of the most populous cells in the airway, suggesting that they coordinate the immune response in tandem (1) (Figure 1.1). The lung lining fluid secreted by EC provides a number of regulatory molecules that are vital to host defense, and the mucociliary escalator provides a mechanism to remove pathogens and particles from the airway (17). Mac phagocytose antigens to prevent the development of specific immune responses and exist in a subdued phenotype with poor antigen presentation capabilities to prevent acquired immune responses unless truly necessary (23, 26, 27). The interaction between Mac and EC at baseline, and how signals from EC may modify Mac phenotype will be explored later in this dissertation (Chapter 5). Additionally, underlying the epithelium is a population of DC that constantly sample the airway for injurious stimuli (21). The interaction between these resident immune cells underlies host defense and is crucial for immunologic homeostasis.

Upon activation of innate immune receptors, resident immune cells produce a number of mediators to recruit inflammatory cells in the airway and activate other resident cells in the lung (Figure 1.2). Resident DC travel to the draining lymph node to present antigens to T and B cells and initiate adaptive immune responses (38). The onslaught of cytokines and chemokines produced by resident cells in the lung recruit circulating immune cells from the blood to the airway, including neutrophils, eosinophils, and basophils (6). The molecular and cellular events that follow limit the infection or injury, but in the process can damage the surrounding tissue. Resolution is another highly coordinated process that involves clearance of damaged tissue and reactive inflammatory cells by apoptosis and phagocytosis, the release of pre-resolving mediators (e.g. lipoxins, resolvins, protectins, and maresins), and reconstitution of the epithelial barrier (41, 73). Failure to resolve inflammation in the airway can lead chronic inflammatory diseases such as asthma, which will be discussed in more detail below (section 1.3) (7, 42, 73, 74). In the next section, the molecular mechanisms that initiate the innate immune response will be explored.

1.2 Initiation of the innate immune response by pattern recognition receptors[1]

Recognition of an injurious stimulus is the first step of innate immunity, and requires activation of pattern recognition receptors (PRRs) (75). PRRs recognize conserved microbial ligands, termed pathogen associated molecular patterns (PAMPs), and endogenous ligands derived from stressed cells and damaged tissues, termed damage associated molecular patterns (DAMPs). (76). Examples of PAMPs include double-stranded RNA (dsRNA) derived from viruses and bacterial components, such as lipopolysaccharides (LPS, also known as endotoxin). DAMPs are endogenous mediators derived from damaged cells and tissue, and can include damaged extracellular matrix components (such as hyaluronic acid, HA) and intracellular components (such as ATP). DAMPs are important mediators of sterile inflammation, as caused

by inhalation of oxidant gases such as ozone (O_3) (77). Stimulation of PRRs by PAMPs and DAMPs activates downstream signaling pathways that culminate in the production of cytokines and chemokines to attract leukocytes and antigen presenting cells (78). In the case of antigen presentation cells such as DCs, activation of PRRs may provide a necessary maturation signal that leads to activation of naïve T lymphocytes in the draining lymph nodes (38).

There are several classes of PRRs, including the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIGI)-I-like receptors (RLRs), AIM2-like receptors (ALRs) and NOD-like receptors (NLRs) (79, 80). TLRs and CLRs are transmembrane associated receptors, whereas RLRs, ALRs, NLRs are cytoplasmic proteins. A number of studies have demonstrated the role of TLR signaling in both pathogen- and pollutant-induced inflammation. More recently, NLRs and the subset that assemble and oligomerize to form the complex known as the inflammasome have been implicated in both pathogenic and sterile inflammatory responses (81). RLRs play an important role in the innate immune response to RNA viruses, such as influenza, and can also form inflammasome complexes (82). CLRs, such as the Dectins and Mannose Receptor (CD206), though important for the recognition of carbohydrates on microorganisms, will not be discussed here (83). [Adapted from (84)]

1.2.1 Toll-like receptors (TLRs)

The TLR family is responsible for sensing PAMPs and DAMPs at membranes and disseminating the signal to intracellular transcription factors, which regulate cytokine and chemokine gene expression. There are currently 13 identified mammalian TLRs (10 in humans and 12 in mice), which are classified as type 1 transmembrane receptors containing an N-terminal, leucine-rich repeat domain, a transmembrane region, and a C-terminal cytoplasmic domain (85). TLRs are expressed by a wide variety of hematopoietic cells (e.g. Mac and DC) as

well as EC (86). Each TLR is associated with specific recognition patterns: extracellular TLRs 1, 2, 4, and 5 sense bacterial components such as lipoproteins and the bacterial wall component LPS, whereas endosomal TLRs 3, 7, 8, and 9 recognize nucleic acids (Figure 1.3). (80) Notably, TLRs have been implicated in the response to a variety of pathogens, pollutants, and DAMPs, indicating that these receptors are not limited to one specific ligand. For example, TLR4 plays a key role in the innate immune response to the air pollutant O_3, an oxidant gas that cannot be directly bound by a receptor, suggesting that DAMPs from O_3-inudced damage of the airway mediate TLR4 activation (87).

Interaction of the TLR with its specific ligand results in the activation of a signaling cascade leading to the production of innate effector molecules and the initiation of the adaptive immune response (88, 89) (Figure1.3). TLRs signal to the cytoplasm through adaptor proteins, such as myeloid differentiation primary response gene (MyD88), toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF), and translocation associated membrane protein (TRAM), all of which harbor a Toll-Interleukin-1 Receptor (TIR) domain for the recruitment of the adaptor protein to the TLR cytoplasmic domain via a TIR-TIR interaction (85). MyD88 is an adaptor protein shared by all TLRs except TLR3, which instead utilizes TRIF to signal to the cytoplasm. The TLR signaling cascades result in the activation of transcription factors in the cytoplasm, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory Factors (IRFs), and activation protein 1 (AP-1), which then induce or inhibit the transcription of genes involved in inflammatory and immune responses (78, 90).

Dysfunction of the innate immune system and TLR signaling has been associated with the pathogenesis of several airway diseases, including acute respiratory distress syndrome

(ARDS), asthma, and chronic obstructive pulmonary disease (COPD) (78). For example, ARDS may be triggered by bacterial or viral infections and non-infectious insults such as environmental exposures or trauma, which may stimulate TLR signaling and initiate an inflammatory response and tissue damage (78, 91, 92). Additionally, TLR4, the endotoxin receptor, has been shown to play a role in the induction of Th2 biased immune response in the lung and the development of asthma (81). Consequently, mutations in several TLRs, including TLR4, have been associated with asthma (78, 93, 94). Clearly, the TLR signaling pathways play an important role in initiating the immune response to PAMPs and DAMPs which, when left unchecked, can lead to tissue injury and airway disease. [Adapted from (84)]

1.2.2 RIG-I like Receptors (RLRs)

The RLR family consists of three receptors: RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation associated factor 5), and LGP2 (laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2) (82). The receptors of this family share a similar structure with three domains: 1) a N-terminal caspase activation and recruitment domain (CARD); 2) a central DExD/H box RNA helicase domain that recognizes RNA; and 3) a C-terminal repressor domain involved in auto-regulation. Notably, LGP2 does not contain a N-terminal CARD domain and is thought to regulate RIG-I and MDA5 activity (95). These receptors are found in myeloid and epithelial cells in the airway. RIG-I and MDA5 serve as cytoplasmic sensors of RNA from viruses such as influenza, and activation of RLRs results in downstream signaling through the adaptor protein IPS-1/MAVS/VISA/CARDIF leading to transcription factors that regulate antiviral gene expression and production of type 1 interferons (IFN) (82). Notably, RIG-I may also form inflammasome complexes with an adaptor protein, ASC (apoptosis speck-like protein containing a CARD domain) and caspase-1 via the CARD

domain and activate processing of IL-1β and IL-18 (96). Inflammasome signaling will be discussed in detail below (section 1.2.4).

1.2.3 Nod-like Receptors (NLRs)

Nucleotide-binding oligomerization domain receptors (NLRs) are a family of cytoplasmic PRRs that that are characterized by the presence of three domains: a C-terminal leucine-rich repeat domain that binds ligands, a central NATCH nucleotide binding domain that is important for oligomerization, and N-terminal effector domain that may be a pyrin, CARD, or BIR domain (97). The N-terminal effector domain determines the subfamily of the NLR: NLRB has a BIR domain and contains one member NAIP; the NLRC family has a CARD domain and contains NOD1, NOD2, NLRC3, NLRC4, NLRC5, and NLRX1; and the NLRP family has a pyrin domain and includes NLR1 through NLRP14 (98). Notably, the members of these subfamilies may differ in mice (99). The ligands recognized by these receptors are highly varied. For example, NLRC4 recognizes microbial bacterial flagellin and type III secretion machinery, NLRP1 recognizes muramyl dipeptide (a bacterial peptidoglycan), and NLRP3 recognizes a wide range of stimuli from influenza to uric acid crystals (100). Engagement of these receptors results in a cascade of intracellular signaling leading in the production of innate immune mediators, and the specific intracellular signaling pathways differ depending on the receptor. For example, NOD1 and NOD2 signal to transcription factors such as NF-kB, NLRC5 serves as a regulator of NF-kB signaling, and several other NLRs (namely NLRP1, NLRP3, NLRC4) form inflammasome complexes (97, 101, 102). A number of diseases are associated with mutations in NLRs, highlighting the importance of NLRs in immunity. For example, activating mutations in NLRP3 are associated with Familial Cold Autoinflammatory Syndrome and Muckle-Wells Syndrome (98). [Adapted from (84)]

1.2.4 Inflammasome signaling

Upon recognition of a PAMP or DAMP, several NLRs, RIG-I, and the DNA-binding IFI200 family member absent in melanoma 2 (AIM2) may form a multi-protein complex termed the inflammasome. The inflammasome is composed of a PRR, pro-caspase-1, and the adaptor protein Pyd and card domain containing (PYCARD) (103). This complex has been best studied in myeloid cells, particularly Mac and monocytes, though recently there has been increased interest in inflammasome signaling in non-myeloid cells (104).

Upon formation of the inflammasome complex, caspase-1 is autoactivated from the pro-form to the cleaved active form, which can then catalyze the proteolytic processing and release of IL-1β and IL-18. Caspase-1 mediated processing and the release of mature cytokines requires two distinct stimuli (100, 105). First, an inflammatory stimulus activates the transcription of pro-cytokines, such as pro-IL1β and pro-IL18. This signal may be triggered by TLR activation and downstream signaling through the NF-κB or MAPK pathways. The second signal induces formation of the inflammasome complex, cleavage of caspase-1, and then pro-cytokine maturation and release (Figure 1.4). Active caspase-1 may also participate in one of several other effector mechanisms, including induction of a caspase-1 dependent form of programmed cell death termed pyroptosis, activation of NF-kB, and cleavage of proteins involved in glycolysis, apoptosis, and cytoskeleton functions (106, 107).

The exact mechanism of inflammasome activation is not clear. Only AIM2 has been shown to directly bind to its ligand, DNA, prior to inflammasome formation (108). Conversely, NLRC4 and NLRP3 are not thought to directly bind to ligands. NLRC4 has been shown to require co-receptors from NAIP family to recognize the flagella, rod, or needle proteins of bacteria (109). Because NLRP3 senses a variety of PAMPs, DAMPs, and pollutants, NLRP3 is

generally thought to sense general changes in cellular homeostasis rather than directly binding to a ligand (Figure1.4) (100, 110). Activation of the NLRP3-mediated IL-1β/IL-18 processing actually requires three steps: pro-IL-1β and pro-IL-18 transcription, up-regulation of *NLRP3* transcription, and NLRP3-PYCARD-caspase-1 association to form the inflammasome complex (105). Mitochondria are thought to play a key role in NLRP3 inflammasome assembly and activation. Mitochondria may serve as an activation platform for the NLRP3 inflammasome and a source of reactive oxygen species and DNA, both of which have been shown to either directly activate NLRP3 or up-regulate its expression (111-114). Alterations in ion concentrations in the cell have also been associated with NLRP3 inflammasome activation, as low levels of intracellular potassium (K^+) and high levels of intracellular calcium (Ca^+) can activate inflammasome formation (110, 115, 116). Finally, the NLRP3 inflammasome may also be activated by lysosamal damage, as caused by phagocytosis of large crystals or fibers (e.g. asbesotos or silica). Rupture and release of lysosomal contents, such as cathepsin B, may trigger NLRP3 inflammasome activation (117). Thus, a number of questions remain regarding inflammasome activation, and inflammasome activation likely involves a combination of mechanisms that is tailored for specific PRRs and stimuli. [Adapted from (84)]

1.2.5 PRRs in the response to pathogens versus pollutants

Pattern recognition is typically associated with the response to microbial stimuli that may be directly recognized by PRRs via a PAMP, yet PRRs are also implicated in the inflammatory response to a number of pollutants. Neutrophilic influx is a shared characteristic of exposure to both pollutants and the bacterial cell component LPS, leading to the hypothesis that the inflammatory response to these different stimuli may be mediated by a common innate immune mechanism (81). Indeed, studies have shown that the prototypical endotoxin receptor, TLR4, is

important for the inflammatory response to air pollutants (75, 87). Exactly how pollutants activate PRRs such as TLR4 is not as clear. For particulates such as particulate matter or biomass, endotoxin is a common contaminate that can activate TLR4 signaling (118, 119). Yet, other pollutants, such as O_3, induce sterile inflammation and cannot directly bind a PRR, suggesting that pollutant-induced injury may generate DAMPs that activate PRRs (87). For example, O_3 is associated with fragmentation of the extracellular matrix component hyaluronic acid (HA), and these fragments can be recognized by PRRs including TLR2 and TLR4 to induce innate immune responses (91, 120, 121).

Inflammasome signaling has received increased attention as an innate immune pathway induced by a wide array of stimuli, including pathogens, DAMPs, and pollutants. Whereas infection with a virus or bacteria may directly activate AIM2, RIG-I, and other receptors in the cytoplasm and induce inflammasome signaling, the mechanism by which pollutants activate inflammasome signaling is less clear. Some pollutants, such as asbestos fibers and silica crystals, have been shown to induce phagosomal destabilization, lysosomal damage, and the generation of reactive oxygen species in Mac that induce NLRP3 inflammasome formation (117). Other pollutants, such as O_3, more likely activate inflammasome signaling through a secondary mediator or DAMP. Indeed, in murine studies the NLRP3 inflammasome be activated by HA fragments, which may contribute to O_3-induced airway hyperresponsiveness in mice (122, 123). Thus, the inflammasome may be a common pathway by which pollutants and pathogens stimulate similar inflammatory responses.

As indicated above, signaling through PRRs is the first step of the innate immunity to the inhaled environment, be it viruses or pollutants, and the response dictates subsequent adaptive immune responses (3, 80). A number of studies indicate that PRR signaling may be important

mechanism contributing to the pathogenesis of immune diseases, such as allergy and asthma (6). Indeed, viral infection and inhalation of air pollutants are known causes of asthma exacerbation, suggesting innate immunity contributes to asthma pathogenesis. The role of innate immunity in asthma will be discussed below.

1.3 Asthma overview

Over 20 million Americans suffer from asthma, a chronic lung disease characterized by airway inflammation and variable and reversible obstruction of the airway (7). The rates of asthma incidence have continued to increase over the past 50 years, which is thought to be due to a combination of changes in lifestyle and urbanization (124). Asthma is an extremely heterogeneous disease with a diverse etiology and pathophysiology (7). Asthma is thought to be caused by a combination of genetic and environmental factors. A number of susceptibility genes for asthma have been identified that are involved in pattern recognition, T lymphocyte differentiation and function, epithelial cell function, and lung function (125). Yet, not all individuals with familial history or susceptibility genes have asthma, suggesting that interaction with additional environmental factors is necessary (125, 126). Environmental factors that are associated with asthma susceptibility include prenatal maternal smoking, early life viral infection, lack of exposure to infectious agents (known as the “hygiene hypothesis”), and inhalation of air pollutants (124, 127).

The most common form of asthma is allergic asthma, which is induced by allergy to an antigen such as house dust mite (HDM), pollen, or mold and is dependent on the presence of IgE antibodies specific for the allergen. Non-allergic asthma is exacerbated by stimuli such as exercise, cold air, medications (e.g. aspirin), and stress (128). Both forms of asthma are associated with the accumulation of Th2 lymphocytes that secrete the cytokines IL-4, IL-5, and

IL-13. These cytokines induce B cell isotype switching to IgE synthesis, the recruitment of eosinophils, basophils, and mast cells to airway, goblet cell metaplasia, and bronchial reactivity (21). Notably, other helper T lymphocytes can also contribute the pathogenesis of asthma, such as Th17 lymphocytes, which produce IL-17 and attract neutrophils to the airway (71). Chronic inflammation of the airways leads to airway remodeling, obstruction, and airway hyperresponsiveness (AHR) that mediate the asthma symptoms of wheezing and difficulty breathing (7).

Though adaptive immunity, mediated by B and T lymphocytes, is clearly central to asthma pathophysiology, the innate immune system is essential for the initiation and propagation of these adaptive responses and of particular significance during asthma exacerbation (3, 6, 129). The role of innate immunity in asthma development and asthma exacerbation will be briefly introduced below. Primarily allergic asthma development will be discussed here as it is the most common type of asthma.

1.3.1 Innate immunity in asthma development

Both structural airway epithelial cells and innate immune cells, such a DC, are important contributors to the induction and maintenance of Th2-mediated immune responses (21). Increases in airway permeability caused by damage to tight junctions as caused by inhalation of viruses, pollutants, or allergens that contain proteases (e.g. certain HDM species) can lead to increased access of antigen to the underlying tissue or intraepithelial DCs (21, 74). DC that have recognized an antigen then migrate to the draining lymph node, where they present the processed antigen to naïve T lymphocytes (21).

Innate immune PRRs play a key role in priming DC and the airway for the development of Th2 responses. Without the appropriate co-stimulatory molecules to fully activate DC,

presentation of a harmless antigen leads to the induction of tolerance, or unresponsiveness of the adaptive immune system to an antigen (6, 21). In order to be fully active, DCs must be stimulated by PAMP or DAMP activation of innate pattern recognition receptors (TLRs and CLRs) or through proteolytic activity of the allergen (e.g. proteases found in some HDM) that can induce protease activated receptors (PARs) (21, 130). Activation of PRRs induces the production of pro-Th2 cytokines and the expression of co-stimulatory molecules by DCs. Additionally, activation of PRRs on EC induces the release of cytokines, including granulocyte-macrophage colony stimulating factor (GM-CSF), IL-33, and thymic stromal lymphoprotein (TLSP), that prime DC and promote Th2 cell differentiation (131). For example, TSLP produced by EC induces expression of the co-stimulatory molecule OX40L by DCs (132). Thus, there is a clear role for innate immune receptors in the development of asthma, and previous studies have indicated that mice deficient in PRRs such as TLR4 are protected against the development of allergic inflammation (133).

With appropriate activation of DC and the presence of Th2-polarized cytokines in the draining lymph node, particularly IL-4, naïve T cells will differentiate to Th2 lymphocytes (6). Th2 lymphocytes produce cytokines such as IL-4, IL-5, IL-13 that drive allergic inflammation, including B cell isotype switching to IgE synthesis and recruitment or activation of mast cells, eosinophils, and basophils to the airway (6). Degranulation of mast cells, eosinophils, and basophils induces airway smooth muscle contraction and goblet cell metaplasia that are associated with airway obstruction (7). Chronic Th2-skewed airway inflammation causes airway remodeling and permanent changes to the lung structure, such as increased airway smooth muscle mass (6). Thus, the innate immune system is crucial not only for allergen sensitization, but also maintenance of chronic airway inflammation.

Despite the important link between PRRs and asthma development, the contribution of the inflammasome signaling pathway to asthma remains unclear. Due to the involvement of NLRP3 inflammasome in the response to many PAMPs and DAMPs associated with allergic diseases, such as alum and several bacterial and viral species, the NLRP3 inflammasome has been best investigated for a a role in asthma development (134-138). Results from these studies are conflicting and have not identified a clear role for NLRP3 or caspase-1 in the development of allergic inflammation (131, 135,136). Likewise, studies investigating the role of IL-1 receptor (IL-1R) and IL-1 axis signaling in murine models of allergic inflammation are also unclear (131, 133, 134, 137). These studies suggest contribution of inflammasome signaling to asthma development is complex, and may differ depending on the iniator of asthma pathogenesis.

1.3.2 Innate immunity in asthma exacerbation:

Exacerbations of asthma symptoms can lead to emergency department visits, hospitalization, and even fatality in very severe cases (129, 139). There are many causes of asthma exacerbation, including viral infections, allergens, occupational exposures, exercise, stress, and air pollutants (129). In response to these stimuli, structural cells of the airway, such as EC and airway smooth muscle, as well as inflammatory cells, including macrophages, mast cells, eosinophils, and neutrophils initiate an innate immune response, including production of proinflammatory and cytotoxic mediators that lead to acute asthma symptoms (140). Here we will explore two common causes of exacerbation,respiratory viral infections and air pollution, and the innate immune responses that mediate these effects.

Virus-induced asthma exacerbation. The leading cause of an asthma exacerbation is respiratory viral infection. Viruses have been associated with approximately 80% of wheezing episodes in children, and up to three-fourths of wheezing episodes in adults (141-143).

Rhinovirus (RV) is the most frequent cause of virus-induced asthma exacerbation, though other viruses including influenza A virus (IAV) and respiratory syncytial virus (RSV) are also common (142). IAV infection frequently reaches seasonal epidemic and, at times, pandemic proportions and thus is a serious concern for patients with asthma (144).

Airway ECs are the primary target for respiratory viruses such as RV and IAV. *In vitro* and *in vivo* studies have found that the barrier function of EC in asthmatics is impaired due to disruptions in tight junctions (22). Additionally, EC repair and proliferation of basal cells is reduced in the airways of asthmatics (74, 145). Finally, genome-wide expression comparisons of ECs from asthmatics to non-asthmatics found that a number of genes involved in repair and immune responses are differentially expressed by EC from asthmatics (146). Thus, it is not surprising that EC from asthmatics respond differently to viral infection. Upon infection with a virus, pattern recognition receptors such as TLR3 and RIG-I recognize the virus and induce downstream cytokine, chemokine, and antiviral mediators (20). Previous studies have found that EC from asthmatics have deficient interferon (IFN) production after infection and increased production of the inflammatory cytokines IL-1, IL-6, IL-8, and GM-CSF with RV or RSV infection, correlating with the increased pro-inflammatory response to viral infections observed *in vivo* (146-150). Virus-induced damage of EC may increase airway permeability to allergens. Indeed, previous studies have shown a synergistic effect of virus infection and allergen exposure on hospital admissions for asthma (151).

Increased production of cytokines and chemokines by virus-infected EC from asthmatics contributes to the recruitment of inflammatory cells, such as neutrophils, eosinophils and T lymphocytes, to the airways (152, 153). As indicated above, eosinophils are important mediators of allergic asthma, but neutrophilia of the airway is also observed in patients with severe asthma

or during an exacerbation (49). Neutrophils are very reactive and secrete a variety of cytokines and proteases such as neutrophil derived elastase and matrix metalloproteinase 9 that damage the airway and contribute to AHR and increased mucus production (154, 155, 156). Virus-induced asthma exacerbations may also be related to an imbalance of Th1/Th2 responses to the virus. In patients with RV infection, reduced CD4+ T cell production of IFNγ and increased IL-4, IL-5, and IL-13 were associated with more severe RV-induced asthma symptoms (152).

In summary, alterations in EC response, increased airway inflammation, and altered Th1/Th2 response to virus infection are key mediators of virus-induced asthma exacerbation. The specific molecular signaling pathways contribute to altered response to virus must be further investigated.Specifically, the function of inflammasome signaling in virus-induced asthma exacerbations has not been studied despite clear involvement of the inflammasome in the innate immune response to several respiratory infections. such as IAV (157). Moreover, epithelial cells from asthmatics have been shown to have enhanced IL-1 expression after RV infection, suggesting the inflammasome signaling may be altered in airway epithelial cells from asthmatics and thereby contribute to asthma pathogenesis (146). Results shown in Chapter 2 will elucidate the role of inflammasome signaling in the airway epithelial cell innate immune to IAV infection, and how asthma may modified this signaling pathway.

Pollutant-induced asthma exacerbation. Exposure to air pollutants such as particulate matter (PM), diesel exhaust (DE), and ozone (O_3) are associated with increased rates of hospitalization for asthma exacerbation (158-160). Pollutants induce oxidative stress that damages the airway and activates redox-reactive transcription factors, such as NF-kB and AP-1, to enhance inflammatory gene expression (161). Components of damaged cells and tissue serve as DAMPs that can activate innate immune pathways through PRR signaling. Particulate air

pollutants may also serve as carriers for allergens and endotoxin, and thus induce synergistic inflammatory responses (118, 119, 140). Finally, air pollutants may directly affect lung function by activating nocicepetive receptors (162). Though air pollutants may have direct effects on the airway, air pollutants have an even greater adjuvant effect, whereby the inflammation and damage caused by air pollutants may prime for more severe response to a subsequent environmental allergen (163-165).

The effects of DE on asthma and allergy have been best characterized. DE particles (DEP) consist of a carbon core with a large surface area to absorb heavy metals and organic compounds, including allergens (140, 166). DEP has effects on both innate and adaptive immune responses that may contribute to asthma exacerbation. Both *in vitro* and *in vivo* studies suggest that DEP simulates B lymphocyte production of IgE, which then bind to receptors on mast cells and basophils to induce degranulation (167, 168). DEP also enhances Th2-cytokine production, particularly IL-4 and IL-5, as well as induction of IL-8 (a potent neutrophil chemoattractant) and GM-CSF among other mediators (169, 170). The effects of DEP on airway inflammation are even greater in combination with exposure to an allergen, suggesting that DEP may serve as an adjuvant for allergic inflammation and Th2-skewed responses (163, 171) .

Epidemiological studies suggest a clear association between exposure to O_3 and asthma exacerbations (81, 160). O_3 induces both a nociceptive response that affects lung function and an inflammatory response in the airway, and these effects may be more severe in individuals with asthma (81). The effects of O_3 on lung function are observed immediately after exposure and are likely mediated by stimulation of nociceptive receptors and/or by stimulation of C fibers in the lung that induce smooth muscle contraction and increased airway resistance (172, 173). Interestingly, the lung function effects of O_3 seem to be independent of airway inflammation,

suggesting separate mechanisms mediate these effects (81, 174). O_3-induced airway inflammation is thought to be mediated by oxidative stress and damage of the airway epithelium and resident cells. O_3 may directly damage cells of the airway, but due to its high reactivity O_3 likely does not penetrate beyond the epithelial layer or deep into the lung (77). O_3 also reacts with lipid or protein components of the lung lining fluid and induces reactive intermediates such as oxidized lipids and proteins that can travel further into the lung (175-177). Oxidized lipids as well as damaged tissue and cellular debris can serve as DAMPs for PRRs (178, 179). For example, fragments of the extracellular matrix component hyaluronic acid (HA) found in the airway after exposure to O_3 activate TLR4 and the NLRP3 inflammasome to induce inflammation and AHR (121, 123, 179, 180). O_3-induced airway damage is greater in asthmatics compared to healthy volunteers and asthmatics have enhanced O_3-induced inflammatory cytokine levels in the airway, such as IL-1β and IL-6 that correlates with increased neutrophilia in the airway with O_3 (181-183). Additionally, an increase in eosinophils has been observed in the airway of allergic asthmatics after O_3 exposure (182, 184).

Epidemiological studies indicate that there may be a 24-48 hour lag period between O_3 exposure and asthma-related exacerbation, suggesting that increased airway sensitivity and inflammation may prime individuals for enhanced response to an inhaled allergen (81, 185). Indeed, previous controlled exposure studies have found that allergic asthmatics have heightened airway reactivity to allergens after exposure (165, 185, 186). These effects may be due to alterations in inflammatory cell phenotype. Previous studies have shown that Mac and monocytes have altered expression of PRRs, co-stimulatory/antigen presentation molecules, and immunoglobulin receptors after exposure to O_3, and that expression of CD23, FceRI, and TLR4 were further increased in asthmatic subjects (81, 182, 187). Furthermore, a murine study found

that O_3 served as an adjuvant for OVA-induced airway inflammation and activated expression of the co-stimulatory molecule CD86 on DCs (188).

In summary, inhalation of O_3 or other pollutants induce inflammation that may exacerbate the chronic airway inflammation associated with asthma and prime the airway for an enhanced response to a subsequent inhaled stimulus, such as an allergen. As air pollutants are associated with oxidative damage in the airway, DAMPs likely play a particularly important role in the initiation of inflammation in the airway and the chronically inflammed asthmatic airway may be more susceptible to this damage. Many DAMPs found in the airway after exposure to air pollutants, such as HA, may activate inflammasome signaling. Interestingly, asthmatics subjects exposed to O_3 *in vivo* have been shown to have enhanced HA in the airway after exposure to O_3, which was correlated with elevated IL-1β (181, 182). Thus inflammasomem signaling and DAMPs may be important contributors to pollutant-induced asthma exacerbation. In chapters 4 and 5, the contribution of DAMPs to the innate immune response to O_3 will be investigated.

1.4 Summary

The respiratory mucosal host defense system is composed of a complex network of innate and adaptive cells that together must distinguish between innocuous and injurious inhaled stimuli. Resident immune cells, such as EC and Mac, are crucial for determining the very initial responses to inhaled stimuli and recruiting the appropriate inflammatory cells to the airway. The innate immune response informs the development of adaptive immunity. Failure to respond to a pathogenic stimulus may result in susceptibility to infection, whereas excessive response to a harmless stimulus may lead to diseases such asthma.

In this dissertation, we explored the role of inflammasome signaling in the innate respiratory mucosal host defense system and how these pathways may contribute to asthma

pathogenesis. Given that the inflammasome may be a common signaling pathway shared by pathogens and pollutants, we chose to investigate this pathway in the context of two known causes of asthma exacerbation: viral infection and inhalation of the oxidant air pollutant O_3. Viral components serve as PAMPs and can be directly recognized by PRRs, whereas O_3 is a model of sterile inflammation that activates PRRs via DAMPs. In chapters 2 and 3, the role of the inflammasome in virus-induced asthma exacerbation and the development of allergic inflammation will be discussed. In chapters 4 and 5, we investigated the role of DAMPs and the inflammasome in the innate immune response to O_3, and how the interaction between resident EC and Mac influence the response to O_3. Our results uncover new insights on the innate immune response to viruses and pollutants in humans that may contribute to asthma pathogenesis, and suggest that inflammasome signaling may have diverging functions depending on the stimulus and the cell type.

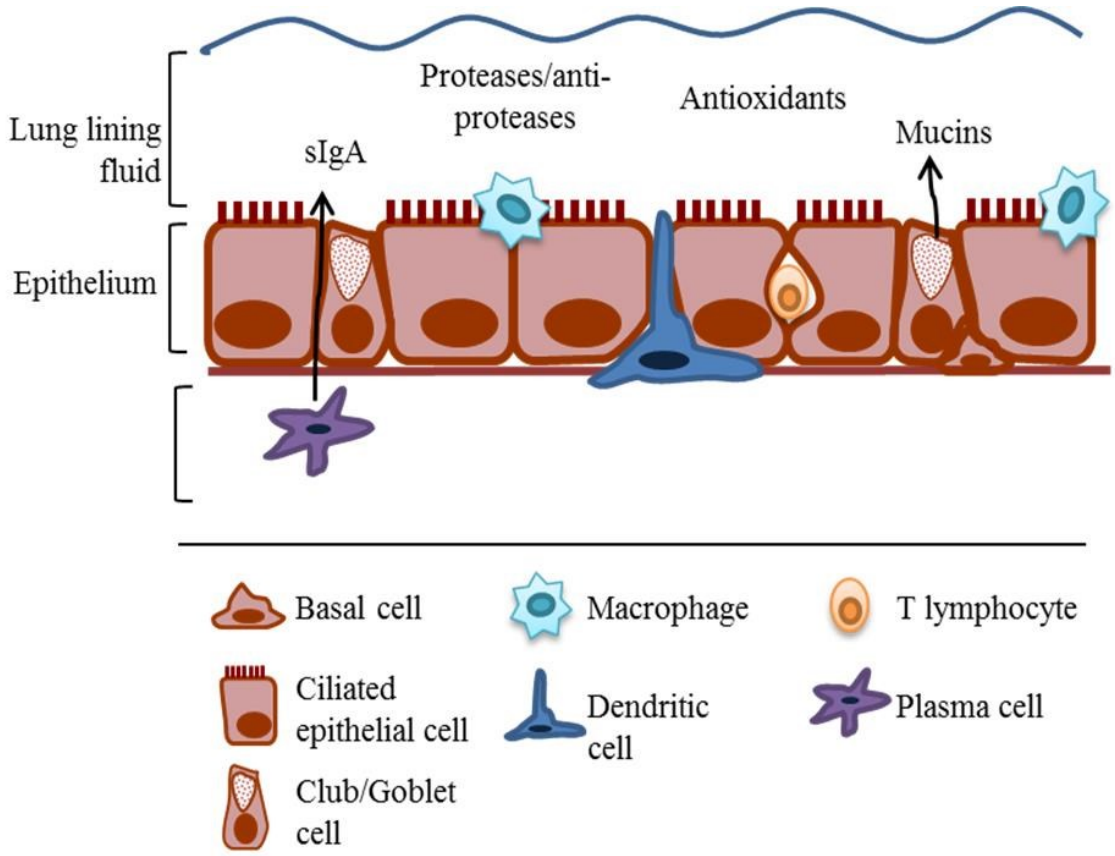

Figure 1.1 Respiratory epithelium at homeostasis. The respiratory epithelium of the bronchial mucosa is composed of four main types of airway epithelial cells: 1) ciliated epithelial cells; 2) goblet (mucus-producing) cells; 3) club (secretory) cells; and 4) basal (precursor) cells. Epithelial cells secrete a number of mediators that make up the lung lining fluid, including mucins, antioxidants, proteases, and anti-proteases, and transport secretory immunoglobulin A (sIgA) from plasma cells to the lumen. Macrophages patrol the airway and clear debris, particulates, and pathogens. Dendritic cells lie under the epithelium, and extend processes to "sample" the airway. Finally, T lymphocytes may be found within the epithelium, though the role of these cells in host defense is unclear.

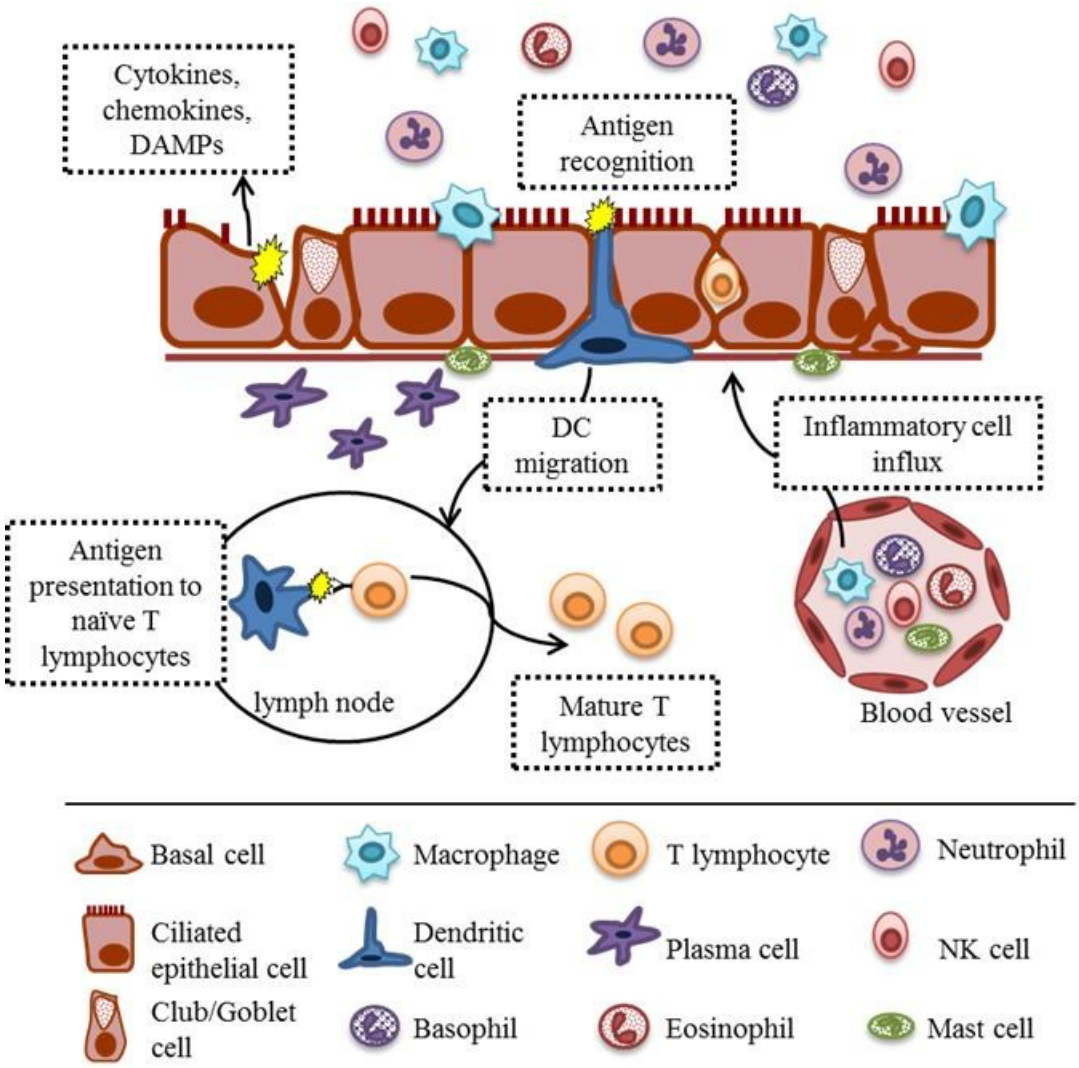

Figure 1.2. Respiratory mucosal host defense to an antigen. Damage to epithelial cells by an injurious stimulus causes the release of damage associated molecular patterns (DAMPs), cytokines, and chemokines to alert immune cells of the injury. The stimulus is recognized by DC as an antigen, leading to DC migration to a draining lymph node where antigen is presented to naïve T lymphocytes. Mature T lymphocytes travel to the site of injury to preform effector functions. Innate immune cells (neutrophils, basophils, eosinophils, mast cells, NK cells) respond to cytokines and chemokines and travel from the blood stream to the airway. B lymphocytes proliferate and differentiate to plasma cells that produce antibody specific for the antigen.

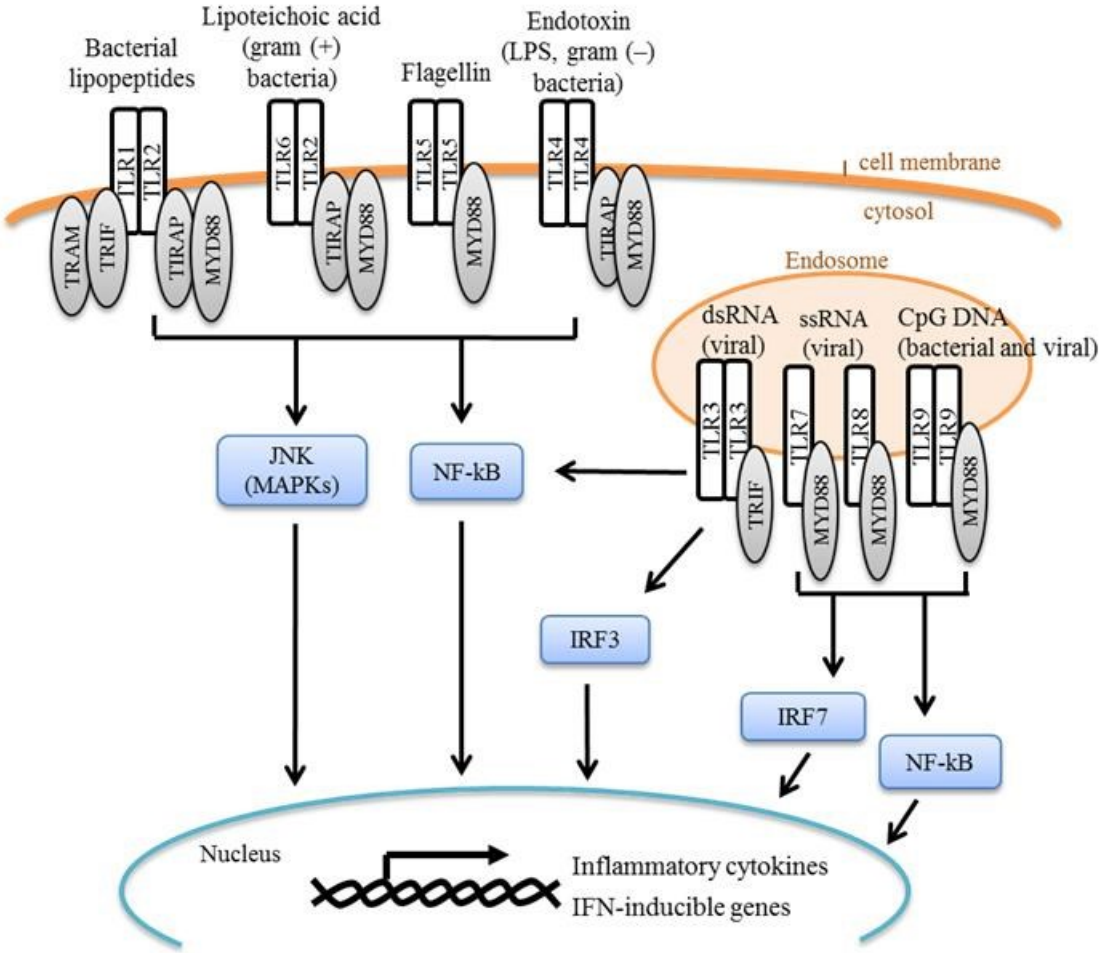

Figure 1.3. Toll-like receptor (TLR) signaling. TLRs are membrane-associated pattern recognition receptors. TLRs may reside on the cell membrane or the endosomal membrane. Each TLR recognizes specific pathogen-associated molecular patterns or damage-associated molecular patterns. Examples of prototypical stimuli are indicated. Active TLRs signal through adaptor proteins such as TRAM, TRIF, TIRAP, and MYD88 to activate MAPK, NF-kB, IRF3, or IRF7 signaling and transcription of inflammatory cytokines and IFN-inducible genes. Image adapted from Bauer, RN; Diaz-Sanchez, D; Jaspers I. Effects of air pollutants on innate immunity: the role of toll-like receptors and nucleotide-binding oligomerization domain–like receptors. J Allergy Clin Immunol. 2012 Jan; 129(1):14-24 with permission from Elsevier.

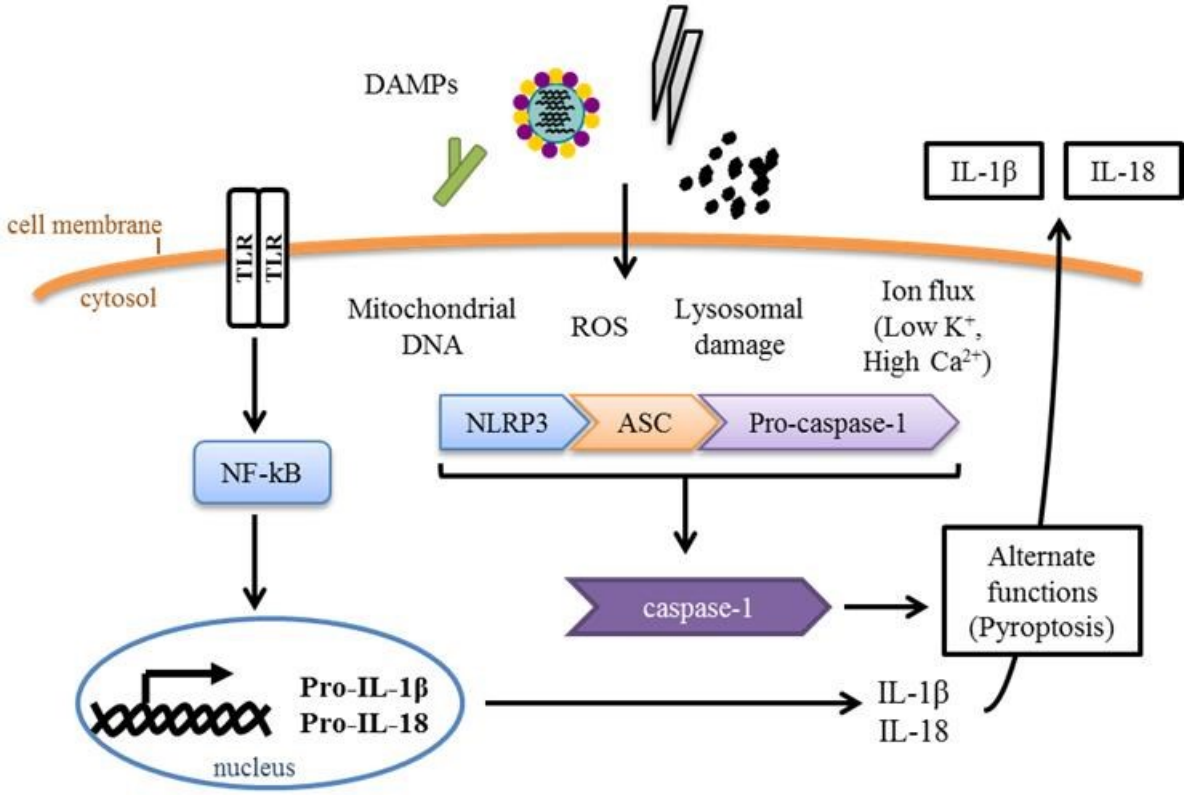

Figure 1.4. Activation of the NLRP3 inflammasome. The NLRP3 inflammasome can respond to a wide range of stimuli, including silica and uric acid crystals, asbestos fibers, bacteria, viruses, particulates, and a number of DAMPs. NLRP3 likely does not bind to these stimuli, but rather senses changes in cellular homeostasis (free mitochondrial DNA, reactive oxygen species (ROS), lysosomal damage, and changes in ion concentration). The NLRP3 inflammasome consists of an adaptor protein (ASC) and pro-caspase-1. Upon formation of the inflammasome, caspase-1 becomes active and can then cleave pro-IL-1β and pro-IL-18 to an active form that can be secreted from the cell or can perform several alternative functions, including initiation of pyroptosis. Image adapted from Bauer, RN; Diaz-Sanchez, D; Jaspers I. Effects of air pollutants on innate immunity: the role of toll-like receptors and nucleotide-binding oligomerization domain–like receptors. J Allergy Clin Immunol. 2012 Jan; 129(1):14-24.

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Chapter 2: Influenza enhances caspase-1 in bronchial epithelial cells from asthmatics and is associated with pathogenesis[1]

2.1 Introduction

Most acute asthma exacerbations are caused by respiratory viral infections and the resulting innate immune response (1-6). Epidemiological studies of the 2009 H1N1 influenza A virus (IAV) pandemic revealed an association between asthma diagnosis and increased morbidity and mortality from infection, yet the mechanisms by which underlying asthma enhances IAV-induced responses are poorly understood (7-10).

The primary sites for IAV infections are airway epithelial cells (AECs). The first step in the AEC response to viral infections is recognition of the virus by pattern recognition receptors (PRRs), such as NOD-like receptors (NLRs) and RIG-I- like receptors (RLRs), which activate downstream signaling cascades to initiate expression of cytokines and chemokines (11, 12). Several NLRs and RIG-I that oligomerize with caspase-1 and PYCARD to form the inflammasome complex have been implicated in the innate immune response to viruses, including IAV (12, 13). Formation of the inflammasome complex induces auto-activation of caspase-1, which catalyzes the proteolytic processing of pro-IL-1β and pro-IL-18. Active caspase-1 may also participate in several alternative mechanisms, including a pro-inflammatory form of programmed cell death termed pyroptosis (14). Alterations in inflammasome signaling are associated with inflammatory diseases including Crohns' disease, gout, and atopic dermatitis

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(15); however, involvement of the inflammasome in virus-induced asthma exacerbation is unknown.

Human bronchial epithelial cells (HBEC) from asthmatics have modified innate immune responses to viral infection. A genome-wide expression study comparing rhinovirus (RV)-infected HBEC from asthmatics and non-asthmatics demonstrated that HBEC from asthmatics had altered RV-induced expression of immune response genes, including *IL1B* (16). In addition, HBEC from asthmatics have been shown to have deficient RV-induced interferon (IFN) production (17, 18) and increased production of the inflammatory cytokines IL-6, IL-8, and GM-CSF with RSV infection (19), correlating with the increased pro-inflammatory response to viral infections seen *in vivo* (20).

Since inflammasome signaling is important for antiviral response to IAV in AECs (21), and virus-induced IL-1β expression differs in HBEC from asthmatics and non-asthmatics (16), we hypothesized that in the asthmatic epithelium, altered expression of inflammasome and innate immune signaling components contributes to virus-induced asthma pathogenesis. Using differentiated primary HBEC, we compared IAV-induced activation of inflammasome and innate immune responses in HBEC from asthmatics and non-asthmatics. Our data show that IAV-induced expression of inflammasome and innate immune signaling components is enhanced in HBEC from asthmatics. Specifically, caspase-1 expression and localization differed in IAV-infected HBEC from asthmatics and associated with enhanced, albeit low levels, of IAV-induced IL-1β production. Using tracheal epithelial cells (MTEC) from caspase-1 deficient (*Casp1* $^{-/-}$) mice, we found that caspase-1 affects expression of several innate immunity genes and viral replication. Our results demonstrate an important role for caspase-1 in the response to IAV

infection at the level of the epithelium, which may be independent of IL-1β production and is enhanced in HBEC from asthmatics.

2.2 Materials and Methods

Subject characterization. Refer to Tables 2.1-2.3 for subject information and a list of allergens included in the allergy skin prick test. All asthmatics had positive skin prick test results or history of allergic rhinitis. 54.5% of the non-asthmatic volunteers had positive skin prick test results or history of allergic rhinitis. Mild asthma status was characterized by history of asthma symptoms (e.g. cough or wheeze) two times or less a week and no current use of inhaled steroids. All asthmatics were considered "mild" except for subject 4, who was considered "moderately asthmatic" due to use of an oral steroid. Subject 4 first entered the human subjects database in 2003 and during this initial screening visit was characterized as "moderately asthmatic" due to the frequency of symptoms (3-4 times a week). The subject went on oral steroids and albuterol in 2003. When the subject enrolled in this study (2009), the subject had not used the oral steroid or albuterol medications in over two months. This subject did not appear to be an outlier in any endpoints. Non-asthmatics had no history of asthma symptoms.

Human bronchial epithelial cell (HBEC) culture. Primary HBEC were obtained from non-asthmatic (n=11) and asthmatic (n=13) adult volunteers by cytologic brushing during bronchoscopy using a protocol approved by the UNC-Chapel Hill School of Medicine Institutional Review Board. HBEC were expanded to passage two in bronchial epithelial growth medium (BEGM; Cambrex Bioscience Walkersville, Inc., Walkersville, MD) and differentiated as described before (22).

Animals and murine tracheal epithelial cell (MTEC) isolation. C57BL/6 *Casp1 -/-* mice were purchased from Jackson Laboratories (Bar Harbor, ME). Female *Casp1 -/-* or wild type

matched littermates were used throughout the study. All experimental procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. MTEC isolation and culture was performed as described by You et al. (23). MTEC were expanded to passage one in Ham's F-12 medium (Invitrogen, Carlsbad, CA) before use.

Influenza infection. HBEC were infected with Influenza A/Bangkok/1/79 (H3N2) (24-25) diluted in Hank's Buffered Saline Solution (HBSS, Invitrogen). MTEC were infected with mouse-adapted Influenza A/PR/8/34 (H1N1) (26-27) diluted in culture medium. Both viruses were obtained from Dr. Melinda Beck (Department of Nutrition, University of North Carolina, Chapel Hill), propagated in 10-day-old embryonated hens' eggs, and collected from the allantoic fluid. For infection of HBEC and MTEC, 500,000 cells were infected with approximately 50 hemagglutination units (HAU). Control treated HBEC received HBSS alone, and MTEC received media alone.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated from HBEC from asthmatics (n=7) and non-asthmatics (n=8) using TRizol (Invitrogen) according to manufacturer instructions. First-strand cDNA synthesis and qRT-PCR were performed as previously described (28, 29). Primers and probes for caspase-1, PYCARD, capase-4, NOD2, RIG-I, IP-10, and β-actin were commercially available (Applied Biosystems, Foster City, CA). Sequences for primers and probes designed "in-house" are as follows: Human: IFN-β: 5'-FAM-AGCAGCAATTTTCAGTGTCAGAAGCTCCTG-TAMRA-3' (probe), 5'-CAACTTGCTTGGATTCCTACAAAG-3' (sense), and 5'-AGCCTCCCATTCAATTGCC-3' (antisense); Hemagglutinin (HA) of influenza A/Bangkok/79/1: probe, 5′-FAM TGATGGGAAAAACTGCACACTGATAGATGC-TAMRA-3′; sense, 5′-CGACAGTCCTCACCGAATCC-3′; antisense, 5′-TCACAATGAGGGTCTCCCAATAG-3′.

Mouse: Matrix 1 (M1) gene of influenza A/PR/8/34: 5'-FAM-TTT GTG TTC ACG CTC ACC GT-TAMRA-3'(probe), and (sense) 5′-AAGACCAATCCTGTCACCTCTGA-3′, and (antisense) 5′-CAAAGCGTCTACGCTGCAGTCC-3′; IL-6: 5'-FAM-CCAGCATCAGTCCCAAGAAGGCAACT-TAMRA-3'(probe), and sense 5'-TATGAAGTTCCTCTCTGCAAGAGA-3' (sense), and 5'-TAGGGAAGGCCGTGGTT-3' (antisense); IFNβ: 5'-FAMAGGGCGGACTTCAAGATCCCTATGGA-TAMRA-3' (probe), and 5'-TGAATGGAAAGATCAACCTCACCTA-3' (sense), and 5'-CTCTTCTGCATCTTCTCCGTCA-3' (antisense). Differences in expression were determined using the ΔΔCt method and *B-actin* for normalization.

qRT-PCR array. Total RNA isolated from a subset of HBEC from asthmatics (n=3) and non-asthmatics (n=3) was purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was prepared using a RT First Strand Kit (SABiosciences, Frederick, MD) and analyzed using the human inflammasome RT2 Profiler PCR Array System (SABiosciences). Gene expression results were normalized to *GAPDH*, analyzed using the ΔΔCt method, and probed for genes with ≤1.5-fold difference in expression.

Cytokine quantification. The apical surface of HBEC were washed with HBSS and analyzed for concentrations of IL-6, IL-1β, and TNFα using a commercially available ELISA kit (Meso Scale Discovery, Gaithersburg, MD). Lower limits of detection were: IL-1β= 0.5 pg/ml; IL-6= 0.22 pg/ml; TNFα= 0.49 pg/ml.

Immunofluorescence microscopy. HBEC from asthmatics (n=3) and non-asthmatics (n=3) were fixed with 4% paraformaldehyde (Sigma-Aldrich) and prepared for immunofluorescence as described previously (30). Primary antibodies for Caspase-1 (1:200, Cell Signaling, Danvers, MA) and PYCARD (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) were incubated

overnight at 4°C. Secondary antibodies were goat anti-mouse-Alexa 488 (for PYCARD) or donkey anti-rabbit- Alexa 568 (for CASP1) (Molecular Probes , Invitrogen). Vectashield plus DAPI (Vector H-1200; Vector Laboratories, Burlingame, CA) was added to mount the transwell membrane. A Nikon C1si confocal microscope with a 60x oil lens and Nikon EZ-C1 3.8 software were used to acquire z-stack images (Nikon Instruments, Melville, NY), which were processed using the NIS-Elements software (Nikon). Images shown are composites of all z-stack slices. Equal adjustments were performed on all images.

Lactate dehydrogenase (LDH) assay. To assess cytotoxicity, the basolateral supernatents from HBEC were analyzed for concentrations of LDH using a commercially available kit (Takara Bio Inc., Otsu, Shiga, Japan).

UV-inactivation of influenza A virus. UV inactivated, replication deficient Influenza A/PR/8/24 was generated by exposing the virus inoculum on ice to 120mJ UV light for 20 minutes using a Stratlinker 1800 (Stratagene, La Jolla, CA). Humaggultination assay was performed to ensure the virions remained intact and M1 gene expression was used to verify replication deficient virus.

Influenza virus titer. Influenza virus titers in apical washes (HBEC) or supernatants (MTEC) were determined by 50% tissue culture infections dose (TCID50) in Madin-Darby canine kidney cells (MDCK) and by hemagglutination as previously described (31).

Array Analysis. Array results were analyzed using the Comparative Marker Selection tool in GenePattern (www.broadinstitute.org/cancer/software/genepattern/) (32). Differences in gene expression between HBEC from asthmatics and HBEC from non-asthmatics were determined using the ΔΔCt method. Briefly, threshold cycle (Ct) value for the housekeeping gene (GAPDH) were subtracted from the Ct value for the gene of interest to determine the ΔCt value. Ct values

that were "undetermined" were replaced with Ct=40 for calculation purposes. For each pair-wise set of samples to be compared, the difference in ΔCt values between the two samples were calculated for the genes of interest to determine the ΔΔCt value. The fold change in gene expression was calculated as 2-ΔΔCt.The fold difference in expression between HBEC from asthmatics and HBEC from non-asthmatics was determined by dividing the average asthmatic 2-ΔΔCt by the average non-asthmatic 2-ΔΔCt. For the array analysis, the p-value was determined by GenePattern comparative marker selection tool with a p-value cut-off of 0.05. 1000 permutation tests were carried out using the signal-to-noise (SNR) ratio analysis and smoothed p-values were determined for each gene. SNR is defined by the equation SNR = (μA – μB) / (σA + σB), where μ represents average sample intensity and σ represents standard deviation.

Statistical analysis. For other non-array gene expression, cytokine analysis, and viral titer data, individual differences between asthmatics and non-asthmatics or *Casp1-/-* and wild type mice were assessed by analysis of variance (ANOVA) with a Tukey post-hoc test (*$p < 0.05$, ** $p< 0.01$, ***$p < 0.001$). Factorial ANOVA was used to determine the interaction between IAV infection and asthma status or mouse genotype (# $p<0.05$, ## $p< 0.01$, and ### $p<0.001$). Endpoints with no detection at baseline (MTEC IL-6 and M1 expression and viral titer) were assessed using unpaired T-test for individual differences between *Casp1 -/-* and wild type MTEC 24 hours post-IAV infection (@ $p<0.05$). All graphs show mean ± SEM.

2.3 Results

HBEC from asthmatics have enhanced IAV-induced production of cytokines

To determine whether HBEC from asthmatics have altered IAV-induced cytokine production, HBEC from asthmatics and non-asthmatics were infected with IAV for 6 or 24 hours and assessed for cytokine secretion (Figure 2.1A-C; Table 2.4-2.5). HBEC from asthmatics secreted significantly more IAV-induced IL-6, TNFα, and IL-1β, with the greatest increase observed 24 hours post-infection. Notably, the subject characterized as "moderately asthmatic" (Table 2.1) had neither an ablated nor a particularly heightened response to IAV infection.

HBEC from asthmatics have differential expression of inflammasome- and innate immunity-related genes

Based on the elevated production of IL-1β by HBEC from asthmatics (Figure 2.1A), we investigated alterations in inflammasome signaling. Using an inflammasome-specific qRT-PCR array, encompassing 84 genes involved in innate immunity and inflammasome signaling, we compared baseline and IAV-induced gene expression between HBEC from asthmatics and non-asthmatics (Table 2.6 and Table 2.7). The expression of eight genes was significantly altered at baseline in HBEC from asthmatics (Table 2.6). Of these genes, all but *CCL5* were down-regulated in HBEC from asthmatics. 24 hours post-IAV infection, HBEC from asthmatics had altered expression of seventeen genes, all of which were enhanced (Table 2.6).

To confirm and expand upon the array results, we performed gene-specific qRT-PCR analysis of several genes with significant differences in HBEC from asthmatics using an increased number of subjects and expanded time course. Similar to the array results, HBEC from asthmatics had significantly increased expression of *CASP1*, *CASP4*, *RIPK2, NLRC5,* and *NOD2* after IAV infection (Figure 2.2A, C-F). The greatest increase was observed 24 hours post-IAV

infection, correlating with the highest production of cytokines. In both HBEC from asthmatics and non-asthmatics, the expression of *PYCARD*, an adaptor protein which links caspase-1 to one of several inflammasome receptors, was initially reduced with IAV infection, and then returned to baseline levels by 24 hours post-infection (Figure 2.2B). The expression of *PYCARD* was slightly decreased in HBEC from asthmatics throughout the time course, correlating with the array results.

Caspase-1 and PYCARD immunofluorescence microscopy

Inflammasome signaling involves the oligomerization of caspase-1, PYCARD, and a PRR to form the inflammasome complex. To determine whether IAV- induced expression of caspase-1 translates to caspase-1/PYCARD co-localization, we used immunofluorescence microscopy to qualitatively assess the localization of PYCARD and caspase-1 in HBEC from asthmatics and non-asthmatics 6 or 24 hours post-IAV infection (Figure 2.3-2.5). In both groups at baseline, caspase-1 and PYCARD were located diffusely throughout the cell (Figure 2.3A-B 1st and 3rd rows, 2.4A-B 1st row). With IAV infection, we observed the formation of co-localized caspase-1/PYCARD foci, especially in the asthmatics, and particularly at 24 hours post-IAV infection (Figure 2.3A-B 2nd and 4th rows, 2.4A-B 2nd row). The HBEC from asthmatics had large, intense foci of co-localized PYCARD and caspase-1 at 24 hours post-IAV infection, (Figure 2.3A 4th row, 2.4A 2nd row), correlating with the enhanced caspase-1 expression 24 hours post-IAV infection (Figure 2.2A). The co-localization of caspase-1 and PYCARD did not correlate with cytotoxicity, as we did not detect any differences in cytotoxicity throughout the infection time course (Figure 2.6). Our data show that PYCARD and caspase-1 co-localized in response to IAV infection in HBEC, and suggests that the intensity of co-localization appears to differ in HBEC from asthmatics.

HBEC from asthmatics have increased expression of antiviral genes, which correlates with enhanced IAV replication

We next investigated whether HBEC from asthmatics also had enhanced expression of known antiviral genes, particularly interferon-γ induced protein 10 (IP-10), interferon β (IFNβ) and retinoic acid inducible-gene I (RIG-I). Our data show that HBEC from asthmatics had elevated expression of these genes 24 hours post- IAV infection (Figure 2.7A-C).

To determine whether the enhanced innate immune response was due to increased viral replication, we assessed influenza hemagglutinin (HA) transcripts and TCID50 viral titers of HBEC from asthmatics and non-asthmatics(24, 33). Though HBEC from asthmatics had no significant overall differences compared to non-asthmatics (Figure 2.8), there was significant correlation between the expression of IP-10, IFNβ, and RIG-I and influenza HA transcript number, suggesting that the enhanced innate immune response in the HBEC from asthmatics was correlated with greater viral replication (Figure 2.9A-C). Likewise, caspase-1 expression was correlated with influenza HA mRNA levels, suggesting that caspase-1 is involved in the antiviral response (Figure 2.9D).

MTEC from Casp1-/- mice have diminished antiviral response to IAV infection and reduced viral replication

Our findings in HBEC from asthmatics indicated the enhanced expression of caspase-1 was associated with heightened innate immune response to IAV. To further assess whether caspase-1 was causally linked to antiviral defense against IAV infection, we examined the expression of innate immune genes by MTEC from *Casp1 -/-* and wild type mice, which were cultured and infected with IAV *ex vivo*. Our results show that *Casp1 -/-* MTEC had reduced IAV-induced expression of IFNα, IFNβ, IL-6, and IP-10 compared to wild type (Figure 2.10),

suggesting that the presence of caspase-1 is necessary for optimal expression of these genes. The baseline and IAV-induced expression of RIG-I was decreased to a similar extent in *Casp1 -/-* MTEC, suggesting that caspase-1 expression may regulate baseline expression of RIG-I, and that these effects persist during IAV infection.

To determine whether decreased viral replication was associated with the diminished innate immune gene expression response in *Casp1-/-* MTEC, we assessed viral replication 24 hours post-IAV infection. Compared to wild type, the *Casp1 -/-* MTEC had reduced levels of influenza matrix 1 (M1) RNA and lower viral titers (Figure 2.11). To determine if viral replication was necessary for the diminished innate immune gene expression response in *Casp1 -/-* MTEC, wild type and *Casp1 -/-* MTEC were infected with UV-inactivated (replication deficient) IAV, wild type IAV, or media control and assessed for expression of IP-10, which had the most robust IAV-induced expression (Figure 2.10), and influenza M1 RNA (Figure 2.12). No M1 transcripts were detected from cells infected with UV-inactivated IAV. As observed in Figure 2.10, wild type-IAV infected *Casp1 -/-* MTEC had significantly lower IP-10 expression compared to wild type MTEC. When infected with UV-inactivated virus, the *Casp1 -/-* and wild type MTEC had similar expression levels of IP-10, suggesting that viral replication was necessary for the diminished innate immune gene expression in *Casp1 -/-* MTEC.

2.4 Discussion

Respiratory viral infections are the leading cause of asthma exacerbations. Innate antiviral defense pathways are altered in the asthmatic epithelium, yet involvement of the inflammasome in virus-induced asthma exacerbations is unknown. We compared IAV-induced activation of inflammasome and innate immune signaling between differentiated HBEC from asthmatics and non-asthmatics, and found that HBEC from asthmatics had modified baseline and

IAV-induced expression of genes involved in innate immune and inflammasome signaling. In particular, we showed that caspase-1 expression was enhanced in HBEC from asthmatics, correlating with enhanced, albeit low levels, of IAV-induced IL-1β production. Using MTEC from *Casp1* -/- and wild type mice, we found that IAV-infected *Casp1* -/- MTEC had decreased expression of innate immunity genes and viral replication compared to wild type. These results establish an important role for caspase-1 in the AEC response to IAV, which may involve inflammasome-independent functions and is enhanced in the asthmatic epithelium.

AEC-derived cytokines and chemokines contribute to the inflammatory cell influx and airway hyperresponsiveness associated with asthma exacerbation. AECs from asthmatics have altered production of cytokines and chemokines in response to RV and RSV (16, 17, 19). Consistent with these observations, we found that baseline and/or IAV-induced levels of innate immune cytokines and chemokines (IL-1β, TNFα, IL-6, CCL5, IP-10, and IFNβ) were enhanced in HBEC from asthmatics. Previous studies have shown that these cytokines are increased in asthmatic airways *in vivo* either at baseline or following challenge and contribute to virus-induced asthma exacerbation (34-39). Our findings add to the literature suggesting that baseline differences in HBEC from asthmatics may prime the epithelium for an exaggerated innate immune response to viral infection.

Many of the cytokines and chemokines with enhanced expression in HBEC from asthmatics are under NF-kB regulation. NF-kB activity has previously been shown to be enhanced in AECs from asthmatics (40, 41).We found that expression of NFKBIB, an inhibitor of NF-kB signaling, was reduced in HBEC from asthmatics at baseline (Table 2.6), which may contribute to the enhanced innate immune response to IAV infection. Interestingly, caspase-1 has been shown to activate NF-kB via RIPK2, a CARD-containing kinase (42) that, similar to

caspase-1, had enhanced IAV-induced expression in HBEC from asthmatics (Table 2.6, Figure 2.2E). Additionally, Nod2 activation of NF-kB signaling has been shown to induce CCL5 release in murine macrophages (43). Our findings indicate that IAV-induced expression of Nod2 was enhanced in HBEC from asthmatics (Table 2.6, Figure 2.2D), suggesting that Nod2 may be a regulator of IAV-induced CCL5 release that is enhanced in HBEC from asthmatics.

The enhanced IAV-induced production of IL-1β suggested that HBEC from asthmatics may have modified inflammasome activity. Other studies have demonstrated involvement of the inflammasome and, more specifically, NOD-like receptor protein 3 (NLRP3), in defense against IAV infection (21, 44, 45). Allen et al. found that AECs secrete IL-1β, albeit at much lower concentrations than monocytes (21). We similarly observed low levels of IL-1β secretion, suggesting that AECs are not a major source of this cytokine. Though the canonical function of the inflammasome is activation of caspase-1 for the proteolytic processing of pro-IL-1β and pro-IL-18, caspase-1 has alternative functions involved in the activation of NF-kB signaling, cell death, cellular metabolism, and cell repair (14). Therefore, caspase-1 may play alternative roles in non-myeloid cells, such as AECs, which do not produce high levels of IL-1β.

Based on this knowledge, we used a less biased approach to identify inflammasome-related pathways activated by IAV infection. The most consistent differences in IAV-induced gene expression were the increased expression of *CASP1* and *CASP4* in HBEC from asthmatics. Caspase-4, a member of the caspase-1 subfamily, is involved in inflammatory responses to IAV infection (46-48). The murine homolog of caspase-4, caspase-11, may be involved in caspase-1 activation and inflammasome-mediated cell death (49, 50).

Activation of the inflammasome pathway involves oligomerization of caspase-1 and PYCARD with a PRR to form the inflammasome complex. The formation of large caspase-

1/PYCARD foci has previously been shown in macrophages and monocytes (51-53). In resting monocytes and macrophages, PYCARD and caspase-1 exist diffusely throughout the cell (50, 52). When stimulated by an agonist, PYCARD forms large cytosolic foci, which are often associated with NLRs and caspase-1 (50). Using immunofluorescence microscopy, we demonstrated IAV-induced co-localization of caspase-1 and PYCARD in HBEC. In agreement with the gene expression and cytokine data, we observed large and intense caspase-1 and PYCARD co-localization with IAV infection especially in asthmatics at 24 hours post-IAV infection.

Our findings in HBEC indicate that caspase-1 is involved in the innate immune response to IAV infection. To further define the role of caspase-1 in antiviral defense, we infected MTEC from *Casp1 -/-* and wild type mice with IAV. Notably, the mouse-adapted influenza virus influenza A/PR/8/34 strain was used to infect MTEC and may differ in infectivity compared the human influenza A/Bangkok/1/79 strain. Our results indicate that IAV-infected MTEC and HBEC had similar patterns of innate immune gene expression. We found that 24 hours post-IAV infection, *Casp1 -/-* MTEC had decreased viral replication, correlating with reduced expression of several innate immune genes. Interestingly, enhanced caspase-1 expression in HBEC was correlated with increased influenza HA transcript quantity, suggesting that caspase-1 may be a determinate of viral replication. Previous studies have shown that *Casp1-/-* mice infected *in vivo* with IAV had reduced quantities of innate immune cytokines and chemokines in the bronchoalveolar lavage, which correlated with increased severity of pneumonia and mortality (44). In contrast to our study, this study did not detect a difference in lung viral titers measured at 3 or 6 days post-IAV infection. Thus, though caspase-1 appears important for IAV-induced expression and secretion of cytokines and chemokines, caspase-1 involvement in viral replication

remains unclear. Notably, the *Casp-1 -/-* mouse model also harbors a mutation in the *Casp11* gene, rendering the mice *Casp1*/*Casp11* double knockouts (48). Our findings in HBEC show that expression of caspase-4 (the human homolog of caspase-11) is up-regulated following IAV infection. Future studies are necessary to delineate the functions of caspase-1 and caspase-4 in antiviral response.

Though our results suggest that caspase-1 is important for the innate immune response to IAV, whether caspase-1 activity is related to inflammasome complex formation remains unclear. We were unable to detect expression of many NOD-like receptors by the qRT-PCR array, including NLRP3, which was previously shown to be expressed in AECs following IAV infection (21, 44). While protein expression cannot be excluded, these results indicate that, in AECs, the NLRP3 inflammasome may play a less prominent role than caspase-1 and other complexes in the response to IAV.

Our findings suggest that HBEC from asthmatics have an enhanced innate immune response to IAV infection. We found that HBEC from asthmatics had baseline and virus-induced differences in gene expression, and that enhanced innate immune gene expression correlated with viral replication. However, we found no overall differences in viral replication. These findings are consistent with a recent genome-wide expression study demonstrating that HBEC from asthmatics have many baseline differences in gene expression also present in cells with RV infection (16). Yet, this study and others using HBEC have shown either enhanced or no difference in RV replication in asthmatics compared to non-asthmatics (16, 17, 54). Therefore, HBEC from asthmatics may not necessarily have a more severe viral load, but rather a more sensitive and severe innate immune response, contributing to airway inflammation and pathogenesis.

Collectively, our results demonstrate that caspase-1 is important for the AEC innate immune response to IAV infection, which is enhanced in HBEC from asthmatics. Similar to other studies, our findings suggest that caspase-1 regulates the expression and secretion of cytokines besides IL-1β and IL-18, and thus may have other non-inflammasome related functions contributing to innate immunity (44). Since viral infections are the leading cause of asthma exacerbations, understanding the contribution of modified innate immune mechanisms to asthma pathogenesis is essential for the development of relevant therapies.

Table 2.1 Subject Characteristics

SUBJECT NUMBER	AGE	SEX	RACE	SKIN TEST	HISTORY OF ASTHMA	HISTORY OF ALLERGIES
1	25	F	W	POSITIVE-+7	Y-MILD	Y
2	27	F	W	POSITIVE-+5	Y-MILD	Y
3	26	F	B	POSITIVE-+4	Y-MILD	Y
4	32	F	H	POSITIVE-+2	Y-MOD	Y
5	22	M	W	POSITIVE-+9	Y-MILD	Y
6	30	M	W	NOT DONE	Y-MILD	Y
7	22	F	H	POSITIVE-+3	Y-MILD	NO
8	20	M	B	POSITIVE-+4	Y-MILD	Y
9	23	M	W	POSITIVE-+5	Y-MILD	Y
10	26	F	MULTI	POSITIVE-+2	Y-MILD	NO
11	23	M	W	NOT DONE	Y-MILD	Y
12	22	F	W	POSITIVE-+4	Y-MILD	Y
13	21	M	H	POSITIVE-+3	Y-MILD	Y
14	24	M	W	POSITIVE-+3	NO	NO
15	20	M	W	POSITIVE-+8	NO	NO
16	22	F	W	NEGATIVE	NO	NO
17	20	F	W	NOT DONE	NO	Y
18	38	M	W	NOT DONE	NO	Y
19	21	F	B	NEGATIVE	NO	NO
20	31	M	B	POSITIVE-+9	NO	Y
21	24	M	W	POSITIVE-+2	NO	Y
22	22	F	W	NEGATIVE	NO	NO
23	19	M	B	NOT DONE	NO	NO
24	23	M	W	NOT DONE	NO	NO

Sex: F= female; M= male
Race: W=white; B= black; H=Hispanic; MULTI= multiple races
Skin test: If positive, the number of antigens the subject was sensitive to is listed.
History of asthma symptoms; Y= yes; MILD= history of mild symptoms; MOD= history of moderate symptoms
History of allergy symptoms; Y=yes

Table 2.2 Summary of subject characteristics

Parameter	Non-asthmatic	Asthmatic
Age (years)	24.54	24.77
Sex (n; female/male)	4/7	7/6
Race (n; black (B)/white (W)/Hispanic (H)/multi (M))	3B/9W/0H/0M	2B/7W/3H/1M
Postive Allergy skin prick test or history of allergic rhinitis (% yes)	54.5%	100%
History of asthma symptoms (% yes)	0 %	100%*

*All subjects were considered "mild asthmatics" except for one, which was characterized as "moderate asthmatic" due to use of oral steroids.

Table 2.3 Antigens for allergy skin test

Antigens for allergy skin test
Mite Pteronyssinus
Mite Farinae
Cockroach
Tree Mix (GS) 10
Grass Mis (GS) 7
Weed
Mold #1
Mold#2
Cat
Guinea Pig
Rabbit
Dog
Rat
Mouse

Table 2.4 Cytokine levels and Influenza HA mRNA 6 hours post-IAV infection

Cytokine levels and Influenza HA mRNA 6 hours post-IAV infection					
Subject #	Asthma	IL-1β pg/ml	IL-6 pg/ml	TNFα pg/ml	HA mRNA ($2^{\wedge -\Delta\Delta Ct}$)
1	Y	0.5	276.8	6.1	15.8
2	Y	0.6	572.6	11.5	9.6
3	Y	0.8	190.0	7.1	3.1
4	Y	0.9	417.7	3.8	0.3
5	Y	1.9	394.5	5.1	0.7
6	Y	1.3	224.8	10.6	0.3
8	Y	1.3	81.5	2.9	1.1
14	N	0.0	19.4	0.4	1.2
15	N	0.0	13.2	0.2	0.4
16	N	0.0	19.3	0.2	0.3
17	N	0.2	69.7	2.0	1.1
18	N	0.0	1.0	0.1	1.0
19	N	0.0	4.2	0.1	2.1
20	N	0.0	0.4	0.2	0.4
21	N	0.1	15.5	0.3	0.8

Table 2.5 Cytokine levels and Influenza HA mRNA 24 hours post-IAV infection

Cytokine levels and Influenza HA mRNA 24 hours post-IAV infection					
Subject #	Asthma	IL-1β pg/ml	IL-6 pg/ml	TNFα pg/ml	HA mRNA ($2^{\wedge -\Delta\Delta Ct}$)
1	Y	5.8	5732.7	354.6	2824.9
2	Y	12.8	7325.3	435.0	2115.8
3	Y	2.5	1316.9	17.7	434.4
4	Y	5.0	1684.7	6.7	60.4
5	Y	11.7	3858.4	24.9	322.5
6	Y	2.3	1126.6	8.6	109.2
8	Y	1.1	193.6	20.6	282.5
14	N	0.2	135.4	2.0	124.9
15	N	0.2	24.4	0.3	55.6
16	N	0.5	150.1	1.7	20.5
17	N	0.9	677.3	14.7	250.4
18	N	0.1	32.0	0.5	92.2
19	N	0.9	385.3	2.5	1169.0
20	N	0.4	15.0	1.6	41.2
21	N	2.3	71.1	1.0	90.6

Table 2.6 Genes with significantly different expression in HBEC from asthmatics vs. non-asthmatics at baseline (control treatment) and 24 hours post-IAV infection

Gene Symbol	Gene Name	Average Non-Asthmatic $2^{-\Delta\Delta Ct}$ [1]	Average Asthmatic $2^{-\Delta\Delta Ct}$ [1]	Average FD[3]	p-value[2]
Baseline (control treatment)					
BCL2	B-cell CLL/lymphoma 2	0.89	0.33	0.39	0.050
CCL5	Chemokine (C-C motif) ligand 5	0.68	3.04	4.50	0.002
IRAK1	Interleukin-1 receptor-associated kinase 1	1.23	0.60	0.49	0.050
MAP3K7IP1	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	0.96	0.63	0.66	0.050
MAPK13	Mitogen-activated protein kinase 13	1.12	0.54	0.48	0.050
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, β	1.14	0.61	0.54	0.050
NLRP1	NLR family, pyrin domain containing 1	1.49	0.67	0.45	0.050
PYCARD	PYD and CARD domain containing	1.15	0.65	0.57	0.050
24 hours post- IAV infection					
BIRC2	Baculoviral IAP repeat-containing 2	0.88	2.41	2.75	0.002
BIRC3	Baculoviral IAP repeat-containing 3	0.56	4.89	8.72	0.002
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interlekin 1β, convertase)	0.75	2.16	2.86	0.002
CASP4	Caspase 4, apoptosis-relted cysteine peptidase	0.73	1.85	2.54	0.002
CASP5	Caspase 5, apoptosis-related cysteine peptidase	0.60	10.90	18.13	0.002
CCL5	Chemokine (C-C motif) ligand 5	0.38	5.56	14.80	0.002
CFLAR	CASP8 and FADD-like apoptosis regulator	1.19	3.11	2.61	0.002
IL12A	Interleukin 12A	0.42	3.44	8.29	0.002
IRF1	Interferon regulatory factor 1	0.74	1.51	2.04	0.002
IRF2	Interferon regulatory factor 2	1.04	1.84	1.77	0.002
MYD88	Myeloid differentiation primary	0.84	1.67	1.99	0.002

	response gene (88)				
NLRC5	NLR family, CARD domain containing 5	0.65	1.49	2.30	0.002
NOD2	Nucleotide-binding oligomerization domain containing 2	0.92	2.48	2.71	0.002
PANX1	Pannexin 1	0.95	2.92	3.07	0.002
RIPK2	Receptor-interacting serine-threonine kinase 2	0.71	3.14	4.44	0.002
TNF	Tumor necrosis factor (TNF superfamily, member 2)	0.55	7.67	13.86	0.002
TXNIP	Thioredoxin interacting protein	0.94	1.62	1.73	0.002

IAV, influenza A virus; HBEC, human bronchial epithelial cell; FD, fold difference

1. Differences in gene expression between HBEC from asthmatics (n=3) and non-asthmatics (n=3) were determined using the ΔΔCt method. $2^{-\Delta\Delta Ct}$ represents the fold change in gene expression.
2. Significantly different gene expression ($p \leq 0.05$) was determined using signal-to-noise ratio (SNR) analysis.
3. The fold difference (FD) in expression between HBEC from asthmatics and non-asthmatics was determined by dividing the average asthmatic $2^{-\Delta\Delta Ct}$ by the average non-asthmatic $2^{-\Delta\Delta Ct}$. Only genes with greater than 1.5 times higher or lower expression were considered "significantly different."

Table 2.7. Full inflammasome array results at baseline (A) and 24 hours after IAV infection (B)

Table 2.7.A. Baseline ($2^{-\Delta\Delta Ct}$) Differences between HBEC from asthmatics vs. Non-asthmatics

Gene Symbol	Average $2^{-\Delta\Delta Ct}$ Non-Asthmatic baseline[1]	Average $2^{-\Delta\Delta Ct}$ Asthmatic baseline [1]	Fold Difference (Asthmatic/Non-Asthmatic)[2]	p-value[3]
AIM2	0.401	0.057	0.142	0.096
BCL2	0.885	0.326	0.368	0.050
BCL2L1	0.946	1.022	1.081	0.703
BIRC2	0.878	0.995	1.134	0.373
BIRC3	0.588	1.220	2.076	0.060
CARD18	8.844	8.041	0.909	0.902
CARD6	1.118	1.164	1.041	0.699
CASP1	1.084	1.026	0.946	0.920
CASP4	0.964	1.063	1.102	0.435
CASP5	0.409	1.607	3.926	0.190
CASP8	1.446	1.187	0.821	0.633
CCL2	0.546	1.538	2.815	0.513
CCL5	0.675	3.040	4.503	0.002
CCL7	ND	ND	N/A	N/A
CD40LG	ND	ND	N/A	N/A
CFLAR	1.141	1.453	1.274	0.174
CHUK	1.033	0.866	0.838	0.607
CIITA	0.767	0.597	0.779	0.401
CTSB	0.748	0.801	1.071	0.856
CXCL1	0.588	0.917	1.560	0.427
CXCL2	1.322	1.558	1.179	0.687
FADD	1.116	0.801	0.718	0.341
HSP90AA1	1.080	1.242	1.150	0.463
HSP90AB1	1.086	0.987	0.908	0.216
HSP90B1	0.855	1.011	1.183	0.174
IFNB1	28.402	25.254	0.889	0.962
IFNG	ND	ND	N/A	N/A
IKBKB	0.872	0.668	0.765	0.250
IKBKG	1.212	0.815	0.673	0.148
IL12A	0.565	1.672	2.959	0.060
IL12B	5.088	0.697	0.137	0.709
IL18	0.905	0.760	0.840	0.250
IL1B	1.699	3.003	1.767	0.148
IL33	2.004	3.417	1.705	0.391
IL6	47.578	858.135	18.036	0.070

IRAK1	1.227	0.602	0.491	0.050
IRF1	0.762	0.724	0.950	0.872
IRF2	0.940	0.768	0.817	0.216
MAP3K7	0.862	0.986	1.144	0.649
MAP3K7IP1	0.955	0.628	0.657	0.050
MAP3K7IP2	0.933	0.825	0.884	0.721
MAPK1	1.051	0.774	0.737	0.204
MAPK11	0.864	0.668	0.773	0.250
MAPK12	0.861	0.954	1.108	0.782
MAPK13	1.123	0.538	0.479	0.050
MAPK3	0.999	0.728	0.729	0.050
MAPK8	0.940	1.081	1.151	0.449
MAPK9	0.826	0.691	0.836	0.479
MEFV	ND	ND	N/A	N/A
MYD88	1.459	1.010	0.693	0.162
NAIP	0.963	1.272	1.320	0.387
NFKB1	0.821	0.603	0.734	0.216
NFKBIA	0.924	0.773	0.836	0.152
NFKBIB	1.141	0.614	0.538	0.050
NLRC4	0.811	0.404	0.499	0.176
NLRC5	0.942	1.146	1.217	0.683
NLRP1	1.491	0.673	0.451	0.050
NLRP12	ND	ND	N/A	N/A
NLRP3	ND	ND	N/A	N/A
NLRP4	ND	ND	N/A	N/A
NLRP5	ND	ND	N/A	N/A
NLRP6	1.276	2.103	1.648	0.230
NLRP9	1.134	1.064	0.938	0.958
NLRX1	1.179	0.830	0.704	0.150
NOD2	1.859	1.052	0.566	0.172
P2RX7	7.281	3.149	0.432	0.405
PANX1	0.904	0.845	0.936	0.733
PEA15	1.474	0.930	0.631	0.108
PSTPIP1	0.907	0.597	0.659	0.192
PTGS2	0.710	1.970	2.775	0.269
PYCARD	1.150	0.651	0.566	0.050
PYDC1	0.902	1.369	1.517	0.228
RAGE	0.949	0.897	0.945	0.954
RELA	1.036	0.711	0.686	0.050
RIPK2	0.790	0.784	0.993	0.924
SUGT1	1.178	1.104	0.937	0.856
TIRAP	0.820	0.471	0.574	0.150
TNF	29.599	97.748	3.302	0.297

TNFSF11	1.107	1.587	1.434	0.667
TNFSF14	0.624	1.667	2.673	0.202
TNFSF4	0.570	0.337	0.591	0.323
TRAF6	0.959	0.664	0.692	0.257
TXNIP	0.895	0.709	0.792	0.303
XIAP	0.946	0.799	0.844	0.411

ND= not detectable, all subjects had Ct>35 or "undetermined"
N/A= not applicable because gene expression was not detectable
IAV, influenza A virus; HBEC, human bronchial epithelial cells

Table 2.7.B 24 hours post-IAV infection differences ($2^{-\Delta\Delta Ct}$) HBEC from asthmatics vs. non-asthmatics

Gene Symbol	Average $2^{-\Delta\Delta Ct}$ Non-Asthmatic IAV 24hr[1]	Average $2^{-\Delta\Delta Ct}$ Asthmatic IAV 24hr [1]	Fold Difference (Asthmatic/Non-Asthmatic)[2]	p-value[3]
AIM2	0.45	2.25	5.01	0.162
BCL2	1.09	0.94	0.86	0.978
BCL2L1	1.07	0.92	0.86	0.994
BIRC2	0.88	2.41	2.75	0.002
BIRC3	0.56	4.89	8.72	0.002
CARD18	13.91	13.87	1.00	0.643
CARD6	0.91	0.90	0.99	0.886
CASP1	0.75	2.16	2.86	0.002
CASP4	0.73	1.85	2.54	0.002
CASP5	0.60	10.90	18.13	0.002
CASP8	0.54	0.94	1.75	0.707
CCL2	0.50	8.48	17.08	0.527
CCL5	0.38	5.56	14.80	0.002
CCL7	ND	ND	N/A	N/A
CD40LG	ND	ND	N/A	N/A
CFLAR	1.19	3.11	2.61	0.002
CHUK	0.76	0.49	0.65	0.655
CIITA	0.98	1.24	1.26	0.601
CTSB	0.76	0.63	0.83	0.667
CXCL1	0.67	1.11	1.67	0.343
CXCL2	1.06	2.55	2.40	0.343
FADD	0.94	0.72	0.77	0.421
HSP90AA1	1.09	1.09	1.00	0.601
HSP90AB1	1.41	1.05	0.74	0.581
HSP90B1	1.07	1.39	1.30	0.198
IFNB1	0.37	4.56	12.25	0.162
IFNG	ND	ND	N/A	N/A
IKBKB	0.73	0.51	0.70	0.655

IKBKG	1.72	1.01	0.59	0.397
IL12A	0.42	3.44	8.29	0.002
IL12B	0.36	2.02	5.69	0.162
IL18	0.83	0.72	0.87	0.794
IL1B	0.88	2.38	2.69	0.184
IL33	8.21	19.97	2.43	0.184
IL6	0.41	12.01	29.47	0.162
IRAK1	1.44	0.49	0.34	0.238
IRF1	0.74	1.51	2.04	0.002
IRF2	1.04	1.84	1.77	0.002
MAP3K7	1.24	1.31	1.06	0.379
MAP3K7IP1	1.86	1.00	0.54	0.397
MAP3K7IP2	0.96	1.23	1.28	0.379
MAPK1	1.23	1.08	0.88	0.812
MAPK11	1.80	1.08	0.60	0.567
MAPK12	1.19	0.81	0.68	0.603
MAPK13	1.08	0.56	0.52	0.421
MAPK3	0.96	0.68	0.71	0.581
MAPK8	1.17	1.17	1.00	0.812
MAPK9	0.84	0.63	0.75	0.655
MEFV	ND	ND	N/A	N/A
MYD88	0.84	1.67	1.99	0.002
NAIP	1.31	1.46	1.12	0.944
NFKB1	0.73	0.96	1.32	0.537
NFKBIA	0.74	1.72	2.34	0.343
NFKBIB	1.93	1.60	0.83	0.812
NLRC4	0.93	1.67	1.79	0.343
NLRC5	0.65	1.49	2.30	0.002
NLRP1	1.45	1.05	0.72	0.567
NLRP12	ND	ND	N/A	N/A
NLRP3	ND	ND	N/A	N/A
NLRP4	ND	ND	N/A	N/A
NLRP5	ND	ND	N/A	N/A
NLRP6	0.71	3.65	5.18	0.345
NLRP9	1.02	1.27	1.25	0.343
NLRX1	1.38	0.83	0.60	0.581
NOD2	0.92	2.48	2.71	0.002
P2RX7	0.75	0.57	0.76	0.974
PANX1	0.95	2.92	3.07	0.002
PEA15	1.44	0.90	0.63	0.397
PSTPIP1	3.81	3.69	0.97	0.643
PTGS2	0.71	3.28	4.59	0.343
PYCARD	0.95	0.84	0.88	0.994

PYDC1	0.93	1.02	1.09	0.002
RAGE	1.27	1.16	0.91	0.956
RELA	1.13	1.51	1.34	0.002
RIPK2	0.71	3.14	4.44	0.002
SUGT1	1.42	1.13	0.80	0.812
TIRAP	1.14	0.74	0.65	0.812
TNF	0.55	7.67	13.86	0.002
TNFSF11	0.85	3.53	4.14	0.527
TNFSF14	0.91	3.43	3.78	0.345
TNFSF4	5.29	2.93	0.55	0.784
TRAF6	0.76	0.72	0.94	0.902
TXNIP	0.94	1.62	1.73	0.002
XIAP	1.02	1.58	1.54	0.184

ND= not detectable, all subjects had Ct>35 or "undetermined"
N/A= not applicable because gene expression was not detectable
IAV, influenza A virus; HBEC, human bronchial epithelial cells

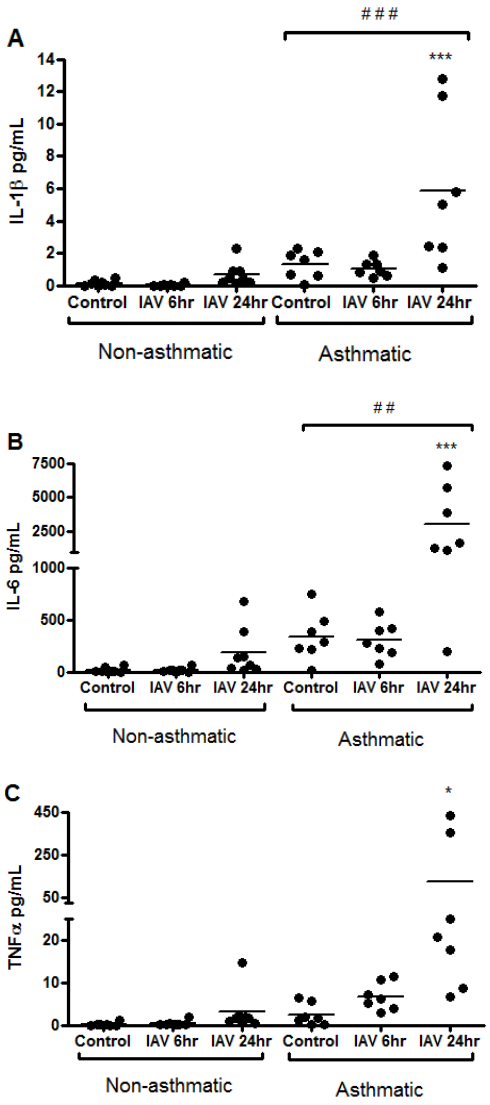

Figure 2.1. HBEC from asthmatics have enhanced production of pro-inflammatory cytokines in response to IAV infection. Apical washes from HBEC from asthmatics (n=7) and non-asthmatics (n=8) were collected 6 or 24 hours post-IAV infection or 24 hours HBSS control treatment and analyzed for (A) IL-1β; (B) IL-6; and (C) TNFα concentrations by ELISA.

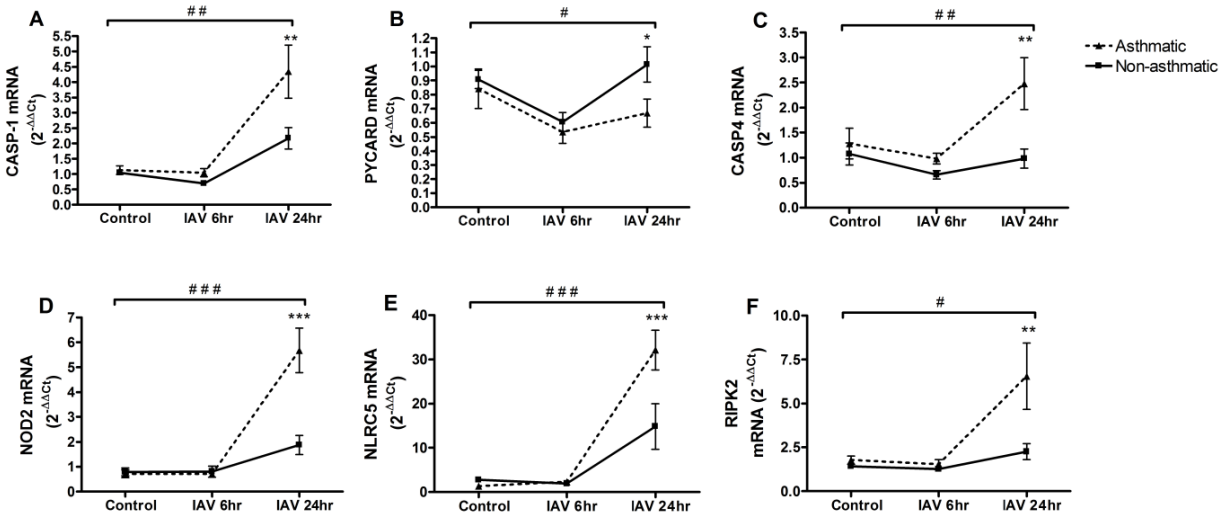

Figure 2.2. Gene- specific qRT-PCR confirmation of inflammasome-related gene expression. Total RNA from HBEC from asthmatics (n=7) and non-asthmatics (n=8) at 6 or 24 hours post-IAV infection or 24 hours HBSS control treatment was analyzed for expression of (A) CASP1; (B) PYCARD; (C) CASP4; (D) NOD2; (E) NLRC5; and (F) RIPK2 by qRT-PCR. Ct values were normalized to β-actin.

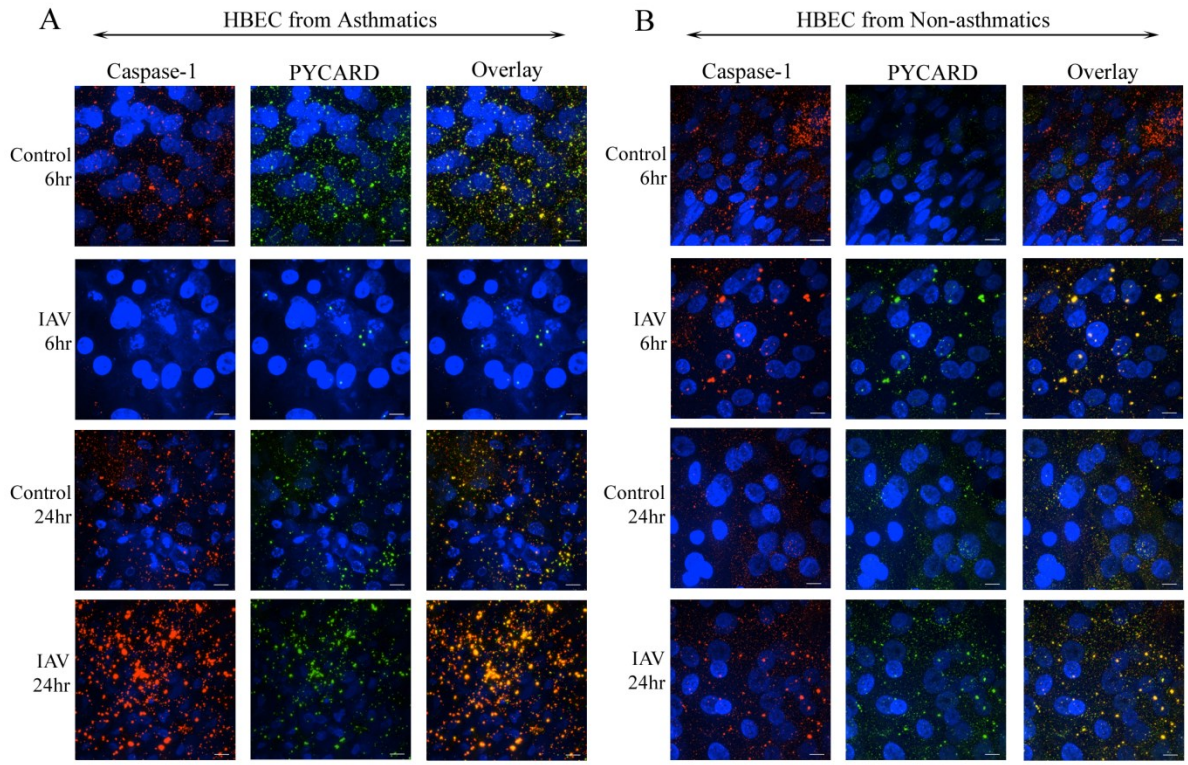

Figure 2.3. Caspase-1 and PYCARD co-localize with IAV-infection. HBEC from (A) asthmatics and (B) non-asthmatics 6 or 24 hours post-IAV infection or HBSS control treatment were probed for *Caspase*-1 (red) and PYCARD (green). DAPI stain identified nucleic acid (blue). Images are representative of 3 asthmatic and 3 non-asthmatic isolates. White bars=10µm.

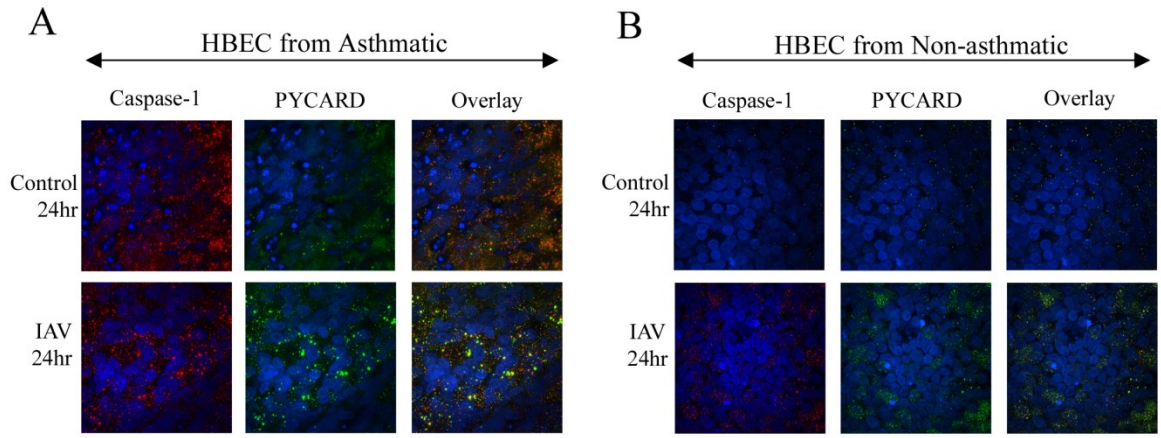

Figure 2.4. Additional immunofluorescence images of caspase-1 and PYCARD localization. HBEC from (A) asthmatic and (B) non-asthmatic 24 hours post-IAV infection or HBSS control treatment were probed for Caspase-1 (red) and PYCARD (green). DAPI stain identified nucleic acid (blue).

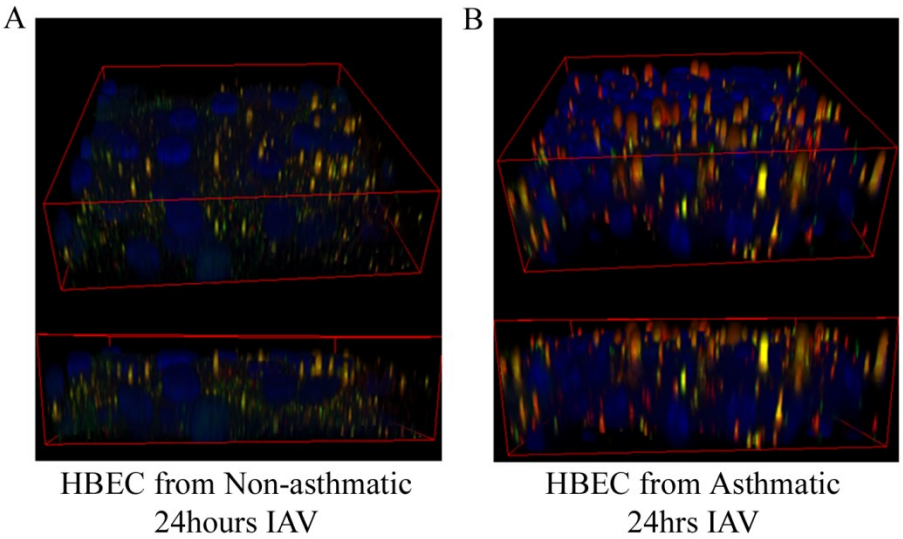

Figure 2.5. 3-dimensional and side view of caspase-1 and PYCARD localization. HBEC from an (A) asthmatic and (B) non-asthmatic 24 hours post-IAV infection were probed for Caspase-1 (red) and PYCARD (green). DAPI stain identified nucleic acid (blue). Images show 3-dimensional and side views of the z-stacks that were used to create the compressed images corresponding with images from Figure 2.3.

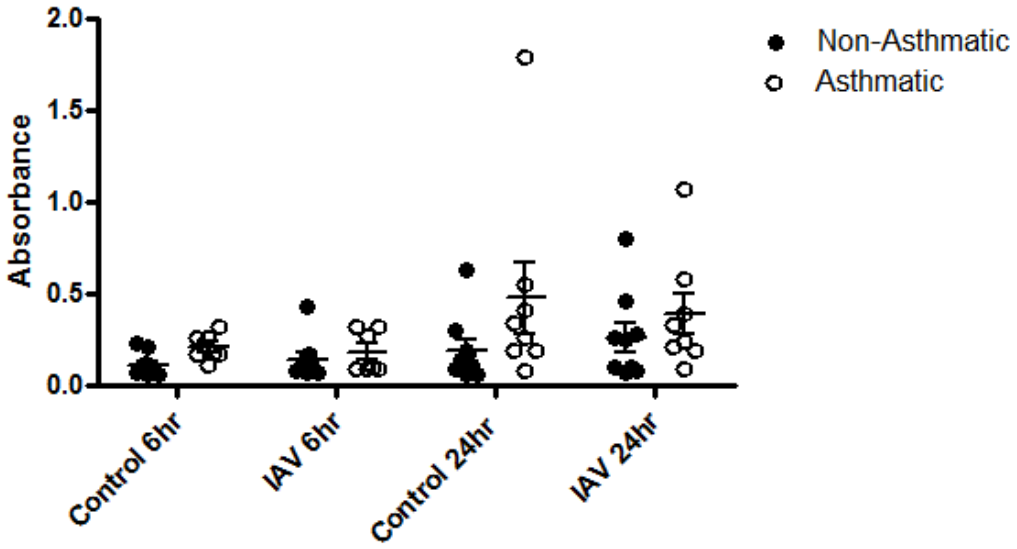

Figure 2.6. HBEC from asthmatics and non-asthmatics have similar levels of cytotoxicity. Supernatants from HBEC from asthmatics (n=4-5) and non-asthmatics (n=6) were assessed for LDH quantities as a measure of cytotoxicity. No statistical differences were detected.

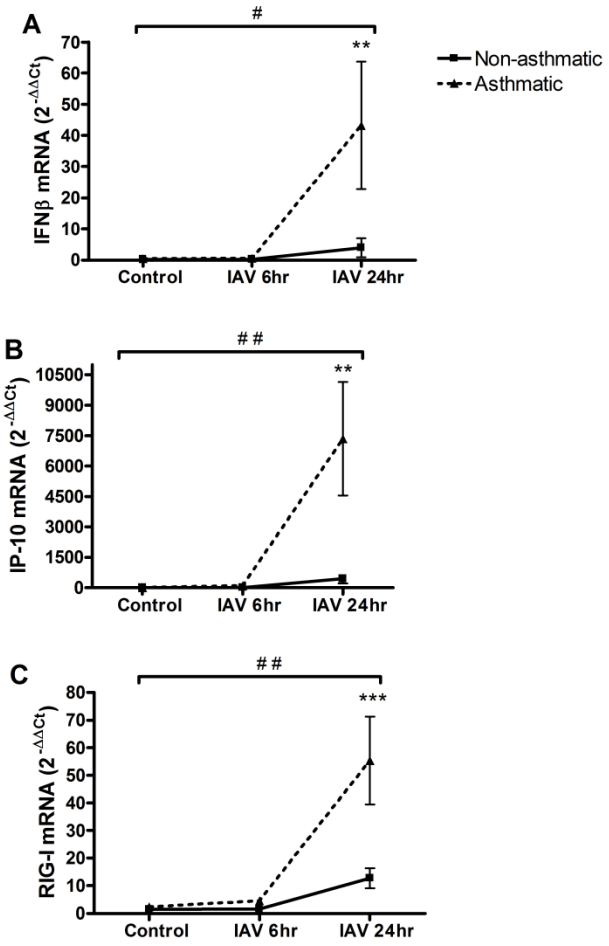

Figure 2.7. HBEC from asthmatics have enhanced expression of antiviral genes. Total RNA from HBEC from asthmatics (n=7) and non-asthmatics (n=8) at 6 or 24 hours post-IAV infection or 24 hours HBSS control treatment was analyzed for expression of (A) IFNβ; (B) IP-10; and (C) RIG-I by qRT-PCR. Ct values were normalized to β-actin.

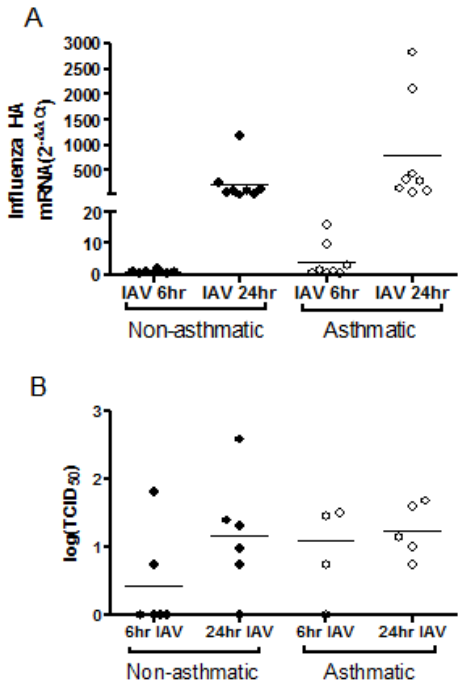

Figure 2.8. Viral replication is not significantly increased in HBEC from asthmatics. (A) Total RNA from IAV-infected HBEC from asthmatics (n=7) and non-asthmatics (n=8) were analyzed for IAV hemagglutinin (HA) RNA by qRT-PCR. Ct values were normalized to β-actin. (B) Vial titer of apical washes from HBEC from asthmatics (n=4-5) and non-asthmatics (n=6) at 6 and 24 hours post-IAV infection.

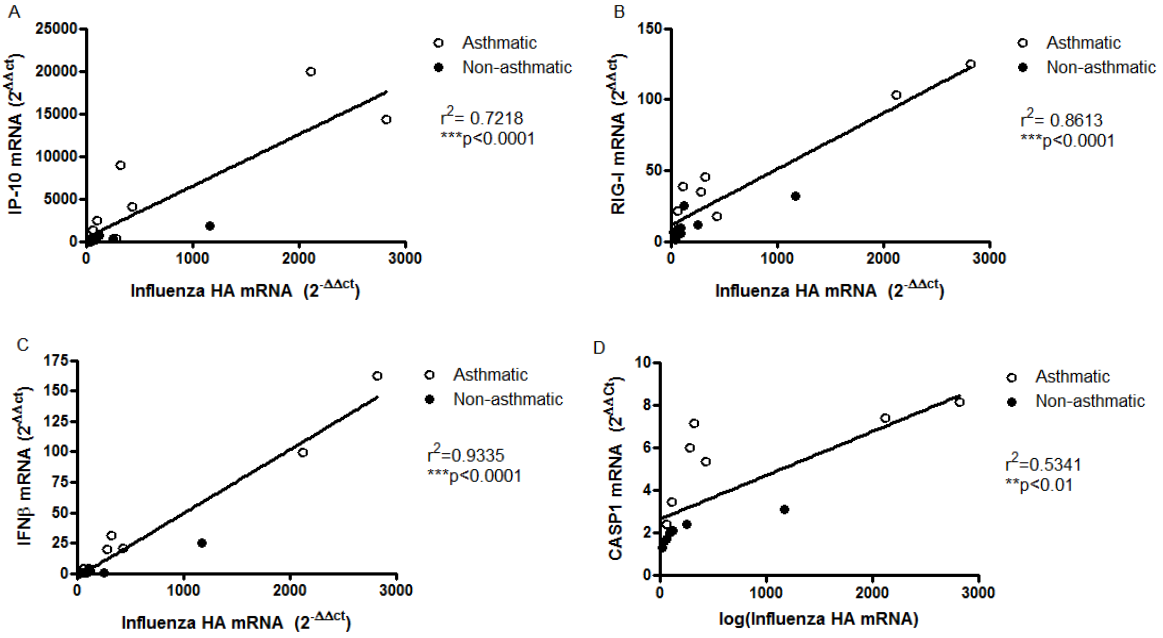

Figure 2.9. Correlation between expression of innate immune genes and quantity of influenza HA transcripts. Total RNA was isolated from HBEC from asthmatics (n=7) and non-asthmatics (n=8). Data were analyzed for correlation between influenza HA and (A) IP-10, (B) RIG-I(C) IFNβ, and (D) CASP1 using Pearson's correlation test (p-value, **p<0.01, ***p<0.001) and linear regression (r^2 value).

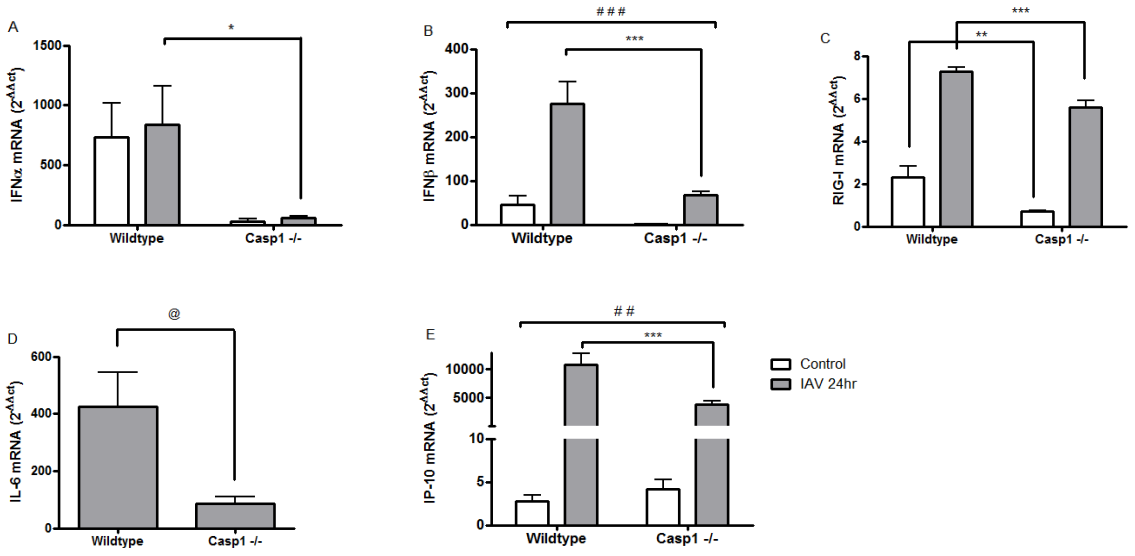

Figure 2.10. *Casp1*$^{-/-}$ MTEC have diminished antiviral gene expression in response to IAV infection. RNA from wild type (n=8) and *Casp1* -/- (n=9) MTEC 24 hours post-IAV infection or control were analyzed for expression of (A) IFNα; (B) IFNβ; (C) RIG-I; (D) IL-6; (E) IP-10 by qRT-PCR. Ct values were normalized to β-actin. @ $p<0.05$ Student's T-test *Casp1* -/- vs. wild type, 24hrs post-IAV infection only; * $p<0.05$, ** $p<0.01$; *** $p<0.001$ ANOVA and Tukey post-hoc test, *Casp1* -/- vs. wild type 24hrs post-IAV and control infection; ## $p<0.01$, ### $p<0.001$ Factorial ANOVA, interaction between genotype and infection.

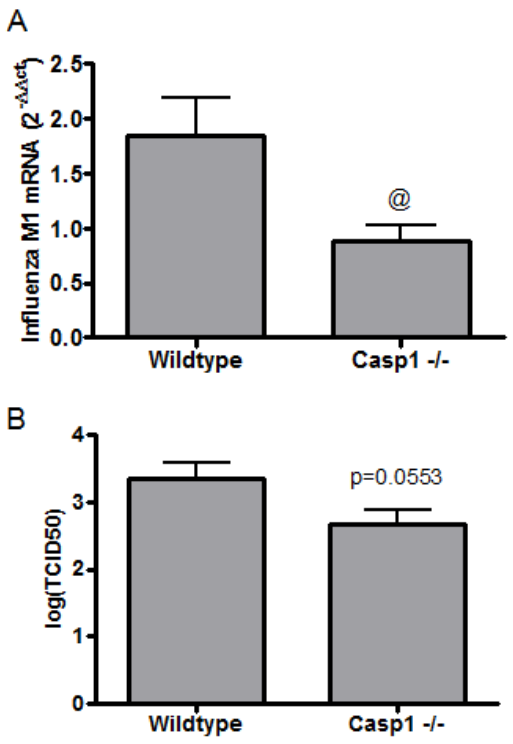

Figure 2.11. *Casp1*$^{-/-}$ MTEC have diminished viral replication. (A) Total RNA from *Casp1 -/-* (n=9) and wild type (n=8) MTEC 24 hours post-IAV infection or media control treatment were analyzed for IAV matrix 1 (M1) RNA by qRT-PCR. Ct values were normalized to β-actin. (B) Vial titer was assessed using media supernatents from *Casp1 -/-* (n=9) and wild type (n=8) MTEC 24 hours post-IAV infection.

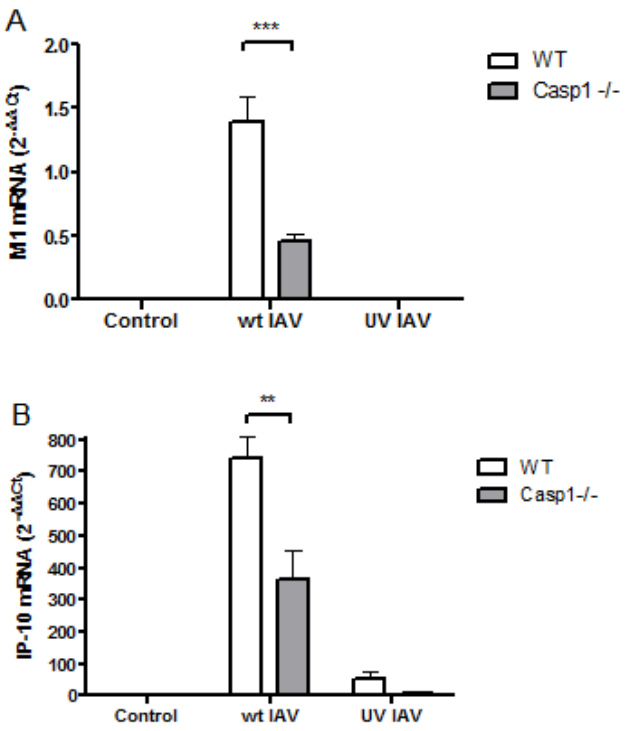

Figure 2.12 Viral replication is necessary for the reduced innate immune response in *Casp1*$^{-/-}$ MTEC. MTEC were infected with wild type IAV (wt IAV) or UV-inactivated IAV (UV20 IAV) for 24 hours. Total RNA was isolated from *Casp1 -/-* (n=6) and WT (n=6) MTEC and probed for (A) M1 and (B) IP-10 expression by qRT-PCR.

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Chapter 3: Caspase-1 deficiency modifies *in vivo* development of house dust mite-induced allergic airway disease[1,2]

3.1 Introduction

Asthma is a chronic inflammatory disorder of the conducting airways that is associated with airflow obstruction and airway hyperresponsiveness (AHR) (1). The incidence of asthma has increased significantly over the past 50 years, which may be attributed to changes in urbanization, diet, and lifestyle (1, 2). Despite the increasing prevalence of asthma, the genetic and environmental factors that contribute to the development asthma remain unclear (3). Since allergic asthma is the most prevalent form of the disease, several murine models of allergic asthma have been developed to investigate the specific mechanisms contributing to the development and pathophysiology of asthma (4). The most common approaches to mimic allergen-induced lung inflammation in mice consist of sensitization with ovalbumin (OVA) with/or without an adjuvant such as Alum or sensitization to house dust mite (HDM) antigen (4).

Airway inflammation and type 2 helper (Th2) T lymphocyte-mediated adaptive immunity to an innocuous antigen are key characteristics of atopic asthma (5). More recently, the role of the innate immune system in the initiation and propagation of Th2 responses has received

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increased attention (1). Both innate structural cells, including airway epithelial cells, and inflammatory cells, such as mast cells and dendritic cells (DC), are now considered important contributors to the induction and maintenance of Th2 lymphocyte-mediated immune responses (6, 7). Initiation of atopic asthma is thought to begin with sensitization of the airway to allergens such as HDM, animal dander, or pollen, through enzymatic activity of the allergen (e.g. proteases found in some HDM) that activate protease-activated receptors and/or though recognition by innate pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) (7, 8). DC in the airway recognize, process, and present the antigen to T lymphocytes in the lymph node (7). Additionally, activation of PRRs on epithelial cells and other innate immune cells in the airway causes the release of cytokines, including granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-33 and thymic stromal lymphoprotein (TLSP), that prime DC and promote Th2 cell differentiation (9). Th2 lymphocytes produce cytokines such as IL-4, IL-5, and IL-13 that drive allergic inflammation, including B cell isotype switching to IgE synthesis and recruitment or activation of mast cells, eosinophils, and basophils (1).

Considering the important role of innate immune receptors in allergen sensitization, a number of studies have investigated the specific contribution of PRRs to the initiation of allergic airway inflammation. Previous studies have indicated that mice deficient in TLR4 or its adaptor molecule MyD88 have reduced HDM-induced allergic inflammation (9-11). Yet, further analyses indicate the necessity for MyD88 in asthma development may be dependent on HDM sensitization, as some studies that sensitized mice using OVA with an adjuvant via intraperitoneal injection did not observe MyD88-dependent allergic inflammation (12, 13). Additionally, the nucleotide-binding domain leucine-rich repeat-containing receptor (NLR) family of PRRs may also contribute to the development of asthma. Several NLRs can form

inflammatory signaling complexes called inflammasomes that consist of an adaptor protein (PYCARD) and the protease caspase-1 (14). Upon formation of the inflammasome, caspase-1 becomes active and then cleaves pro-IL-1β and pro-IL-18 to their active forms. IL-1β has been proposed to promote Th2 cell proliferation (15), and IL-18, though typically associated with Th1 responses, may also enhance IgE production (16). Of all of the NLRs, the NLRP3 inflammasome has been most investigated as a mediator of asthma development due to its ability to respond to many PAMPs and DAMPs associated with allergic inflammation, such as uric acid, alum, ATP, and several bacterial and viral species (12, 13, 17, 18). However, involvement of NLRP3 in asthma pathogenesis is controversial. A recent exhaustive study of several murine models of asthma in *Nlrp3*$^{-/-}$ mice could not confirm a role for NLRP3 in the development of allergic inflammation (17). Similar to NLRP3 and MyD88, studies investigating IL-1 receptor (IL-1R) signaling in murine models of allergic inflammation have also yielded conflicting results, suggesting an unclear role for IL-1 axis signaling in the development of allergic inflammation (9, 10, 12, 19).

Caspase-1 is a cysteine protease that is activated upon formation of the inflammasome complex and thus plays a key role in the innate immune response to a number of viruses, bacteria, and DAMPs that can trigger asthma symptoms (20). Beyond IL-1β and IL-18 processing, caspase-1 has several other functions, including activation of NF-kB (21) , induction of a pro-inflammatory form of programmed cell death termed pyroptosis (22), and cleavage of several proteins involved in cell death (e.g. caspase-7), glycolysis (e.g. GAPDH and α-enolase), and cytoskeletal function (23, 24). Despite the ability of caspase-1 to regulate the innate immune response to many sources of asthma exacerbation, the involvement of caspase-1 in asthma development is unclear. Two previous studies have investigated caspase-1 in asthma

development: one study used an OVA model with alum adjuvant and found that *Casp1*$^{-/-}$ mice had significantly reduced airway inflammation and OVA- specific IgG1 and IgG2c (13); conversely, a second study used a low-level HDM sensitization model and found that *Casp1*$^{-/-}$ mice sensitized had similar levels of airway inflammation to wild type mice (9). With these studies in mind, we sought to elucidate the role of caspase-1 in the development allergic inflammation using a well characterized model of high dose acute HDM sensitization (17, 25, 26). We found that caspase-1 deficiency reduced AHR in response to methacholine challenge, but enhanced neutrophilic airway inflammation, therefor suggesting that caspase-1 may play divergent roles in the development of allergic inflammation.

3.2 Materials and Methods

Experimental animals. Adult female C57BL/6 wildtye (WT) and *Caspase 1*-deficient (*Casp1*$^{-/-}$) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used at 3 to 4 months of age. All experiments were conducted on age and sex matched mice. The *Casp1*$^{-/-}$ mouse strain has been previously described (27). All experimental procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. Genotypes were confirmed by PCR as previously described using the primers: 5'- GCGCCTCCCCTACCCGG-3' (mutant), 5'-GAAGAGATGTTACAGAAGCC-3' (common), 5'-CATGCCTGAATAATGATCACC-3' (wild type).

Induction of Allergic Airway Inflammation with house dust mite (HDM). Mice were anesthesized with isoflurane and treated intranasally to 25µg of HDM (*Dermatophagoides pteronyssinus 1*, Greer Laboratories, Lenoir, NC) in 20µl of sterile PBS (Life Technologies, Carlsbad, CA) or 20µl PBS control once a day for 14 consecutive days similar to previous studies (17, 25, 26). Mice were harvested 72 hours after the last HDM challenge. Notably, the HDM contained 23.01 EU/mg of HDM. See schematic figure 3.1.

Lung function assessment. Lung function was assessed by the forced oscillation technique as previously described (17, 28-30). Briefly, mice anesthetized with pentobarbital sodium were tracheostomized and mechanically ventilated using a computer-controlled small animal ventilator (flexiVent, Scireq, Montreal, Canada). Mice were treated with aerosolized sterile saline or methacholine (Sigma, St. Louis, MO) at increasing doses (10 mg/mL, 20 mg/mL, and 40 mg/mL) and lung resistance (R_L), central airway resistance (R_{aw}), and alveolar tissue dampening (G) was measured every 10 seconds. Max values for each methacholine dose were graphed.

Serum and bronchoalveolar lavage collection. Mice were euthanized and blood was collected by cardiac puncture. Serum was separated by centrifugation and stored at -80°C. The left mainstem bronchus was clamped and the right lung lobes were lavaged 5 times with 1 mL of PBS each time. The right lobe was then removed, flash frozen in liquid nitrogen, and stored for protein or RNA isolation. Total cell counts in the lavage fluid of each mouse were obtained using a hemacytometer. A 200ul of broncoalveolar lavage (BAL) fluid was centrifuged onto slides using a Cytospin (Shandon, Pittsburgh, PA) and stained with Diff -Quik® solution (American Scientific, McGraw Park, PA) for cell differential assessment. At least 200 cells were counted from each slide and cell counts were adjusted for total cell number. The lavage fluid was centrifuged and cell-free BAL fluid was stored at -80°C.

Immunohistochemistry. The left lung was unclamped and fixed by inflation and submersion in 10% formalin (Sigma). The whole lungs were embedded in paraffin and three 5 μm sections were taken every 100 μm. Histopathology was evaluated using hematoxylin and eosin (H&E; Richard Allan, Richland, MI) stained lung sections and scored on a scale of 1 (none) to 5 (severe) for the following parameters: large airway inflammation, alveolar inflammation, perivascular inflammation, and overall impression of severity of disease. Three slides from each mouse lung was read by two independent scorers while blinded to genotype and treatment. Scores were averaged to generate a semi-quantitative histology score. Mucin production and goblet cell metaplasia was assessed by Alcian Blue and Periodic Acid Schiff (AB-PAS) staining. For each slide (representing one animal), eight representative images of the large airway were acquired at 10x magnification. The percentage of positive PAS stained cells within the area of the airway was measured by ImageJ (National Institutes of Health) and a computer-based color deconvolution module as previously described (31).

Enzyme linked immunosorbent assay (ELISA). Total levels of IgE in the serum were assessed using a commercially available kit (Total IgE, BD Biosciences, Franklin Lakes, NJ). Lung homogenates were prepared from approximately 100mg snap-frozen lung tissue homogenized in HBSS using the Lysing Matrix D tubes (Millipore, Billerica, MA) and the Bio101 FastPrep system (MP Biomedicals, Santa Ana, CA). Homogenates were centrifuged and supernatants were assessed for protein levels. Protein levels of IL-12, KC, IL-1β, IL-5, and IL-10 were assessed in serum, cell-free BAL fluid supernatants and lung homogenates using commercially available ELISAs (Mesoscale Discovery, Rockville, MD). Cytokine levels were normalized to mg tissue used for the homogenate. If the majority of samples were below the lower limit of detection (LLOD) as indicated in table 3.1, the cytokine was deemed non-detectable (ND). Otherwise, readings with zero values or that were below LLOD were substituted with $\frac{LLOD}{\sqrt{2}}$ (32).

RNA isolation and quantitative real time PCR (qRT-PCR). 100mg of harvested lungs were homogenized in Trizol for RNA extraction using Lysing Matrix D tubes (Millipore) and the Bio101® FastPrep® system (MP Biomedicals). RNA isolation, first-strand cDNA synthesis, and qRT-PCR were performed as previously described (33, 34). Taqman primers for *Elane, Ctsg, Il1b,* and *B-actin* were commercially available (Applied Biosystems, Life Technologies, Carlsbad, CA). Differences in expression were determined using the ΔΔCt method and *B-actin* for normalization.

Western Blot. Lung homogenates were assessed for total protein concentrations using a Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA). 50 µg of protein per sample were separated by a 15% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad). Proteins were detected with specific antibodies for IL-1β (1:1000) and anti-rabbit horseradish

peroxidase (HRP)-conjugated secondary antibody (1:2000) (Santa Cruz Biotechnology, Dallas, TX) in 5% milk in 1XTBS-0.05%Tween buffer (Sigma). For loading control, nitrocellulose membranes were stripped with Blot Restore (Millipore) and probed for β-Actin (1:5000) and anti-mouse HRP-conjugated secondary antibody (1:10,000) (Santa Cruz Biotechnology). Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and images were acquired with a Fujifilm LAS-3000 imager (Fuji Film Global Tokyo, Japan).

Statistical Analysis. Each experimental group had at 5 to 12 animals. One HDM-sensitized Casp1$^{-/-}$ mouse was removed from analysis because of low IgE levels, low cytokine levels, and low cell counts that suggested the animal was not successfully sensitized to HDM. Data was analyzed by factorial ANOVA, followed by Bonferroni post-hoc test to determine significant differences among the experimental groups for cell counts, cell differentials, serum IgE, lung function, and gene expression analyses. Student's T-test was used to assess differences between HDM-sensitized *Casp1*$^{-/-}$ and WT mice for histopathology scores and cytokine assessment. $p < 0.05$ was considered statistically significant. Data are shown as mean ± SEM.

3.3 Results

Casp1$^{-/-}$ mice have enhanced airway inflammation with HDM sensitization

Casp1$^{-/-}$ or wild type (WT) C57Bl/6 mice were sensitized to *Dermatophagoides pteronyssinus 1*(Der p1) HDM or PBS control intranasally once a day for 14 days. 72 hours after the last HDM sensitization, the mice were challenged with methacholine for lung function assessment and harvested for samples (Figure 3.1). We measured total serum IgE as an indicator of allergic inflammation. Our results revealed that HDM-sensitization induced increased serum IgE in both *Casp1$^{-/-}$* and WT mice, and that there was no significant differences in serum IgE between the genotypes (Figure 3.2).

In line with previous characterization of the *Casp1$^{-/-}$* mouse model, we found that PBS-treated *Casp1$^{-/-}$* and WT mice have similar levels of cellularity in the lung (Figure 3.3) (27, 35). With HDM-sensitization, *Casp1$^{-/-}$* mice had significantly enhanced inflammatory cell influx in the airway compared to WT mice (Figure 3.3A). Further differential analysis revealed that *Casp1$^{-/-}$* mice had increased airway neutrophils (PMN) with HDM sensitization compared to WT (Figure 3.3B). Histological assessment of the lungs indicated that HDM sensitization severely increased allergic inflammation in both genotypes. We observed increased inflammation around the large airways, alveoli, and blood vessels and damage to the airway in both WT and *Casp1$^{-/-}$* mice (Figure 3.4). Upon further evaluation, histopathology scoring of the lungs revealed that HDM-sensitized *Casp1$^{-/-}$* mice had a trend for overall increased severity of inflammation in the lung compared to HDM-sensitized WT mice (p=0.0525) and significant increases in perivascular and alveolar inflammation (Figure 3.5). In summary, our results indicate that the severity of HDM-induced inflammation, particularly neutrophilia, was increased in the lungs from *Casp1$^{-/-}$*

mice, suggesting that caspase-1 is protective against the development of HDM-induced airway inflammation.

Casp1$^{-/-}$ and WT mice have similar levels of HDM-induced goblet cell metaplasia

Goblet cell metaplasia and increased mucin production are characteristic features of allergic airway disease progression in mice that contribute to airway obstruction (36). We assessed mucin production and goblet cell numbers by Alcian Blue and Periodic Acid Schiff (AB-PAS) staining of the lungs from HDM and PBS-treated WT or *Casp1$^{-/-}$* mice. Both *Casp1$^{-/-}$* and WT mice had increased mucin production and goblet cell numbers with HDM sensitization (Figure 3.6A). We used a computer-based color deconvolution module to score the average percentage of goblet cells (%PAS+ cells) per airway area and found a non-significant trend ($p=0.108$) for increased goblet cell number in the lungs of HDM-sensitized *Casp1$^{-/-}$* mice compared to WT (Figure 3.6B). These data suggest that caspase-1 does not play a major role in determining HDM-induced goblet cell metaplasia.

HDM-sensitized Casp1$^{-/-}$ mice have increased IL-1β in lung homogenates

Allergic asthma is often associated with a shift in the balance of Th1 toTh2 cell immunity, with increased levels of Th2 cytokines such as IL-4 and IL-5 contributing to a shift towards Th2 lymphocyte differentiation (1). We compared levels of several Th1 (IL-1β, keratinocyte chemoattractant (KC), total IL-12) and Th2 (IL-4, IL-5, and IL-10) cytokines in BAL fluid, lung homogenates, and serum from HDM-sensitized *Casp1$^{-/-}$* and WT mice. Our results indicate that there were no differences between the genotypes in HDM-induced Th1 and Th2 cytokine levels in the BAL fluid or serum (Table 3.1). In the lung homogenate, lungs from HDM-sensitized *Casp1$^{-/-}$* mice surprisingly had significantly increased levels of IL-1β, but a trend for reduced total IL-12 ($p=0.07$) and no difference in KC levels. IL-1β is produced in a pro-

form that must be cleaved by proteases such as caspase-1 or neutrophil elastase before release from the cell. We assessed pro-IL-1β (*Il1b*) gene expression in lung homogenates and found no effect of HDM or caspase-1 deficiency *Il1b* mRNA (Figure 3.7A). We then measured IL-1β cleavage in the lung homogenates by western blot and found that *Casp1*$^{-/-}$ and WT had similar levels of pro- and cleaved IL-1β in both PBS and HDM-treated lung homogenates (Figure 3.7B). Notably, the lung homogenates used for this analysis had signs of degradation, as evidenced by multiple bands detected for the β-actin loading control. Moreover, we assessed gene expression of neutrophil elastase (*Elane*) and cathepsin G (*Ctsg*), two neutrophil-derived proteases that have been previously shown to cleave IL-1β, in the lung homogenates and found no differences in expression between HDM-sensitized *Casp1*$^{-/-}$ or WT mice (Figure 3.8A-B). In summary, we found that HDM-sensitized *Casp1*$^{-/-}$ mice have increased IL-1β in the lung tissue compared to WT, but no evidence of increased IL-1β production or processing.

Casp1$^{-/-}$ *mice have reduced HDM-induced airway hyperresponsiveness*

A common characteristic of asthma is increased airway hyperresponsiveness (AHR) to inhaled stimuli, which is thought to be related to airway narrowing caused by both inflammation and structural changes in the lung (37, 38). Narrowing of the airway is associated with increased airway resistance, and can be measured using forced oscillation techniques in mice (30). Using a computer-controlled small animal ventilator (flexiVent®), we compared changes in total lung resistance (R_L), large airway resistance (R_{aw}), and alveolar tissue damping (G, closely related to alveolar tissue resistance) in response to the bronchoconstricting agent methacholine between PBS or HDM-treated *Casp1*$^{-/-}$ and WT mice. Our results indicate that *Casp1*$^{-/-}$ and WT mice have similar levels of basal airway resistance in both PBS and HDM-treated groups (Figure 3.9A-C). With increasing doses of methacholine, R_L, R_{aw}, and G increased in the HDM-sensitized mice,

but not in PBS-treated, demonstrating the development of AHR in response to HDM. At the highest does of methacholine (40 mg/mL), HDM-sensitized *Casp1*$^{-/-}$ had significantly reduced R_L and R_{aw} compared to HDM-sensitized WT mice. Together these results suggest that caspase-1 may contribute to development of AHR, despite playing a protective role in HDM-induced airway inflammation.

3.4 Discussion

Given the many functions that caspase-1 plays in innate immunity and the increasing appreciation for the role of innate immune pathways in asthma development, we sought to determine how caspase-1 contributes to the development of allergic inflammation. We compared the development of allergic asthma in caspase-1 deficient (*Casp1*$^{-/-}$) and wild type (WT) mice using an acute model of house dust mite (HDM)-induced allergic inflammation. Our results indicate that *Casp1*$^{-/-}$ mice have enhanced HDM-induced inflammation in the lung, which primarily affects perivascular and alveolar tissue and may be mediated by enhanced neutrophilia. Surprisingly, we found that *Casp1*$^{-/-}$ mice have elevated IL-1β in the lung tissue, but we could not detect a corresponding increase in *Il1b* gene expression nor pro-IL-1β cleavage. Finally, our results indicate that HDM-sensitized *Casp1*$^{-/-}$ mice have reduced airway hyperresponsiveness (AHR) in response to methacholine challenge compared to WT mice. Together, our results indicate that caspase-1 may play a dual role in HDM-induced allergic inflammation, whereby caspase-1 is protective against airway inflammation, but contributes to AHR.

Based on the role of caspase-1 in IL-1β and IL-18 processing and release, and the implications for IL-1 signaling in the development of allergic inflammation, we hypothesized that caspase-1 deficiency would reduce allergic inflammation in response to HDM-sensitization (15). Surprisingly, we observed enhanced airway inflammation and increased levels of IL-1β in the lung tissue from HDM-sensitized *Casp1*$^{-/-}$ mice (Figure 3.3 and Table 3.1). Subsequent analysis revealed that there was no effect of HDM-treatment on *Il1b* gene transcription in either genotype (Figure 3.7A). Western blot analysis indicated that HDM and PBS-treated mice of both genotypes had similar levels of pro- and cleaved IL-1β, though the lung homogenates used for this analysis had signs of degradation (Figure 3.7B). The increased IL-1β may be due to release

of intracellular stores from dying or damaged cells, which can then be cleaved by other proteases such as neutrophil-derived elastase, cathepsin g, and proteinase-3 and mast cell-derived chymase (39-42). Despite elevated levels of neutrophils in the airways of HDM-sensitized *Casp1*$^{-/-}$ mice, we did not detect an associated increase in gene expression of neutrophil elastase (*Elane*) or cathepsin g (*Ctsg*) (Figure 3.8). Thus, our results indicate that the elevated IL-1β in the lung tissue from *Casp1*$^{-/-}$ mice is likely not due to increased production nor is there is increased processing. Future studies will be necessary to determine if the increased IL-1β may be derived from pre-existing intracellular stores and how this mechanism contributes to the allergic inflammation.

Beyond the processing of IL-1β and IL-18, proteomic analysis of caspase-1 substrates have indicated that caspase-1 regulates activity of a number of proteins necessary for cell survival, including proteins involved in metabolism (e.g. GAPDH, α-enolase), cytoskeleton function (e.g. γ-actin), and cell death (e.g. caspase-7, Bid) (23, 24). Caspase-1 activity is directly linked to induction of a pro-inflammatory form of programmed cell death termed pyroptosis. Pyroptotic cell death has been shown in macrophages (and not in neutrophils) and is thought to eliminate the intracellular environment necessary for pathogen replication (43). Pyroptosis shares several features of apoptosis, including DNA fragmentation, but differs in that the process results in lytic release of intracellular contents that must then be cleared by immune cells (22). In addition to pyroptosis, caspase-1 has been shown to regulate a number of other forms of cell death, including apoptosis (44). For example, caspase-1 inhibition or deficiency is protective against septic shock and B cell apoptosis, an effect which was not evident in IL-1β/IL-18 double knockout mice (45). Thus, caspase-1 mediated cell death may be a pathway necessary for inflammatory cell clearance, and loss of this function could contribute to enhanced inflammatory

cell accumulation in response to HDM sensitization. Additionally, caspase-1 is an activator of NF-kB, a transcription factor of many genes involved with inflammation and innate immunity (23, 24, 46). The loss of caspase-1 mediated NF-kB activation could contribute to an altered inflammatory response to HDM. Clearly, future studies are necessary to confirm and delineate the mechanisms contributing to caspase-1 protection against HDM-induced allergic inflammation.

Differential analysis of immune cells in the lung revealed that HDM-sensitized *Casp1*$^{-/-}$ mice increased numbers of neutrophils in the airway (Figure 3.2). Though airway eosinophilia is considered a pathological hallmark of asthma, neutrophils are increasingly recognized as drivers of asthma pathogenesis (47). Airway neutrophilia is most commonly observed in patients with severe or steroid-resistant asthma or undergoing acute asthma exacerbations (48, 49). Neutrophils secrete a variety of cytokines and proteases such as neutrophil derived elastase, cathepsin g, and matrix metalloproteinase 9, which are elevated in the airways of asthmatics and may contribute to airway remodeling, AHR, and increased mucus production (50, 51, 51, 52). Our studies indicate that gene expression of neutrophil elastase and cathepsin G, though enhanced by HDM sensitization, were not significantly altered by caspase-1 deficiency (Figure 3.8). Though it is possible that protein levels or the expression of other proteases, such as matrix metalloproteinases, may differ with caspase-1 deficiency, these results suggest that the elevated neutrophils in the airways of *Casp1*$^{-/-}$ mice were not correlated with increased expression of certain neutrophil-derived proteases.

Several studies suggest that neutrophilic and eosinophilic asthma may be driven by different subsets of CD4+ T helper lymphocytes. Eosinophilic allergic inflammation is thought to be primarily driven by Th2 lymphocytes that produce cytokines such as IL-3, IL-5, and IL-13

to promote eosinophil maturation, recruitment, and survival, whereas Th17 lymphocytes produce IL-17A that attracts neutrophils to the airway (1, 53, 54). In our study, the levels of Th2 cytokines IL-4 and IL-5 were very low or non-detectable in the BAL fluid, lung homogenates, and serum samples of both genotypes (Table 3.1). Though we did not investigate other Th2-promoting cytokines, such as IL-13, these results suggest that the acute HDM sensitization did not induce Th2-skewed immune responses. The poor Th2 response to HDM sensitization may be related to the C57BL/6 mouse strain used for these studies. C57BL/6 mice have been previously shown to have Th1-prone inflammatory response and poor development of airway hyperresponsiveness, whereas other mouse strains, such as the BALB/c, are Th2-skewed (55-57). We were able to detect significant levels of KC and IL-12 in the BAL fluid, serum, and lung homogenates, and IL-1β in the lung homogenates only. IL-1β in concert with IL-6 has been shown to enhance Th17 lymphocyte differentiation, whereas IL-12 inhibits Th17 polarization (58). Lung tissue from *Casp1*$^{-/-}$ mice had significantly elevated IL-1β and a trend for reduced total IL-12 ($p=0.07$), indicating a pro-Th17 milieu in the airways (Table 3.1). Future studies will be necessary to assess development of Th17-mediated allergic inflammation in the airways of *Casp1*$^{-/-}$ mice and how this may correlate with airway neutrophilia.

In opposition to the protective role for caspase-1 in HDM-induced allergic inflammation, our results indicate that caspase-1 may promote HDM-induced AHR. Both airway inflammation and structural changes of the airway are important contributors to AHR. Th2 lymphocyte production of IL-4, IL-5, and IL-13 induce eosinophil and mast cell migration. These cells produce pro-inflammatory mediators such as eosinophil granule proteins, chymase, histamine, leukotrienes, and cytokines that damage the airway, prime airway smooth muscle (ASM), and induce neural pathways leading to bronchoconstriction (59, 60). In addition, structural increases

in ASM mass and altered ASM mechanics, as well as extracellular matrix deposition, are associated with AHR in asthmatics (38, 61). To determine the effects of caspase-1 deficiency on AHR, we used a methacholine challenge to induce bronchonstriction and measured airway resistance as an indicator of AHR. Methacholine acts directly on histamine H1 receptors on ASM to induce bronchoconstriction, and thus is a better measure of structural ASM changes in the lung compared to other agents of provocation such as adenosine monophosphate that act indirectly by stimulating inflammatory cells to produce mediators that induce AHR (62). We observed that in response to methacholine challenge, HDM-sensitized *Casp1*$^{-/-}$ mice have reduced R_L, or total resistance of the lung, suggesting that caspase-1 may contribute to structural changes in the lung. Total resistance of the lung (R_L) can be partitioned into resistance of the large airways (R_{aw}) and resistance from alveolar tissue (G). HDM sensitized *Casp1*$^{-/-}$ mice had reduced R_{aw}, but not G. These results suggest that increased inflammation in the perivascular and alveolar tissue of HDM-sensitized *Casp1*$^{-/-}$ mice was not associated with increased G. We did not detect significant differences in inflammation of the larger airways in HDM-sensitized *Casp1*$^{-/-}$ mice, indicating that the reduced R_L and R_{aw} may be due to structural effects in the large airways. A recent study found that caspase-1 was not expressed in tracheal ASM from human donors, suggesting that caspase-1 may have an indirect effect on ASM, possibly by altered ASM microenvironment, or may contribute to airway remodeling that leads to AHR (63). Future studies using chronic HDM sensitization to induce airway remodeling will be necessary to assess the contribution of caspase-1 to structural changes of the lung and AHR.

In this study, we present findings that caspase-1 may play divergent roles in the development of high level HDM-induced allergic inflammation, with caspase-1 potentially protecting against allergic inflammation, but conversely contributing to AHR. Two previous

reports indicate that caspase-1 deficiency either protected against OVA-induced allergic inflammation (Eisenbarth *et al*.) or had no effect on low-level HDM sensitization (Willart *et al.*). Each of these studies used different models of allergen sensitization: Eisenbarth *et al.* sensitized mice with intraperitoneal OVA and an alum adjuvant on day 0 and day 10, intranasally with OVA on days 21, 22, and 23, and collected samples on day 25; Willart *et al.*sensitized mice to 1 µg intranasal HDM (unspecified type), challenged with 10 µg HDM on days 7-12, and collected samples 3 days after the last challenge. In the study presented here, we sensitized mice to 25 µg intranasal Der p1 HDM for 14 days, and collected samples 3 days after the last challenge. OVA sensitization with an adjuvant has been criticized because OVA is not a natural trigger of asthma in humans. Moreover, chronic OVA administration in mice leads to tolerance (4). Conversely, HDM is a common allergen that induces asthma symptoms in humans, meaning it is a more relevant model allergen for sensitization (64). Additionally, long-term administration of HDM can mimic some aspects of chronic asthma and airway remodeling (4). However, commercial HDM extracts can vary considerably in preparation and composition, including marked differences in endotoxin content (65). Thus, the differences between OVA and HDM, sensitization protocols, and HDM preparation may significantly contribute to the variable findings of *Casp1*$^{-/-}$ allergic sensitization in different studies.

A shortcoming of this and most previous *Casp1*$^{-/-}$ studies is the recent revelation that the commonly used *Casp1*$^{-/-}$ mouse model also harbors a deficiency in caspase-11 (*Casp11*), the ortholog of human caspase-4 and caspase-5, due to a mutation in 129 mice that was backcrossed into the C57BL/6 *Casp1*$^{-/-}$ mouse strain (66, 67). A recent study showed that most of the known inflammasome agonists, such as ATP, dsDNA, flagellin, and monosodium urate, activate inflammasome signaling and caspase-1-mediated IL-1β secretion independently of caspase-11

(66). However, caspase-11 deficiency, but not caspase-1 deficiency, was protective against endotoxic shock, suggesting that early reports that *Casp1*$^{-/-}$ mice were protected from endotoxic shock were due to an unidentified deficiency in caspase-11 (66, 68). Moreover, caspase-11 can signal independently of NLRP3 and caspase-1 to induce a form of cell death similar to pyroptosis (69). Thus, caspase-11 and caspase-1 have non-redundant functions, and future studies will be necessary to delineate the true effects of caspase-1 deficiency on the development of allergic inflammation.

In summary, the data presented here indicates that caspase-1 protects against the development of allergic inflammation, but contributes to AHR with high dose HDM-sensitization. However, these findings are preliminary and future studies will be necessary to better characterize the inflammatory cells and T cell subtypes, assess inflammatory cell death and clearance, and determine the contribution of caspase-1 to structural changes in the lung. Though we believe that this data may indicate a role for caspase-1 in the development of allergic sensitization, this effect may be of even greater significance during asthma exacerbation. We have previously shown that caspase-1 expression is enhanced in bronchial epithelial cells from asthmatic volunteers following influenza infection, suggesting that caspase-1 may contribute to virus-induced asthma exacerbation (70). Moreover, viral infections early in life have been previously associated with the development of asthma (71, 72). Given the important role of caspase-1 in the response to a number of viruses, bacteria, pollutants, and DAMPs that can induce and exacerbate asthma, understanding the contribution of caspase-1 to asthma development and exacerbation will ultimately better our understanding of asthma pathogenesis.

Table 3.1. Summary of cytokine levels in bronchoalveolar lavage fluid (BAL) fluid, lung homogenate, and serum.

	Total IL-12	KC	IL-1β	IL-5	IL-10
			BAL fluid (pg/mL)		
WT HDM	298.9 ± 106.3 (n=10)	16.30 ± 4.554 (n=10)	ND	ND	ND
Casp1 -/- HDM	275.8 ± 79.07 (n=6)	17.43 ± 4.537 (n=6)	ND	ND	ND
P-value	0.8810	0.5662	N/A	N/A	N/A
			Lung homogenate (pg/100mg tissue)		
WT HDM	572.0 ± 183.9 (n=10)	38.98 ± 8.780 (n=10)	3.550 ± 1.437 (n=10)	ND	ND
Casp1 -/- HDM	100.6 ± 38.58 (n=6)	53.08 ± 22.81 (n=6)	17.80 ± 7.856 (n=6)	ND	ND
P-value	0.0723	0.5073	0.0382*	N/A	N/A
			Serum (pg/mL)		
WT HDM	853.4 ± 187.4 (n=10)	47.16 ± 7.612 (n=10)	ND	4.390 ± 1.506 (n=10)	28.56 ± 11.99 (n=10)
Casp1 -/- HDM	851.1 ± 214.9 (n=6)	62.68 ± 6.931 (n=6)	ND	2.940 ± 0.9622 (n=6)	9.997 ± 1.645 (n=6)
P-value	0.9940	0.1897	N/A	0.5018	0.2333
LLOD (pg/ml)	**5.3**	**2.9**	**2.1**	**0.7**	**11**

Lower limit of detection (LLOD), Bronchoalveolar lavage (BAL) fluid, Not-detectable (ND), Not applicable (N/A).
P-value determined by Student's T-test. *p<0.05.
Unless all samples of a group were below LLOD, zero values were substituted with $\frac{LLOD}{\sqrt{2}}$

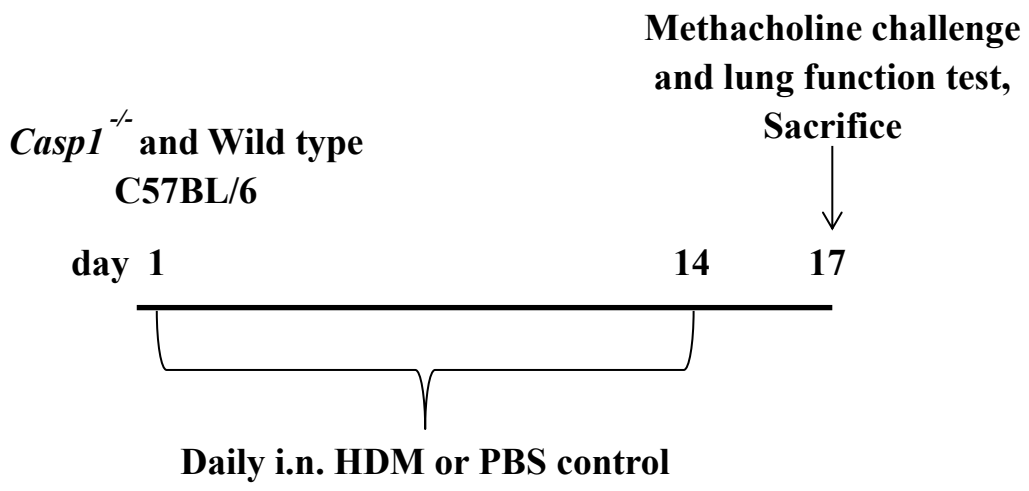

Figure 3.1. Schematic of house dust mite (HDM) sensitization protocol. Casp1$^{-/-}$ or wild type (WT) C57BL/6 mice were exposed intranasally (i.n.) to 25µg of HDM (Dermatophagoides pteronyssinus 1) in 20µl of sterile PBS or 20µl PBS control for 14 consecutive days. Mice were challenged with methacholine for lung function assessment and harvested 72 hours after the last HDM challenge.

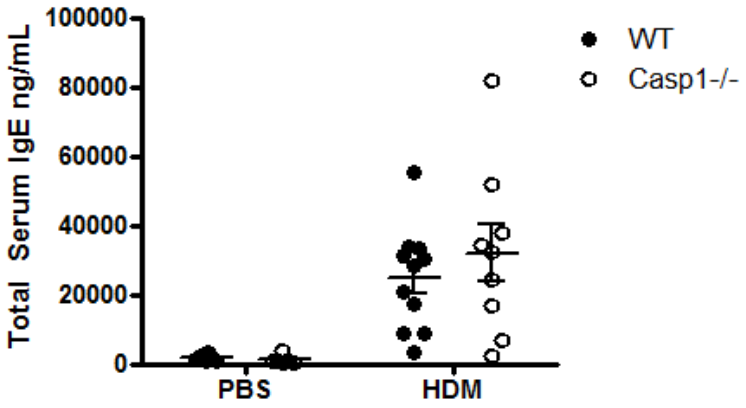

Figure 3.2. No differences between Casp1$^{-/-}$ and WT mice in HDM sensitization-induced serum IgE levels. Total serum IgE was measured by ELISA. n=8 WT PBS, 11 WT HDM, 7 Casp1$^{-/-}$ PBS, 9 Casp1$^{-/-}$ HDM.

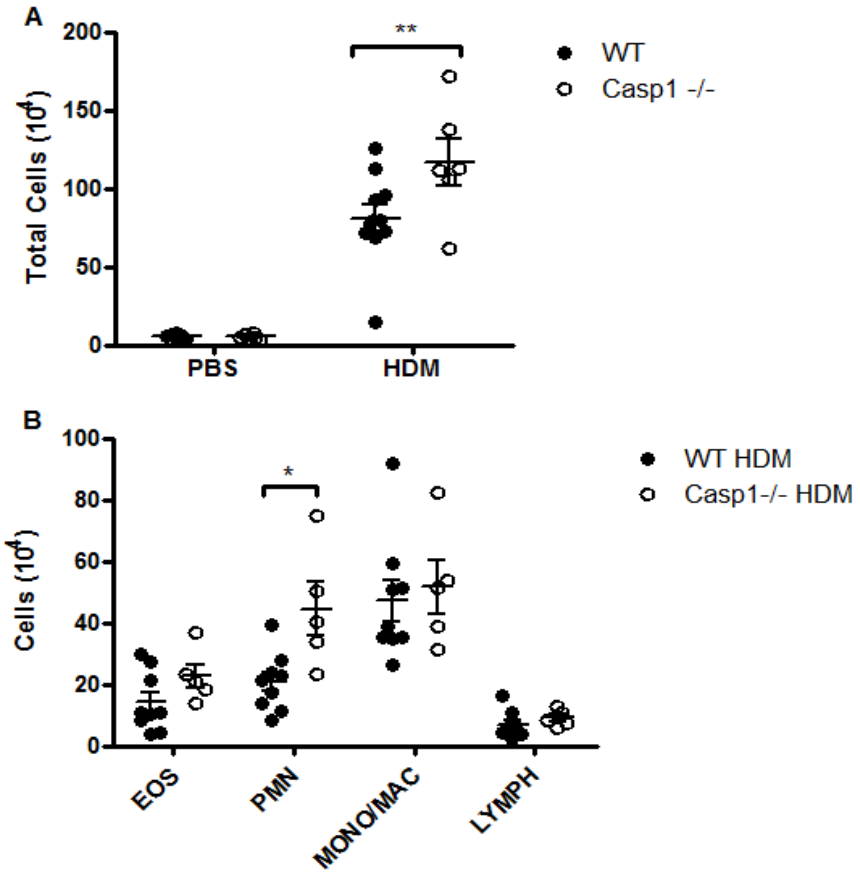

Figure 3.3. Casp1$^{-/-}$ mice have enhanced neutrophil influx with HDM sensitization. A) Total cell count from bronchoalveolar lavage fluid of HDM-sensitized or PBS treated Casp1$^{-/-}$ and WT mice. n= 9 WT PBS, 11 WT HDM, 6 Casp1$^{-/-}$ PBS, 6 Casp1$^{-/-}$ HDM. **B)** Cells in the bronchoalveolar lavage fluid were assessed for number of eosinophils (EOS), neutrophils (PMN), monocytes/macrophages (MONO/MAC) and lymphocytes (LYMPH) from HDM-sensitized Casp1$^{-/-}$ and WT mice. Counts were adjusted for total cell number. n= 9 WT HDM, 5 Casp1$^{-/-}$ HDM. P-value determined by factorial ANOVA with Bonferroni post-hoc test. *$p<0.05$, **$p<0.01$.

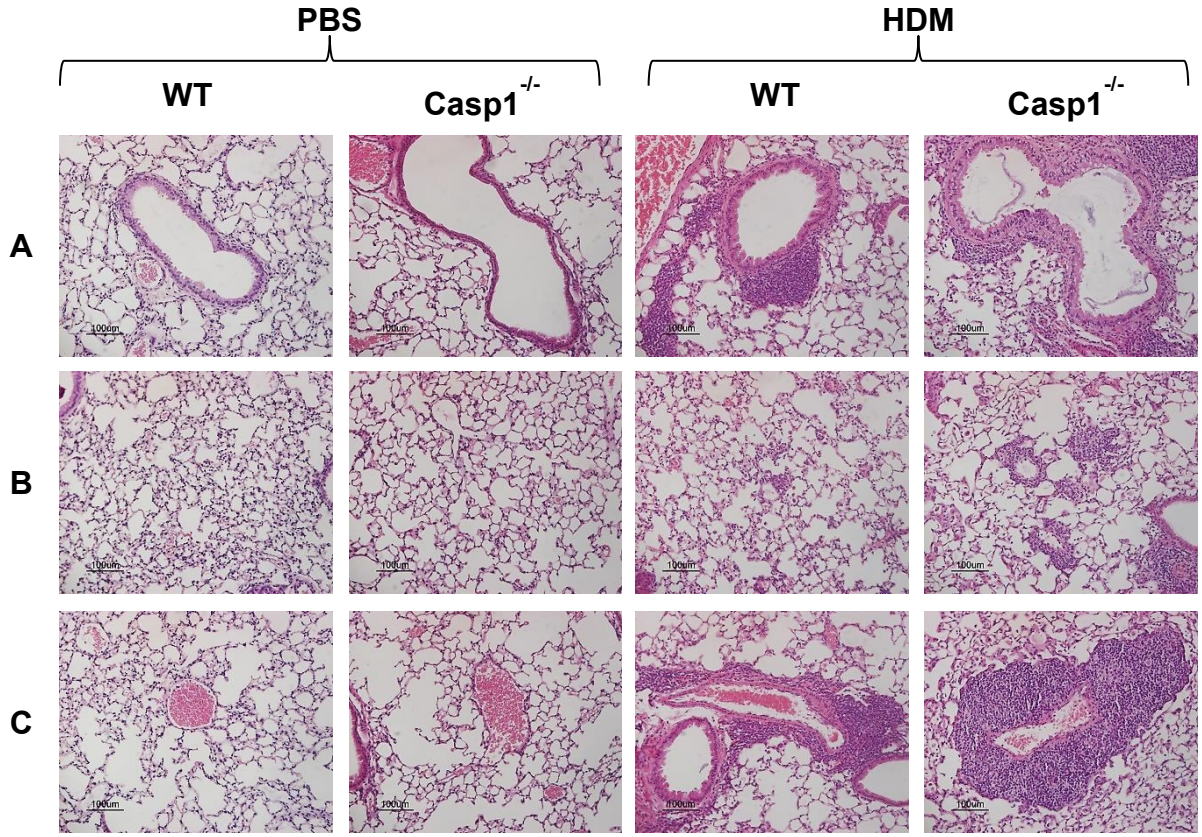

Figure 3.4. Histopathology assessment of lung inflammation and damage. H&E stained lung slices from PBS-treated and HDM-sensitized Casp1$^{-/-}$ and WT mice. Images were acquired at 20x magnification. **A)** Large airway inflammation; **B)** Large airway inflammation; **C)** Perivascular Inflammation. Bars represent 100 micron (μm).

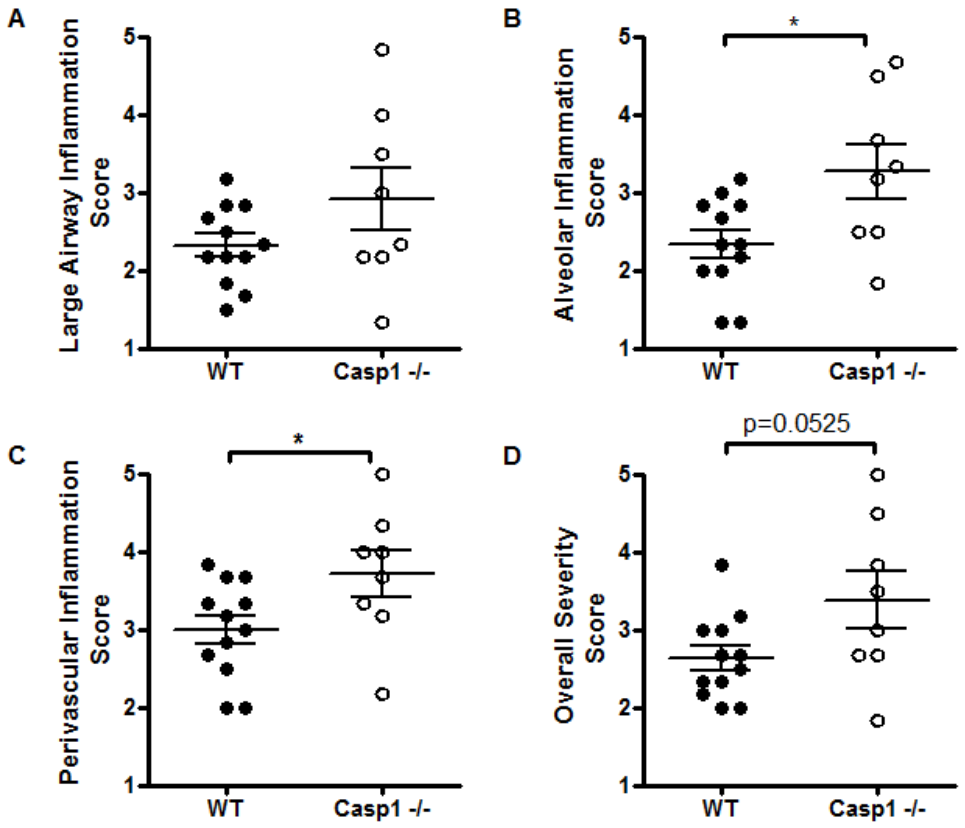

Figure 3.5. Lungs from HDM-sensitized Casp1$^{-/-}$ mice have enhanced inflammation. H&E stained lung slices from HDM-sensitized Casp1$^{-/-}$ and WT mice were assessed for **A)** large airway inflammation, **B)** alveolar inflammation, **C)** perivascular inflammation, and **D)** overall severity of inflammation on a scale of 1 (none) to 5 (severe). n=12 WT HDM, 8 Casp1$^{-/-}$ HDM. P-value determined by Student's T-test. *$p<0.05$.

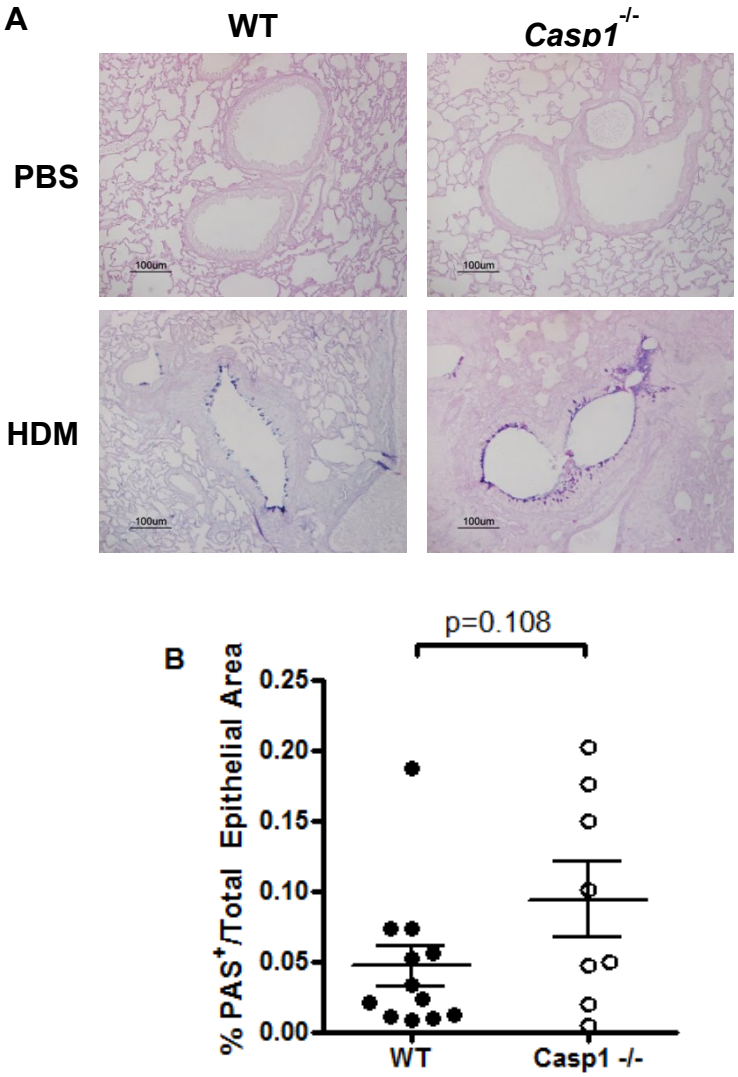

Figure 3.6. Assessment of goblet cell metaplasia. A) Alcian Blue and Periodic acid Schiff (AB-PAS) stained lung sections from PBS-treated and HDM-sensitized Casp1$^{-/-}$ and WT mice. Images were acquired at 20x magnification. Bars represent 100 micron (µm). **B)** Comparison of the quantification of the average percentage of PAS+ cells within an airway in lungs from HDM-sensitized WT and Casp1$^{-/-}$ mice. n=12 WT HDM, 8 Casp1$^{-/-}$ HDM. P-value determined by Student's T-test. *$p<0.05$.

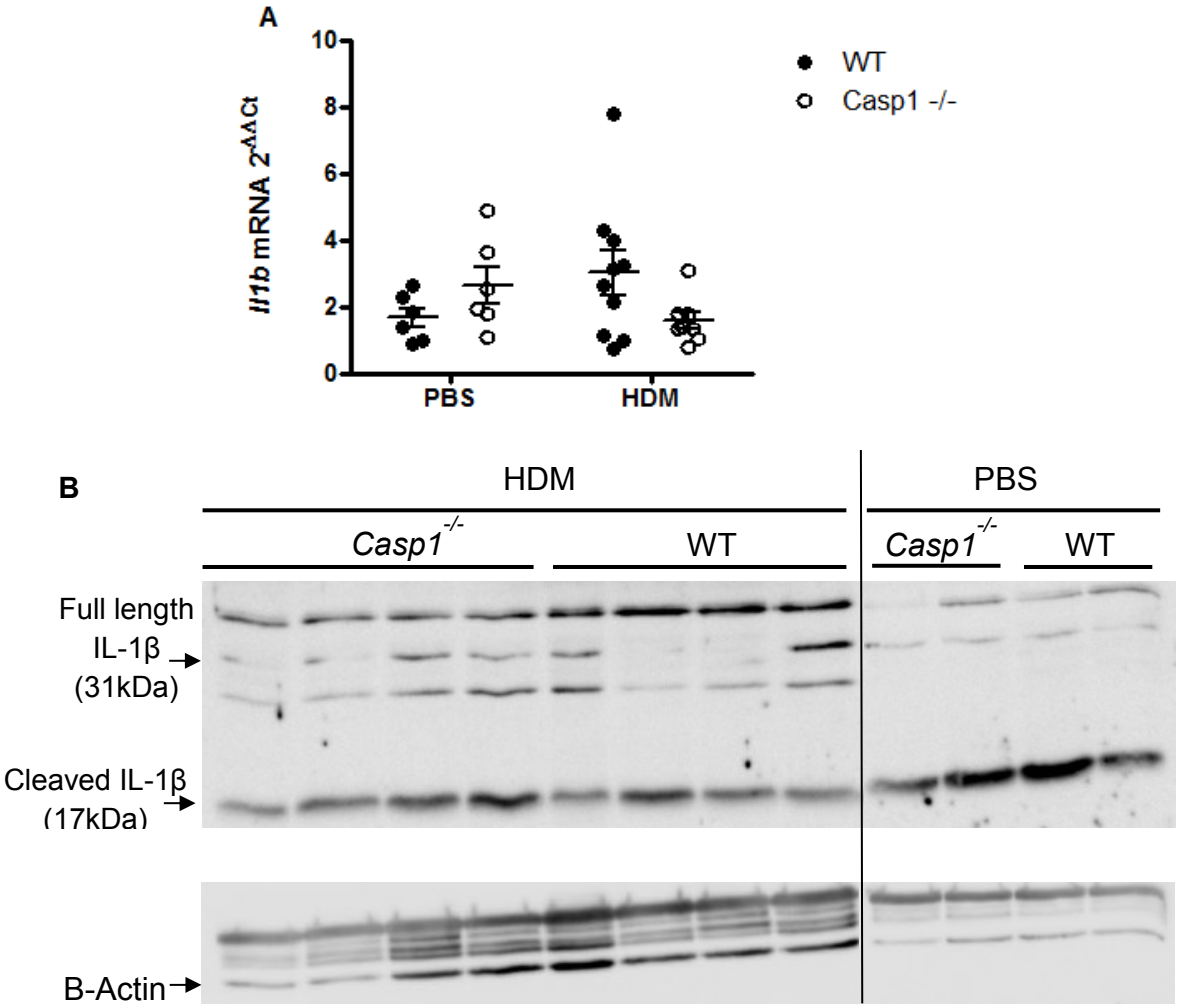

Figure 3.7. HDM-sensitization and caspase-1 deficiency do not affect *Il1b* gene expression or pro-IL-1B cleavage. A) *Il1b* gene expression was assessed in lung homogenates by qRT-PCR. n=6 WT PBS, 10 WT HDM, 6 *Casp1*$^{-/-}$ PBS, 8 *Casp1*$^{-/-}$ HDM. **B)** IL-1β protein in lung homogenates was assessed by western blot. Line indicates that the HDM and PBS samples were run on different gels.

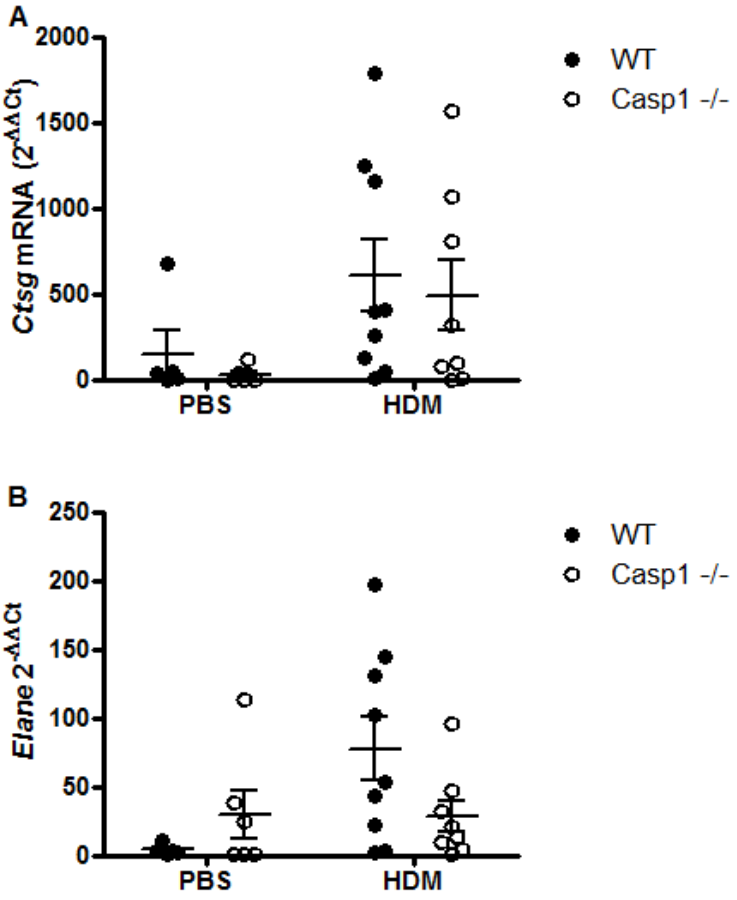

Figure 3.8. WT and Casp1$^{-/-}$ have similar expression of *Neutrophil elastase* and *Cathepsin G* in lung tissue. A) *Cathepsin G (Ctsg)* and **B)** *Neutrophil elestase (Elane)* mRNA expression in lung tissue homogenate from PBS-treated and HDM-sensitized Casp1$^{-/-}$ and WT mice. n=5 WT PBS, 9 WT HDM, 6 Casp1$^{-/-}$ PBS, 8 Casp1$^{-/-}$ HDM.

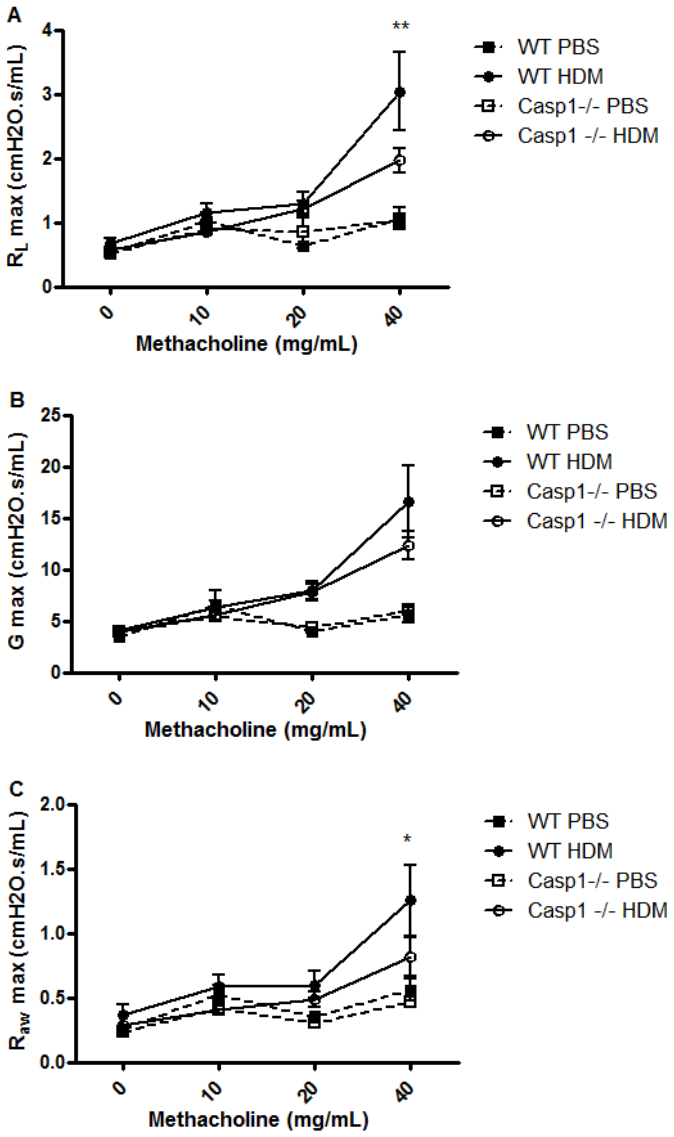

Figure 3.9. HDM-sensitized Casp1$^{-/-}$ mice have reduced airway resistance with methacholine challenge. A) Total lung resistance (R_L), **B)** tissue dampening (G), and **C)** large airway resistance (R_{aw}) was assessed by forced oscillation technique in PBS-treated or HDM-sensitized Casp1$^{-/-}$ and WT mice. n= 9 WT PBS, 11 WT HDM, 7 Casp1$^{-/-}$ PBS, 8 Casp1$^{-/-}$ HDM. P-value determined by factorial ANOVA with Bonferroni post-hoc test. *$p<0.05$, **$p<0.01$.

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Chapter 4: Investigation of damage associated molecular patterns and activation of the inflammasome in the airway of healthy volunteers after *in vivo* ozone exposure[1,2]

4.1 Introduction

Inhalation of ozone (O_3) causes airway inflammation that can exacerbate preexisting diseases, such as asthma and chronic obstructive pulmonary disease, and increases susceptibility to respiratory infections (1-3). The inflammatory response to O_3 suggests an active innate immune response to O_3 in the lung. Previous studies have implicated the pattern recognition receptors (PRRs) toll-like receptor (TLR) 2, TLR4, CD14, NOD-like receptor protein 3 (NLRP3), and CD44 in the innate immune response to O_3 (4-9). These receptors recognize pathogen associated molecular patterns (PAMPs), such as the bacterial cell wall component lipopolysaccharide (LPS), and damage associated molecular patterns (DAMPs), which are endogenously-derived molecules produced by damaged cells or tissue, such as adenosine triphosphate (ATP) or damaged extracellular matrix components (10-12). Since O_3 is an oxidant gas that induces sterile inflammation, activation of PRRs is likely mediated indirectly by a DAMP (13). O_3 induces oxidative stress that can directly damage the airway and react with the lung lining fluid to create reactive intermediates such as oxidized lipids, causing the release of

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pro-inflammatory cytokines and chemokines that enhance migration of inflammatory cells, such as neutrophils, to the airways (14). In the process of responding to the injury, neutrophils generate more reactivate mediators, such as proteases and reactive oxygen species,that can cause further airway damage (15). Damage of the airway caused either directly by O_3 or indirectly by immune-mediated damage is associated with the presence of DAMPs in the airway, including ATP and hyaluronic acid (HA) fragments (6, 16, 17).

The inflammasome is an innate signaling complex composed of a PRR [often a NOD-like receptor (NLR)], pro-caspase-1, and, in some cases, the adaptor protein PYCARD that has been implicated in the innate immune response to a number of DAMPs and pollutants (18). Formation of the complex leads to cleavage of pro-caspase-1 to an active form, which can then preform one of several functions, including the processing and release of IL-1β and IL-18 and a pro-inflammatory form of programmed cell death termed pyroptosis (19). Caspase-1–mediated processing of IL-1β and IL-18 requires two distinct signals (20). First, TLR activation induces downstream signaling through NF-kB to enhance transcription of pro–IL-1β and pro–IL-18. The second signal induces formation of an inflammasome complex and activation of caspase-1 that then cleaves the pro-cytokines to an active form. The NLRP3 inflammasome has been shown to mediate O_3-induced airway inflammation and airway hyperresponsiveness in mice (8). Additionally, several murine studies have found a key role for IL-1-dependent signaling in the response to O_3 (21-23). Though these findings suggest that O_3-induced oxidative damage may activate inflammasome signaling, contribution of the inflammasome to the human innate immune response to O_3 has not been shown.

Inflammasome signaling has been primarily characterized in macrophages and monocytes (24). Pulmonary macrophages (Mac) compose the majority of resident innate immune

cells in the lung at baseline conditions, suggesting that Mac are key initiators of the innate immune response to O_3 (25). Ablation of Mac in the lung with gadolinium chloride prevents O_3-induced inflammation and lung injury, demonstrating Mac are key mediators of the innate immune response to O_3 (26). In this study, we assessed the innate immune response of healthy volunteers exposed to O_3 *in vivo* and tested the hypothesize that DAMPs are early mediators of the innate immune response to O_3 that activate innate pattern recognition and inflammasome responses in Mac to initiate the inflammatory response to O_3.

4.2 Materials and methods

In vivo O_3 exposure. Subjects for this study were healthy adults with no history of smoking within two years of the study. See table 4.1 for subject characteristics. Subjects were recruited by Westat Corporation and underwent a screening procedure with complete medical history, physical exam, and pregnancy test for females. Subjects were excluded if they experienced respiratory illness or symptoms within 6 weeks of bronchoscopy and if they had a positive pregnancy test. Informed consent was obtained from all study participants. The study protocol was approved by the U.S. Environmental Protection Agency and the University of North Carolina at Chapel Hill institutional review board. The study design was a randomized, double-blinded, crossover study of the response of healthy volunteers to inhaled O_3. Subjects were randomly exposed to clean air and, in a separate exposure, 0.3 parts per million (ppm) O_3 for 2 hours with exercise and a minimum of 2 weeks separation between each exposure. The exposures took place in stainless steel chambers at the EPA Human Studies Facility on the campus of the University of North Carolina at Chapel Hill. The generation of O_3 and the exposure chambers were as previously described (27). Minute ventilation was maintained at 25 L/min/m^2 body surface area by adjusting the exercise level. Ventilatory parameters, electrocardiac signals, heart rate, and blood oxygen saturation were monitored throughout the exposure and spirometric lung function and symptom scores were assessed immediately before and after the exposure period for subject safety. The subjects underwent bronchoscopy with bronchoalveolar lavage (BAL) using sterile saline 1 or 24 hours after exposure as previously described (28). BAL samples were kept on ice immediately after aspiration and the cells were pelleted by centrifugation (300 x g for 10 minutes at 4° C). Cell-free BAL fluid was stored at -80°C until analysis.

Measurement of glutathione and purine metabolites in BAL fluid. Mass spectrometric (MS) analysis for purine metabolites (AMP, adenosine, and uric acid) and glutathione (GSH) was performed using a previously described method modified to include detection of GSH and reduced glutathione disulfide (GSSG) (17). Briefly, an internal standard solution of isotopically labeled urea, AMP, adenosine, and amino acids was added to the BAL fluid samples and the mixture was filtered through a 10k Da size selection filter (EMD Millipore, Billerica, MA) to remove macromolecules. Compounds were separated by liquid chromatography using 0.1% formic acid and methanol gradients on a C-18 column (Acquity UPLC HSS T3, Waters, Milford, MA), with positive mode MS detection performed in multiple-reaction monitoring mode scanning for transitions in mass-to-charge ratio (m/z) from parent to product ions for AMP, adenosine, and uric acid as described (17). The method was modified to add the transitions 308.1→179.1 for GSH and 307.1→355.1 for glutathione disulfide (GSSG). Metabolite concentrations were defined as ratio of the peak area from the metabolite to that of the internal standard with the closest column run time, and concentrations quantified by comparison to standard solutions run in parallel.

Measurement of oxysterols in BAL fluid. To 1 mL BAL fluid, 10 µl of alkynyl_secoB (50 ng/µl), 1mL NaCl (0.9%), 10 µl of triphenylphosphine (TPP) and butylated hydroxytoluene (BHT) (25 mg TPP and 10 mg BHT in 10 mL MeOH), 2 mL of MeOH, and 3 mL of iso-octane were added. The mixture was vortexed vigorously for 2 minutes and separated by centrifugation. The collected organic layer was evaporated to dryness in a SpeedVac™ concentrator and resuspended in 100 µl MeOH. For liquid chromatography-multiple-reaction monitoring (LC-MRM) analysis, the resuspended samples were chromatographed by RP-HPLC using a UPLC BEH C18 column (1.7 µm, 2.1 mm × 100 mm) in Waters Acquity UPLC system equipped with

an autosampler (Waters, Milford, MA). The oxysterols were separated by 95% solvent B in isocratic method with a flow rate of 200 µL/min, and the mobile phase solvents consisted of 2 mM NH_4OAc (solvent A) in water and 2 mM NH_4OAc in MeOH (solvent B). The injection volume was 10 µl using a partial loop with needle overfill mode. MS detections were performed using a TSQ Quantum Ultra tandem mass spectrometer (ThermoFisher, Waltham, MA), and data was acquired and analyzed using a Thermo Xcalibur™ 2.2 software package. Alkynyl_secosterol B was used as an internal standard for the analysis. The cholesterol and oxysterols form $[M+NH_4]^+$ ions in positive ion mode (29). The transitions monitored were *m/z* 436→383 for secosterol A and B (SecoA and B), *m/z* 404→369 for cholesterol, m/z 418→365 for alkynyl_secosterolB (a_secoB), *m/z* 420→385 for cholesterol epoxide α and β (CholEp α and β). See Figure 4.1 for structures.

Cytokine analysis. Levels of IL-8 and IL-1β in the BAL fluid were measured using a commercially available enzyme-linked immunosorbent assay (ELISA, BD Biosciences, San Jose, CA). IL-8 and IL-1β in the media supernatants from BAL cells with *ex vivo* ATP stimulation were measured using a commercially available ELISA kit (Meso Scale Discovery, Gaithersburg, MD).

Hyaluronic Acid (HA) Analysis. Concentrations of hyaluronic acid (HA) in the BAL fluid were measured using a commercially available kit (Hyaluronan DuoSet; R&D, Minneapolis, MN).

LDH Assay. To assess cytotoxicity, the BAL fluid and media supernatants from BAL cells were analyzed for concentrations of LDH using a commercially available kit (Takara Bio Inc., Otsu, Shiga, Japan).

Ex vivo ATP stimulation. BAL cells were pelleted by centrifugation and washed twice with RPMI 1640 (Life Technologies, Carlsbad, CA). Cells were resuspended in RPMI supplemented with 10% FBS (Life Technologies) and 1% Penicillin-Streptomycin (Life Technologies) and were kept at 37°C until use within 1 hour. 100,000 cells were aliquoted to sterile 5mL polystyrene culture tubes (Evergreen Scientific, Los Angeles, CA) and incubated with 1mM of ATP (Sigma Aldrich, St. Louis, MO) or PBS vehicle control (Life Technologies) for 30 minutes at 37°C. The cells were centrifuged at 500g for 10 minutes and the media supernatants were stored at -20°C until analysis. The cells were immediately used for flow cytometry assessment of caspase-1 activity.

Flow Cytometry. For surface marker analysis, BAL cells were resuspended in staining buffer [PBS, 1% heat-inactivated FBS, and 0.09% Sodium azide (Sigma, St. Louis, MO, USA)] and stained with antibodies for CD14 (Beckman Coulter, Brea, CA), CD44 (BD Biosciences), TLR4 (eBioscience, San Diego, CA), and with Live/Dead® Violet Fixable cell stain (Life Technologies) for 20 minutes at room temperature in the dark. Cells were washed with staining buffer and resuspended in 0.5% paraformaldehyde (Sigma). Caspase-1 activity in BAL cells was assessed following *ex vivo* ATP stimulation using the FLICA™ Caspase-1 Assay Kit (Immunohistochemistry Technologies, Bloomington, MN). Briefly, the cells were labeled using a carboxyfluorescein-YVAD-fluoromethyl ketone, which is bound by active caspase-1, for 1 hour at 37°C in the dark. During the final 20 minutes of incubation, the cells were stained with CD45 and Live/Dead® stain to assess viability. Cells were washed twice with flow cytometry staining buffer and resuspended in 0.5% PFA. Samples were stored at 4°C in the dark and acquired within 24 hours on a BD Biosciences LSRII flow cytometer. Live Mac were identified by forward and side scatter properties, positive CD45 staining, and negative LiveDead staining,

and analyzed for mean fluorescence instensity (MFI) of receptor expression or percentage of FLICA positive cells. Unstained and isotype-matched single color controls (BD Biosciences) were used to control for nonspecific staining and to set analysis gates.

Statistical Analysis. Non-parametric, paired Wilcoxin Signed Rank Test was used to assess the effects of O_3 on cytokines, purine metabolites, HA, oxysterols, GSSH and GSSG in the BAL fluid and surface marker expression by Mac. Two way ANOVA with Bonferroni post-hoc analysis was to assess the effects of O_3 and ATP on caspase-1 activity in *ex vivo* Mac and IL-1β, IL-8, and LDH release by *ex vivo* BAL cells. Linear regression and non-parametric Spearman correlation analysis was used to assess correlation between IL-1β and LDH produced by *ex vivo* BAL cells. Significance was set at $p<0.05$. All graphs show mean ± SEM.

4.3 Results

In vivo O_3 exposure induces early signs of oxidative stress and cytotoxicity

Healthy volunteers were exposed to 0.3 ppm O_3 and, in a separate exposure, clean air for 2 hours with exercise. Though the concentration of O_3 used here, 0.3ppm, is higher than the EPA standard of 0.075 ppm/hour, peak hourly levels of O_3 close to this magnitude have been reported in highly polluted cities such as Mexico City (30). Inhaled O_3 induces oxidative stress that can damage the airway and is combatted by antioxidants in the lung lining fluid such as glutathione (GSH) (31). Oxidation of GSH to glutathione disulfide (GSSG) is a marker of oxidative stress. We measured the effect of O_3 on GSH in the bronchoalveolar lavage (BAL) fluid using mass spectrometry and found enhanced levels of total glutathione (GSSH + GSH) with O_3 exposure and a non-significant ($p= 0.09$) increase in the ratio of GSSG to GSH at 1 hour after O_3 exposure (Figure 4.2 A-B). Next, we measured levels of lactate dehydrogenase (LDH) in the BAL fluid, a marker of cytotoxicity and airway damage. Our results indicate that there was trend for enhanced LDH in the BAL fluid 1 hour after O_3 exposure ($p=0.0504$, Figure 4.2 C). Together, our results suggest that markers of oxidative stress and airway damage can be measured in the airway as early as 1 hour following O_3 exposure.

In vivo O_3 exposure enhances DAMPs in the airway

Damaged cells and tissue release DAMPs such as hyaluronic acid (HA), oxidized lipids, ATP, and uric acid that activate PRR and inflammasome signaling (12, 32-35). Since O_3 induces sterile inflammation, we hypothesize that DAMPs mediate the innate immune response to O_3. We measured levels of total HA, several purine metabolites (adenosine monophosphate (AMP), adenosine, and uric acid), and several oxidized cholesterols (oxysterols) in BAL fluid from healthy volunteers exposed to air or O_3. 1 hour after exposure, we found significantly increased

uric acid, cholesterol epoxide β, and secosterol A and a trend for increased cholesterol epoxide α in BAL fluid from O_3-exposed individuals, but no difference in HA concentrations (Figures 4.3 and 4.4). Conversely, we found only enhanced levels of HA in BAL at 24 hours after O_3 exposure (Figure 4.3 A). Notably, due to limited sample volume we were unable to assess purine metabolites in the 24 hour samples. These results suggest that exposure to O_3 induces time-dependent accumulation of DAMPs in the airway.

In vivo O_3 exposure enhances IL-8, but not IL-1β, in the airway.

Activation of PRR and inflammasome signaling by DAMPs leads to downstream cytokine and chemokine production. We assessed levels of the inflammasome cytokine IL-1β and the neutrophil chemokine IL-8 in the BAL fluid by ELISA. O_3 significantly enhanced levels of IL-8 in airway at both 1 and 24 hours post-exposure (Figure 4.5), whereas IL-1β was undetectable in the BAL fluid in all samples (data not shown).

In vivo O_3 exposure does not alter macrophage expression of CD14, TLR4, and CD44 at 1 hour post-exposure

Pulmonary macrophages (Mac) compose the majority of innate immune cells in the airway at baseline conditions and express a broad range of PRRs to patrol the airway for injurious stimuli (24). Previous studies have implicated CD14, TLR4, and the HA receptor, CD44, as key PRRs involved in innate immune response to O_3 (6, 13, 36, 37). We assessed surface receptor expression of CD14, TLR4, and CD44 by Mac at 1 hour after exposure to Air or O_3 by flow cytometry and found no differences in surface expression (Figure 4.6).

In vivo O_3 exposure primes Mac for enhanced IL-8 production in response to ATP

IL-1β and IL-1R signaling has been associated with many of the adverse health effects of O_3 (22, 23). IL-1β release is dependent on processing by the proteases such as caspase-1,

neutrophil elastase, or mast cell chymase (38). Since the NLRP3 inflammasome has been implicated in the innate immune response to O_3 in mice (8), we measured caspase-1 activity in Mac from air and O_3-exposed volunteers by flow cytometry. Consistent with the undetectable levels of IL-β in the BAL fluid, we found no effect of O_3 on caspase-1 activity in Mac at 1 hour post-exposure (Figure 4.7 C, control samples).

Caspase-1 mediated processing of IL-1β requires two stimuli: induction of pro-IL-β and formation of an inflammasome complex to activate caspase-1. Pro-IL-1β expression is regulated by transcription factors such as NF-kB that can be activated by signaling through TLRs (39). O_3 has been previously shown to activate TLR signaling and NF-kB activation, suggesting that O_3 may prime Mac for subsequent inflammasome activation and IL-1β processing (40, 41). To determine whether O_3 primes the airway for subsequent IL-1β processing by the inflammasome, we treated BAL cells from air and O_3-exposed volunteers at 1 hour post-exposure with the known inflammasome activator ATP *ex vivo* (1mM ATP for 30 minutes) and measured IL-1β production by ELISA and caspase-1 activity in Mac by flow cytometry. We found that ATP significantly enhanced IL-1β only in O_3-exposed BAL cells (Figure 4.7 A), though the level of IL-1β was barely above detection limits (< 4ng/mL) and caspase-1 activity was not altered by ATP stimulation in either Air or O_3-exposed Mac (Figure 4.7 C). ATP stimulation also enhanced production of IL-8 only in O_3 exposed BAL cells (Figure 4.7 B). Notably, ATP stimulation did not significantly enhance LDH release (Figure 4.7 D), but there was a significant positive correlation between enhanced IL-1β and LDH, suggesting that detected IL-1β may be pro-IL-1β released from lytic cells (Figure 4.7 E). In summary, our results indicate that at 1 hour post-exposure, O_3 did not activate or prime caspase-1 activity in Mac, but enhanced Mac IL-8 response to subsequent stimulation with DAMPs such as ATP.

4.4 Discussion

In this study, we tested the hypothesis that airway damage induced by *in vivo* O_3 exposure causes the release of damage associated molecular patterns (DAMPs) that activate inflammasome signaling in Mac and initiate the inflammatory response to O_3 in humans. Our results indicate that inhalation of O_3 induced time-dependent increases in DAMPs, including hyaluronic acid (HA), oxidized cholesterols (oxysterols), and uric acid, that were associated with enhanced IL-8 in the airway. Additionally, we found that pulmonary Mac exposed to O_3 had enhanced IL-8 production in response to *ex vivo* stimulation with the DAMP ATP. However, we found no evidence of O_3- or ATP-induced caspase-1 activity in pulmonary macrophages (Mac) collected 1 hour after exposure and were unable to detect IL-1β in the BAL fluid at any time point, suggesting that *in vivo* O_3 exposure does not activate inflammasome-mediated IL-1β production in humans. Together, our results uncovered novel mediators of the innate immune response to O_3 in the lung that may induce inflammation independently of inflammasome signaling.

Similar to previous studies, we detected signs of oxidative stress and cytotoxicity as early as 1 hour after O_3 exposure, as indicated by increased oxidation of GSH to glutathione disulfide (GSSG) and enhanced lactate dehydrogrenase (LDH) in the BAL fluid (Figure 4.2) (42, 43). Due to technical limitations, we were unable to measure GSH oxidation or LDH release at 24 hours post-exposure, but previous studies in humans have shown increased LDH, increased antioxidant responses, and decreased activity of glutathione peroxidase, an enzyme that neutralizes hydrogen peroxide and lipid hydroperoxides in the presence of GSH, as late as 18 hours after exposure to O_3 (31, 44, 45). Our results show that oxidative damage induced by O_3 is associated with the

presence of DAMPs in the airway, including several oxysterols, HA, and uric acid, and that abundance of these DAMPs vary over time after exposure.

Due to high reactivity, O_3 is not thought to penetrate further than the lung lining fluid or apical surface of the airway epithelium (46). The reaction of O_3 with lipids and proteins in cell membranes and the lung lining fluid generates more stable intermediates that travel deeper into the lung to damage cells and tissue (47, 48). Though primarily studied in the context of atherosclerosis, oxidized lipids, such oxysterols, oxidized phospholipids, and oxidized cholesteryl esters, have received increased attention as mediators of innate immune responses in the lung (33, 49, 50). Our results indicate that O_3 exposure rapidly induces the presence of cholesterol epoxide α, cholesterol epoxide β, and secosterol A in the airway by 1 hour post-exposure, suggesting that oxysterols may be early mediators of the inflammatory response to O_3 (Figure 4.4). Though other oxidized lipids have been identified in the human airway after *in vivo* O_3 exposure, including the lipid peroxidation product 4-hydroxynonenal (51), to our knowledge this is the first study to identify oxysterols in BAL fluid from humans after *in vivo* exposure to O_3. These findings are in agreement with previous studies that found increased oxysterols, including cholesterol epoxide β, in BAL fluid from mice or rats exposed to O_3 *in vivo* that peak 6 hours post-exposure (29, 52, 53). Oxysterols in the lung may be induced by direct interaction of cholesterol with O_3, generation of free radicals that promote cholesterol peroxidation, or reaction oxidant species produced by immune cells such as neutrophils during normal immune responses with cholesterol (29, 52, 54, 55). Since neutrophil influx typically does not occur until 3 hours after exposure to O_3 and remains elevated through 24 hours, the generation of oxysterols at 1 hour post-exposure was not likely mediated by neutrophil-derived oxidants (42).

In addition to oxysterols, we also found that O_3 enhanced levels of the DAMP uric acid, a product of purine nucleoside catabolism, in the BAL fluid at 1 hour post-exposure (Figure 4.3 B) (32). Whereas at low levels uric acid may serve an antioxidant function by scavenging singlet oxygen, peroxyl radicals and hydroxyl radicals, high levels of uric acid form monosodium urate crystals that act as DAMPs and stimulate PRRs such as TLR2, TLR4, and the NLRP3 inflammasome (32, 56). Though we detected enhanced uric acid in the BAL fluid 1 hour post-O_3 exposure, there was no significant correlation between uric acid and markers of inflammation or oxidative damage in the airway, including oxidation of GSH, LDH, or IL-8 in the airway (data not shown). In addition, due to limited sample volume, we were unable to measure uric acid in the BAL fluid at 24 hours post-exposure to determine if the increase in uric acid accumulates over time after exposure. Thus, future studies will be necessary to determine whether uric acid serves as a DAMP or an antioxidant in the response to O_3.

In opposition to oxysterols and uric acid, which were elevated only 1 hour after O_3 exposure, increased hyaluronic acid (HA) was only detected in the BALF 24 hours post-exposure (Figure 4.3A). HA is a component of the extracellular matrix produced by airway epithelial cells and submucosal glands in the lung as long strands (high molecular weight) that have anti-inflammatory effects (57, 58). With tissue injury or oxidative damage, the long strands become fragmented to low molecular weight HA that induces inflammation (58, 59). Low molecular weight HA has been shown to activate O_3-induced TLR4, CD44, and the NLRP3 inflammasome signaling in mice (6, 8, 37). Similar to our findings, previous studies have found that HA does not accumulate until 12 to 24 hours after O_3 exposure in mice, and a human O_3 exposure study found increased HA in asthmatic subjects 6 hours after O_3 exposure, suggesting that HA may be a late mediator of O_3-induced airway inflammation (8, 60). We propose that accumulation of HA

is not caused by immediate O_3-induced damage, but may rather be a consequence of immune-mediated damage, as caused by neutrophil influx, or inflammatory cell dysfunction. For example, Macs have been shown to have altered immunophenotype and phagocytic function after O_3 (3, 7, 36). As clearance of HA occurs via CD44-mediated endocytosis by Mac, Mac dysfunction could lead to accumulation of HA (61). We assessed Mac receptor expression 1 hour after exposure, and found no effects of O_3 on TLR4, CD14, or CD44 expression (Figure 4.6); however, changes in Mac immunophenotype may not occur until later after O_3 exposure when immune-mediated damage has occurred.

The increased presence of DAMPs in the airway after O_3 exposure was associated with enhanced levels of the neutrophil chemoattractant IL-8 at 1 and 24 hours post-exposure, suggesting an ongoing inflammatory response to O_3 as early as 1 hour post-exposure (Figure 4.5). Surprisingly, IL-1β was undetectable in all BAL fluid samples and O_3 exposure had no effect on caspase-1 activity in Mac, suggesting that inflammasome signaling did not contribute to O_3-induced inflammation (Figures 4.7 C). These results are in disagreement with a previous murine study by *Feng et al.* that showed NLRP3, caspase-1, and PYCARD contribute to O_3-induced airway hyperresponsiveness and inflammation and that inflammasome-mediated IL-1β production by Mac was activated by HA fragments at 24 hours post- O_3 exposure (8). Unlike the *Feng et al.* study, we assessed caspase-1 activity only at1 hour post-exposure, when HA levels were not altered by O_3 exposure (Figure 4.3 A). Thus, the inflammasome may not be activated until later after exposure to O_3 when DAMPs such as HA accumulate. However, we also could not detect IL-1β in BAL fluid at either 1 or 24 hours post-exposure. Though it possible that the BAL fluid was too dilute, or that IL-18 or pyroptotic cell death may be a more important

endpoint of O_3-induced inflammasome signaling in humans, our data does not suggest a strong role for the inflammasome in the innate immune response to O_3.

Caspase-1 mediated IL-1β processing requires two steps: 1) activation of TLR and NF-kB mediated pro-IL1β transcription and 2) inflammasome assembly to induce caspase-1 activity. Previous studies have shown O_3 activates TLR4 and TLR2 signaling and NF-kB activity, suggesting that O_3 may prime the cell for inflammasome activation by a subsequent DAMP such as HA or ATP (9, 13, 40). To test this hypothesis, we treated BAL cells from Air and O_3-exposed individuals at 1 hour post-exposure with the known inflammasome stimulator ATP. We found that BAL cells from O_3-exposed individuals produced IL-8 and IL-1β in response to ATP, whereas ATP had no effect on Air-exposed cells (Figure 4.7 A-B). Notably, the levels of IL-1β were very low (< 4ng/mL) and correlated with LDH, suggesting that IL-1β may be released from lytic cells rather than via caspase-1 activity (Figure 4.7 E). Moreover, we did not detect ATP-induced caspase-1 activity in either Air or O_3-exposed Mac, suggesting that ATP may not induce caspase-1 activity in resident pulmonary Mac (Figure 4.7 C). Many studies of human inflammasome signaling have been performed *in vitro* using blood monocytes and the human THP-1 monocyte cell line (20). Whereas monocytes have constitutively activate caspase-1 and rapidly produce IL-1β in response to a single stimulus, macrophages express low levels of caspase-1, require multiple stimuli to secrete IL-1β, and produce IL-1β more slowly than monocytes (20, 62). Thus, additional stimuli or longer incubation with ATP may be necessary to activate caspase-1 and detect IL-1β production by human pulmonary Mac. Together, our results suggest that O_3 primes Mac for an enhanced inflammatory response to a subsequent DAMP, such as ATP, but that this effect is likely not mediated by inflammasome signaling.

In summary, we show here that *in vivo* O_3 exposure of healthy volunteers induces oxidative damage that is associated with enhanced levels of several DAMPs and IL-8 in the airway, but that O_3-induced airway inflammation in humans is likely not mediated by activation of inflammasome-induced IL-1β. Our results indicate that different DAMPs mediate the early and late immune responses to O_3 in the lung. Oxysterols may serve as early initiators inflammation, and are thus likely induced directly by O_3-induced airway damage. Oxysterols have been primarily studied in the context of atherosclerosis, where overload of macrophages with oxidized lipids induces the formation of pro-inflammatory foam cells that contribute to atherogenesis (63, 64). Our results suggest that oxysterols are also important immune mediators in the lung, and future studies should investigate the downstream effects of O_3-induced oxysterols on airway innate immune responses. As the oxysterols subside, HA accumulates late in the response to O_3, suggesting that increased HA is a consequence of immune-mediated inflammation rather than direct O_3-induced damage. Increased knowledge of the function of these mediators in innate immunity will improve our understanding of O_3-induced lung injury and better inform the design of therapeutics to combat oxidant-related lung pathology.

Table 4.1 Subject Characteristics

Total Subject #	Sex	Race	Average Age (± SD)	Average BMI (± SD)
11	1F/10M	9 Caucasian, 1 Hispanic, 1 African American	24.27 (3.069)	25.24 (2.847)

SD= standard deviation; BMI= body max index; F=female; M=male;

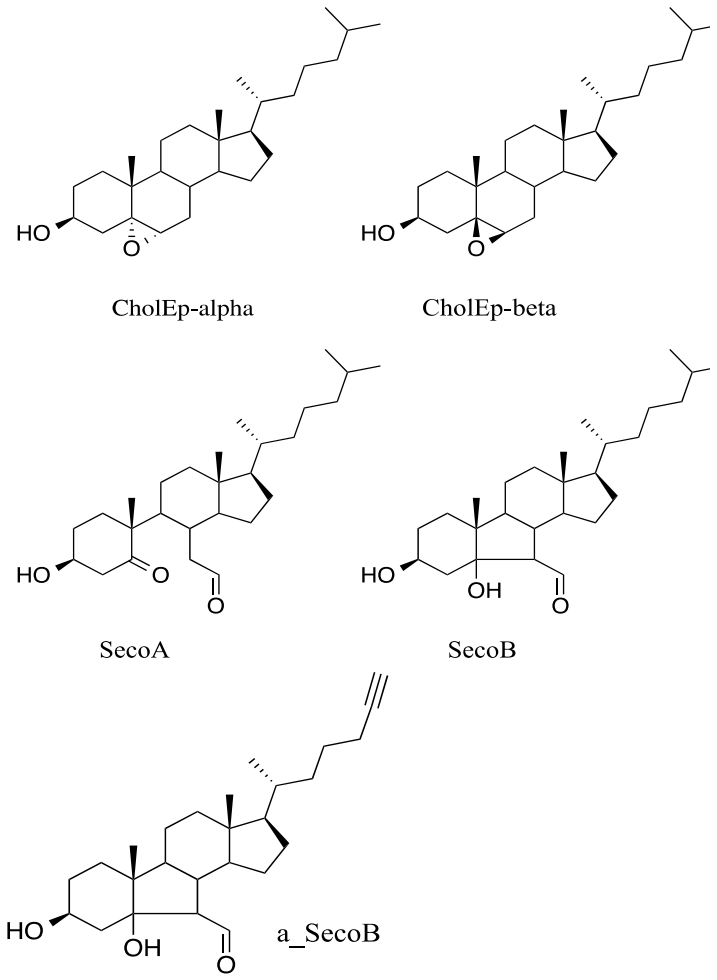

Figure 4.1. Oxysterols analyzed by LC-MRM analysis. A) Cholesterol epoxide α (CholEp-alpha); B) cholesterol epoxide β (CholEp-beta); C) secosterol A (SecoA), and D) secosterol B (SecoB). E) Alkynyl secosterol B (a_SecoB) was used an internal standard for LC-MRM analysis.

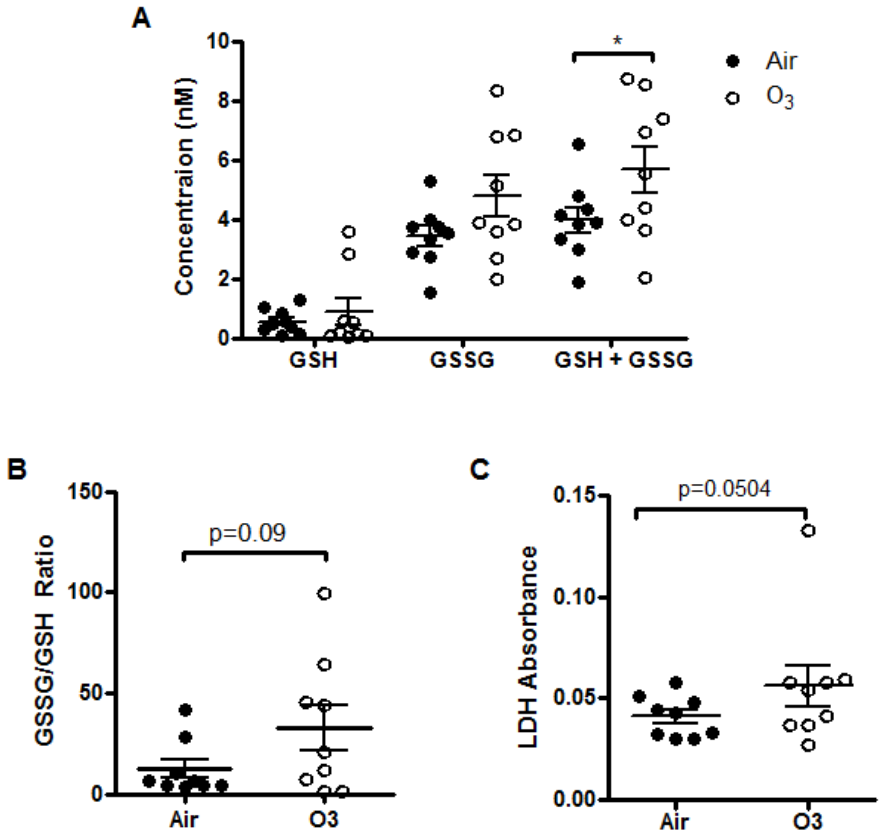

Figure 4.2. Trend for increased gutathione (GSH) oxidation and lactate dehyrodrogenase (LDH) the airway at 1 hour post-O_3 exposure.(A) Levels of oxidized glutathione (GSSG) and GSH in the BAL fluid collected 1 hour after exposure 0.3ppm O_3 or air for 2 hours were measured by mass spectrometry and (B) the ratio of GSSG to GSH was assessed as a marker of oxidative stress. (C) BAL fluid collected 1 hour after exposure to O_3 or Air was assessed for LDH quantities as a measure of cytotoxicity. n=9 subjects. P-value determined by Wilcoxin Signed Rank Test.

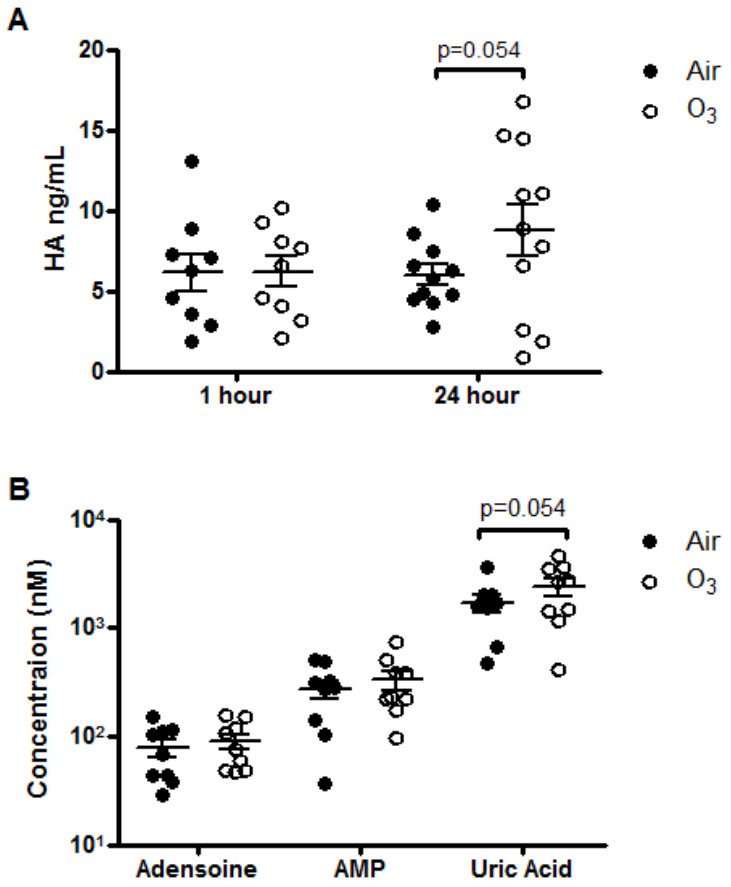

Figure 4.3. O_3 exposure enhances hyaluronic acid and uric acid in the airway. (A) Hyaluronic acid in the BAL fluid at 1 and 24 hours after exposure to 0.3ppm O_3 or air for 2 hours was measured by ELISA. (B) Concentration of the ATP metabolites adenosine, AMP, and uric acid in the BAL fluid 1 hour after exposure to O_3 or air were assessed by mass spectrometry. n=9-11 subjects. P-value determined by Wilcoxin Signed Rank Test.

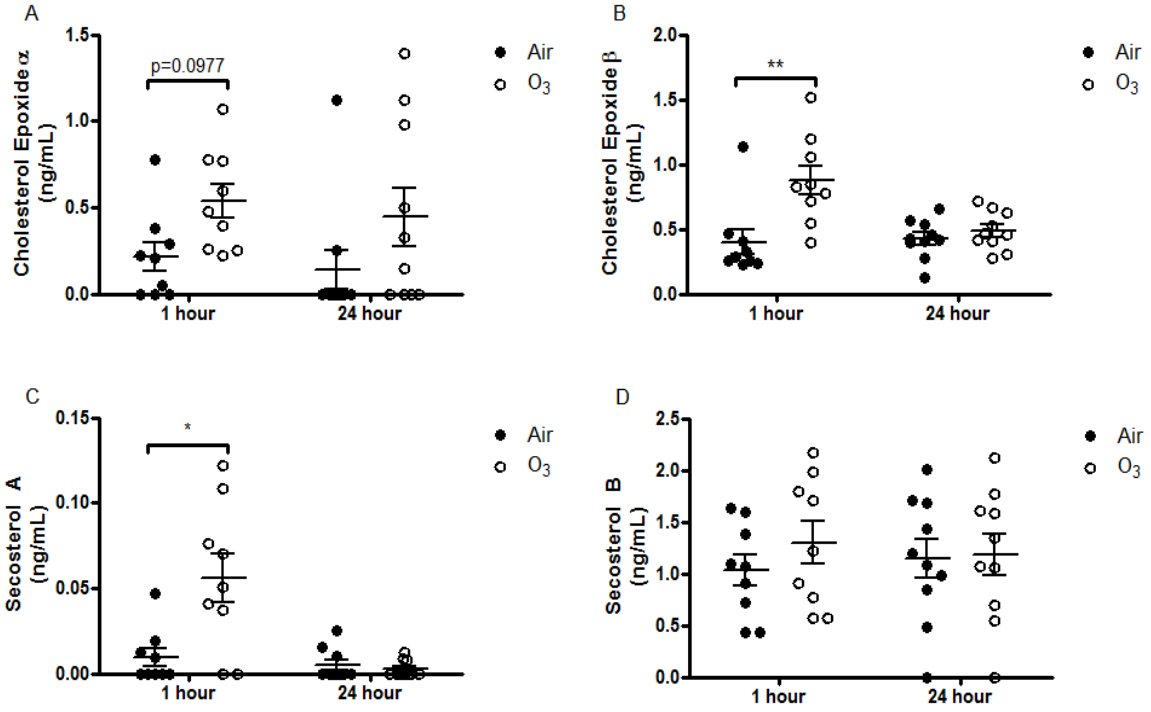

Figure 4.4. O_3 exposure enhances levels of oxysterols in the airway. Quantities of **A)** cholesterol epoxide α, **B)** cholesterol epoxide β, **C)** secosterol A; and **D)** secosterol B in the BALF were measured by liquid chromatography-multiple-reaction monitoring. BALF was obtained by bronchoscopy at 1 or 24 hours after exposure to air or 0.3ppm O_3 for 2 hours. n=9 (1 hour) or 10 (24 hour) subjects. P-value determined by Wilcoxin Signed Rank Test. *$p<0.05$.

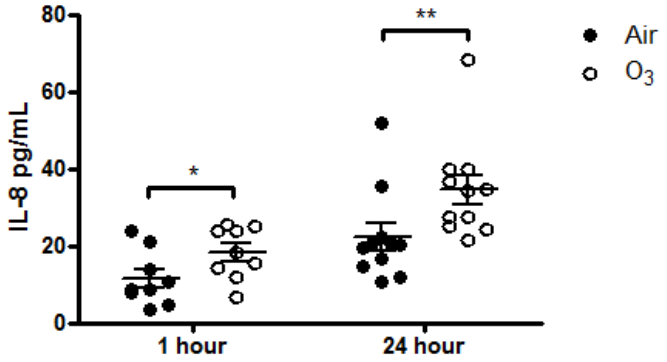

Figure 4.5. O_3 enhances IL-8 in the bronchoalveolar lavage fluid. Quantities of IL-8 in the BAL fluid collected at 1 and 24 hours after exposure to 0.3ppm O_3 or air for 2 hours were measured by ELISA. n=9-11 subjects. P-value determined by Wilcoxin Signed Rank Test. *$p<0.05$.

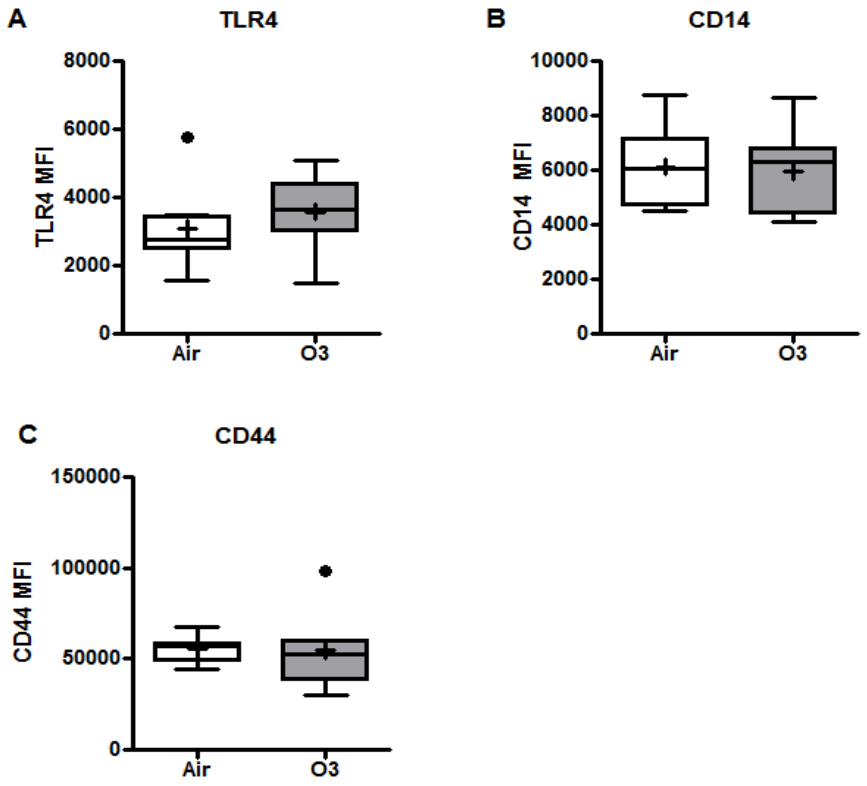

Figure 4.6. O_3 exposure did not modify surface expression of CD14, TLR4, and CD44 by Mac at 1 hour post-exposure. Surface expression of (A) TLR4, (B) CD14, and (C) CD44 by live Mac collected 1 hour after exposure from volunteers exposed 0.3ppm O_3 or Air for 2 hours was assessed by flow cytometry and expressed as mean fluorescent intensity (MFI). n=9 subjects.

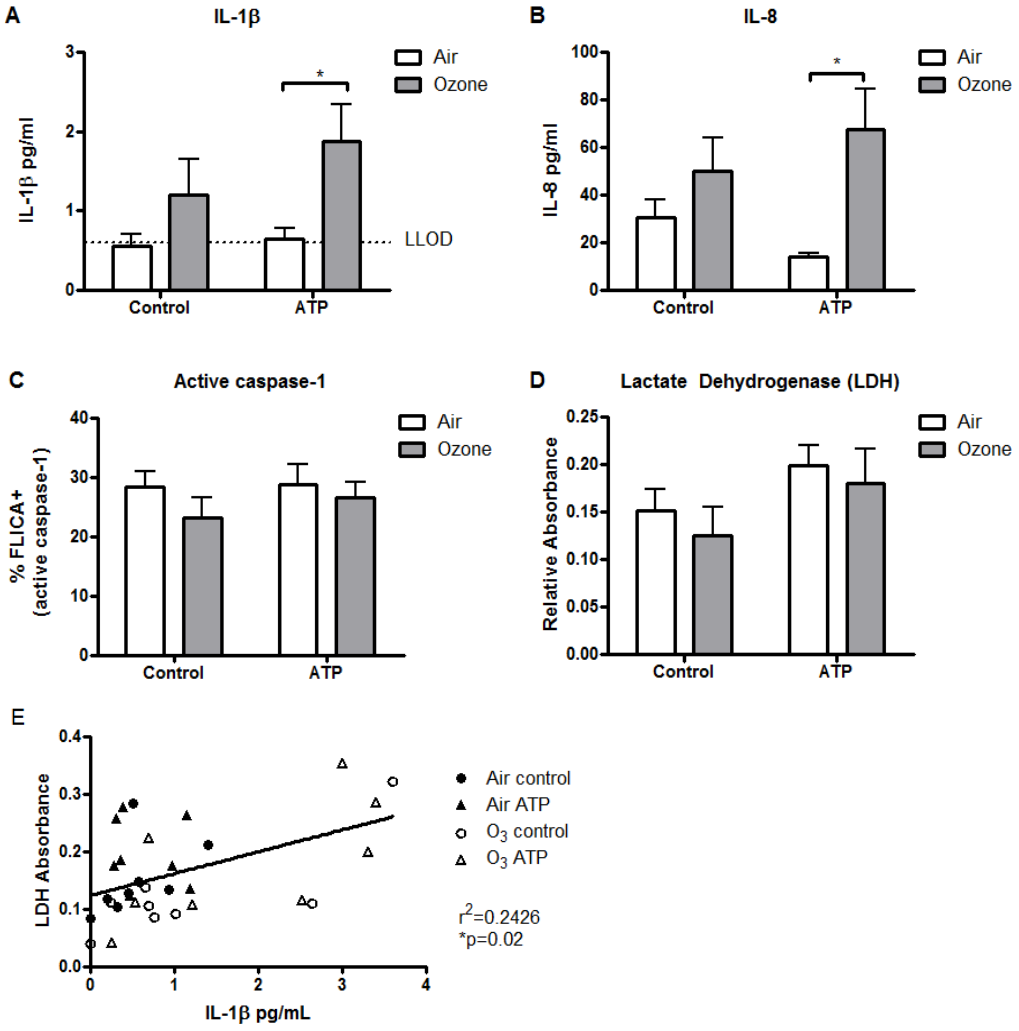

Figure 4.7. O_3 exposure primes Mac for an enhanced inflammatory response to ATP. Bronchoalveolar lavage cells collected 1 hour after exposure to 0.3ppm O_3 or Air were stimulated *ex vivo* with ATP (1mM) or vehicle control (PBS) for 30 minutes. (A) Caspase-1 activity was measured as % FLICA positive by flow cytometry. Production of (B) IL-1β, (C) IL-8, and (D) lactate dehydrogenase (LDH) was measured in the media supernatants from *ex vivo* stimulated bronchoalveolar cells. (E) Correlation between LDH and IL-1β in the media supernatants. LLOD, lower limit of detection. P-value determined by Two-way ANOVA with Bonferfoni post-hoc analysis.*$p<0.05$. n=8 subjects.

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Chapter 5: Airway Macrophage and Epithelial Cell Interaction Modifies the Innate Immune Response to Ozone[1,2]

5.1 Introduction

Inhalation of O_3 causes immediate nociceptive decreases in lung function and increased airway inflammation that can exacerbate preexisting diseases, such as asthma and chronic obstructive pulmonary disease (1, 2). Despite regulations to reduce ambient O_3, in 2008 approximately 36.2% of the U.S. population lived in counties that did not meet the National Ambient Air Quality 8-hour O_3 standard (0.075ppm averaged per 8 hours) (3). Though the adverse health effects of O_3 are well known, the cellular mechanisms by which O_3 alters immune responses in the lung remains unclear.

The induction of airway inflammation following exposure to O_3 suggests an ongoing innate immune response in the lung. Since O_3 is an oxidant gas, the innate immune response to O_3 is not likely mediated by recognition through discreet receptors (4). Rather, previous studies suggest that O_3 reacts with components of the airway lining fluid and cellular membrane, such as surfactants and phospholipids, generating reactive intermediates that damage the respiratory epithelium and induce endogenous danger signals that initiate innate immune responses (2, 5, 6). For example, short fragments of hyaluronic acid (HA), an extracellular matrix

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glycosaminoglycan produced by airway epithelial cells and submucosal glands, have been shown to serve as danger signals in the response to O_3 (7-9). Levels of HA are elevated in the airways of mice 24 hours after exposure to O_3 (7) and 4-6 hours after *in vivo* O_3 exposure in sputum from atopic asthmatic (10). HA fragments can induce airway hyperreactivity and activate inflammatory responses via pattern recognition receptors including toll-like receptor (TLR)2, TLR4, and CD44 (7, 11, 12).

Pulmonary macrophages (Mac) and epithelial cells are two of the most abundant cell types in the lower and conducting airways and thus serve as crucial first responders to O_3-induced airway damage (13). The airway epithelium acts as both a physical barrier against the inhaled environment and orchestrator of the innate immune response (14). Acute O_3 exposure has been previously shown to damage epithelial cells, leading to increased airway permeability, cell death, and the release of cytokines/chemokines and danger signals that can activate local immune cells, such as Mac (2, 15, 16). Airway Mac reside along the airway epithelium and act as key members of the innate immune system by clearing pathogens and debris via phagocytosis, killing pathogens via respiratory burst, and releasing cytokines and chemokines to regulate the inflammatory response (13, 17). Previous studies have shown that Mac play a key role in O_3-induced lung injury, as Mac numbers increase following O_3 exposure and blocking Mac activity during O_3 exposure in rats reduces airway inflammation (18). Yet, Mac also play a protective role in the response to O_3 by clearing reactive intermediates and cellular debris and releasing mediators that are anti-inflammatory to initiate wound repair (5, 17). This yin-yang characteristic of Mac led to the classification of Mac as either “classically activated,” or proinflammatory, or “alternatively activated”, or anti-inflammatory/wound healing (17). Studies in rats suggest that

inhalation of O_3 is associated with accumulation of both classically and alternatively activated Mac in the lung (17).

The close proximity between airway epithelial cells and Mac suggests that they encounter inhaled stimuli simultaneously and regulate the inflammatory response in tandem. Additionally, the tissue microenvironment has been shown to play a key role in determining Mac phenotype and function (19). Yet, most *in vitro* studies investigating the cellular inflammatory response to O_3 have used monoculture systems, which do not address the interaction between multiple cell types in the airway and have limited applicability to *in vivo* situations (15, 16, 20-23). We developed a co-culture model of human bronchial epithelial cells (16HBE) and primary human airway Mac to test the hypothesis that signals from 16HBE modifies Mac phenotype and response to O_3, and that communication between these two cell types is a key determinate of the inflammatory response to O_3.

5.2 Materials and Methods

Preparation of 16HBE-Mac co-cultures, 16HBE monocultures, and Mac monocultures. 16HBE14o- (16HBE) cells, a SV-40 transformed human bronchial epithelial cell line were a gift from Dr. D.C. Gruenert (University of California, San Francisco, CA) (24). 16HBE were plated on fibronectin coated [(LHC Basal Medium (Life Technologies, Carlsbad, CA), 0.01% bovine serum albumin (Sigma), 1% Vitrocol (Advanced Bio Matrix, San Diego, CA), and 1% human fibronectin (BD Biosciences)] 0.4-µm Transwells (Costar, Corning, NY, USA) and grown submerged in 1X MEM media (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Carlsbad, CA), 1% penicillin-streptomycin, and 1% l-glutamine (Life technologies) for six days and one day at air-liquid interface (ALI) before use. Primary human airway macrophages (Mac) were obtained by bronchoalveolar lavage (BAL) of healthy volunteers in collaboration with the U.S. Environmental Protection Agency using a protocol approved by the UNC-Chapel Hill institutional review board, as described previously (25). 1.5×10^5 BAL cells were added to the apical side of 16HBE or grown alone on transwells for monoculture controls. An equal amount of media was added to the apical side of 16HBE monocultures. Mac were selected by adherence for two hours. Apical media containing non-adherent cells was removed and cultures were incubated 18 hours at ALI before exposure (see schematic Figure 5.1).

In vitro ozone (O_3) exposure. Cell cultures at ALI were exposed to filtered air or 0.4 ppm O_3 for 4 hours in exposure chambers (80% relative humidity, 5% CO_2) operated by the U.S. Environmental Protection Agency. The dose used here was selected for maximal innate immune response to O_3 with relatively minimal cytotoxicity and has been used previously by our group in several other reports (22, 26). Samples were collected 1 and 24 hours after exposure.

Flow cytometry. Cells harvested with Accutase (Millipore, Billerica, MA) and washed with staining buffer [PBS (Life Technologies), 1% heat-inactivated FBS, and 0.09% Sodium azide (Sigma, St. Louis, MO, USA)]. Cells were incubated with antibodies for CD45, CD3, CD206 (BD Biosciences, San Jose, CA), and CD14 (Beckman Coulter, Indianapolis, IN) for 20 minutes at room temperature in the dark and fixed with 0.5% paraformaldehyde (PFA; Sigma). Samples were stored at 4°C in the dark and acquired within 24 hours. For cytotoxicity analysis, the cells were incubated with antibodies for CD45, washed and then incubated with propidium iodide (PI; ImmunoChemistry Technologies, Bloomington, MN). Samples were analyzed using a BD Biosciences LSRII flow cytometer. Isotype-matched controls and unstained cells were used to control for nonspecific staining and set analysis gates. Mac were identified by positive CD45 staining and forward-scatter/side scatter properties.

Phagocytosis assay. Opsonized pHrodo Green *Staphylococcus aureus (S. aureus)* BioParticles® (Life Technologies) were incubated with co-cultures and Mac monocultures on the apical side for 1 hour at 37°C. Cells were harvested, stained with antibodies for CD45, fixed in 0.5% PFA, and analyzed by flow cytometry as above.

Analysis of IL-8 and hyaluronic acid (HA). Apical washes from co-cultures, 16HBE monocultures, and Mac monocultures were analyzed for concentrations of IL-8 (BD Biosciences) and HA (R&D Systems, Minneapolis, MN) using a commercially available assays.

Immunofluorescence. For visualization of the 16HBE-Mac co-culture, BAL cells were labeled with a fluorescein-emitting dye (Vybrant Multicolor Cell-Labeling Kit; Life Technologies) before composition of the co-culture as described above. The co-cultures were fixed with 4% PFA and stained for F-actin cytoskeleton with phalloidin-rhodamine (Sigma). Transwells were mounted with Vectashield mounting media containing DAPI (Vector

Laboratories Burlingame, CA). For cleaved caspase-3 immunofluorescence, co-cultures, 16HBE monocultures, and AM monocultures on transwells were fixed in 4% PFA and probed with anti-rabbit cleaved-caspase-3 (Cell Signaling Technology, Beverly, Massachusetts), anti-mouse-HLA-DR (eBioscience) and appropriate secondary antibodies (Life Technologies). Transwells were mounted with DAPI as above. Images were captured using a Nikon C1Si laser-scanning confocal microscope, and processed using EZ-C1 FreeViewer software (Nikon Instruments, Melville, NY).

Conditioned media experiments. 1 x 10^5 BAL cells were plated on 24-well tissue culture plates, and Macs were selected for by adherence for 2 hours as above. Apical washes from 16HBE mono-cultures exposed to 0.4ppm O_3 or Air for 4 hours were collected at 1 or 24 hours post-exposure in 100ul RPMI media (Life Technologies) as "conditioned media." Mac were incubated with 100ul of the conditioned media or RPMI media control for 4 hours and assessed for phagocytosis, CD14 and CD206 expression, and PI staining by flow cytometry as described above.

Statistical analysis. To assess effects of co-culturing or O_3 on surface receptor expression, phagocytosis, PI staining, and soluble mediator levels, a non-parametric, paired Wilcoxin signed rank test was used. The effect of culture type on O_3-induced PI staining was assessed by non-parametric one way analysis of variance (Kruskall Wallis Test). Graphs show mean ± standard error of the mean (SEM). Significance was set at a $p < 0.05$.

5.3 Results

Characterization of the 16HBE-Mac co-culture model

To examine the interaction between Mac and airway epithelial cells, we developed a co-culture model using primary pulmonary Mac and the 16HBE14o- (16HBE) cell line. Bronchoalveolar lavage (BAL) cells from healthy volunteers, which are comprised of over 80% Mac, 10% lymphocytes (largely T lymphocytes), and small populations of neutrophils, basophils, and eosinophils (27), were added to the apical side of 16HBE, and Mac were selected by adherence (Figure 5.1). Immunofluorescence analysis of the co-culture indicated that the Mac became embedded within the 16HBE monolayer, suggesting close cell-cell interaction between Mac and 16HBE (Figure 5.2 A-B). Flow cytometric analysis of the co-cultures using CD45 and forward scatter/side scatter properties to distinguish Mac from 16HBE revealed that on average 6.9% of all cells in the co-culture were Mac (Figure 5.2 C). A small population of CD3+ T lymphocytes (on average 0.67% of all cells) was also identified in the co-culture. Previous studies have found that activated T lymphocytes can adhere to plastics and epithelial cells (27, 28). For the purposes of this study, we did not further characterize the T lymphocytes, but rather focused our analysis on the effects of co-culture with 16HBE on Mac phenotype and response to O_3.

Co-culture of Mac with 16HBE alters Mac immunophenotype and reduces phagocytic activity

We investigated the effect of co-culturing with 16HBE on Mac immunophenotype and phagocytic activity by flow cytometry. We assessed expression of CD14, a pattern recognition receptor commonly used to identify monocytes and macrophages and previously implicated in the response to O_3 (29), and CD206, the macrophage mannose receptor, which is a maker of alternatively active macrophages (17). We found that Mac in co-culture had enhanced expression

of CD14 and CD206 after both 24 and 48 hours of co-culturing compared to Mac in monoculture (Figure 5.3 A-B, Air exposed). We measured Mac phagocytosis of opsonized *S. aureus* bioparticles and found that co-cultured Mac had reduced phagocytic activity compared to Mac in monoculture (Figure 5.3 C, Air exposed). Notably, the effects of co-culture on Mac phenotype and phagocytic activity were not due to soluble mediators, as exposure of Mac mono-cultures to conditioned media from 16HBE did not alter surface marker expression or phagocytic activity (Figure 5.7, Air exposures). Together, these results suggest that signals derived from 16HBE alter Mac immunophenotype and phagocytic function.

Co-culture of Mac with 16HBE modifies Mac response to O_3

We next assessed how co-culture of Mac with 16HBE modifies Mac immunophenotype and phagocytic activity in the context of O_3 exposure. Expression of CD14 and C206 by Mac was analyzed by flow cytometry 1 and 24 hours after exposure to Air or O_3. Flow cytometric analysis showed that exposure to O_3 significantly enhanced surface expression of CD14 by Mac in monoculture only at 24 hours post-exposure, and that there was similar, albeit non-significant trend for O_3-induced CD14 by Mac in co-culture (Figure 5.3 A). Conversely, O_3 significantly enhanced surface expression of CD206 both at 1 and 24 hours only by Mac in co-culture and had no effect on CD206 expression by Mac in monoculture (Figure 5.3 B).

Previous studies have shown that O_3 exposure reduces Mac phagocytosis, which may contribute to enhanced susceptibility to infections following O_3 exposure (25, 30, 31). Similarly, we found that exposure to O_3 reduced phagocytosis of opsonized *S. Aureus* bioparticles by Mac both in co-culture and in monoculture at 1 and 24 hours post-exposure (Figure 5.4 A). Notably, at 1 hour post-exposure, the reduction in phagocytosis was greater in co-cultured Mac compared to Mac in monoculture (Figure 5.4 B). The effects of co-culture on Mac phenotype and

phagocytic activity following exposure to O_3 were not due to soluble mediators, as exposure of Mac mono-cultures to conditioned media from O_3-exposed 16HBE did not alter surface marker expression or phagocytic activity (Figure 5.7 A-C). Taken together, these results indicate that co-culture modifies Mac immunophenotype and phagocytosis in response to O_3.

Co-culture modifies O_3-induced cytotoxicity of Mac, but not 16HBE

Exposure to O_3 has been previously shown to damage airway epithelial cells, leading to cell death and the release of danger signals that can activate local immune cells, such as Mac (2, 16, 21). Similarly, Mac from O_3-exposed rats had increased markers of cytotoxicity (32). We investigated how co-culture of Mac with 16HBE modified O_3-induced cytotoxicity by propidium iodide (PI) staining, a marker of cell lysis. We found that O_3 significantly enhanced the percentage of PI+ Mac at 1 hour after exposure in Mac both in monoculture and co-culture, but that Mac in co-culture had significantly increased cytotoxicity (Figure 5.5 A). Conversely, neither 16HBE in co-culture nor monoculture had significant increases in PI+ staining following O_3 exposure (Figure 5.5 B). Notably, O_3 did not enhance cleaved caspase-3, in any of the cultures, suggesting that O_3 exposure did not induce caspase-3 dependent apoptosis (Figure 5.6). Similar to above, the effects of co-culture on Mac cytotoxicity were not mediated by soluble mediators, as exposure of Mac mono-cultures to conditioned media from 16HBE did not alter Mac cytotoxicity (Figure 5.7 D).Together our results show that Mac, but not 16HBE, are susceptible to O_3-induced cytotoxicity, which is enhanced by co-culturing Mac with 16HBE.

Co-culture of Mac with 16HBE has no effect on O_3-induced IL-8 production

The chemokine, interleukin (IL)-8, is secreted by both Mac and airway epithelial cells and plays a major role in the recruitment of neutrophils to the site of O_3-induced airway damage

(1). In chapter 4, we assessed levels of IL-8 in bronchoalveolar lavage (BAL) fluid from healthy volunteers collected at 1 and 24 hours after *in vivo* exposure to 0.3ppm O_3 or air for 2 hours, and found significantly enhanced IL-8 at both 1 and 24 hours after exposure (Figure 4.5). Here, we assessed how co-culture of Mac with 16HBE alters O_3-induced IL-8 production. Our results indicate that O_3 significantly enhanced IL-8 production by Mac in monoculture at 1 hour but not 24 hours post-exposure, and that there was a similar, albeit non-significant trend for enhanced IL-8 production in 16HBE-Mac co-cultures and 16HBE monocultures (Figure 5.8A). These results suggest that co-culture of 16HBE and Mac does not modify O_3-induced IL-8 production.

O_3 modifies Mac regulation of hyaluronic acid in co-cultures

Elevated levels of the extracellular matrix protein hyaluronic acid (HA) in the lung contribute to airway hyperresponiveness and pro-inflammatory cytokine production following acute O_3 exposure (7, 12). We assessed how O_3 alters levels of HA in the Mac monocultures, 16HBE monocultures, and 16HBE-Mac co-cultures. Our results indicate that in air-exposed cultures, 16HBE constitutively produce large amounts of HA, whereas HA was largely non-detectable in Mac monocultures (Figure 5.8B). Interestingly, when Mac were co-cultured with 16HBE, the concentration of HA in the cultures was starkly reduced, suggesting that Mac regulate the availability of HA in the co-cultures. Exposure to O_3 did not significantly alter production of HA by 16HBE in monoculture at 1 or 24 hours post-exposure; however, we measured increased concentrations of HA in the 16HBE-Mac co-cultures at 24 hours after exposure. These results were similar to our findings in chapter 4, where we assessed HA levels in BAL fluid from healthy volunteers collected at 1 and 24 hours after *in vivo* exposure to 0.3ppm O_3 or air for 2 hours and found HA levels were increased in the BAL fluid only at 24 hours after

O_3 exposure, albeit not significantly (p=0.054, Figure 4.3A). These results demonstrate that the 16HBE-Mac co-culture model closely resembles the *in vivo* response to O_3 in the airway, and demonstrate that Mac regulation of epithelial cell derived-HA is altered by exposure to O_3.

5.4 Discussion

The adverse health effects of inhaled O_3 are orchestrated by multiple cell types in the lung, including structural cells, such as airway epithelial cells, and resident immune cells, such as pulmonary macrophages (Mac). Most studies that have investigated the innate immune response to O_3 focused on an individual cell type, which does not address the important interaction between epithelial cells and Mac in the innate immune response to O_3. We developed a human bronchial epithelial cell (16HBE)-Mac co-culture model to investigate how the interaction between airway epithelial cells and Mac contributes to the innate immune response observed in humans exposed to O_3. Our results suggest that epithelial cells and Mac exist in a homeostatic state, whereby Mac maintain a quiescent phenotype, as indicated by reduced phagocytic activity and enhanced CD206 expression. O_3 exposure disrupts this homeostatic relationship, leading to enhanced cytotoxicity in Mac and further reduced phagocytosis. Production of the danger signal hyaluronic acid (HA) by epithelial cells alone was not affected by O_3 exposure, but availability of HA both in the *in vitro* co-culture and in bronchoalveolar lavage (BAL) fluid from volunteers exposed *in vivo* was increased with O_3 exposure, indicating that effects of O_3 on Mac phagocytosis potentially manifests in impaired regulation of HA. Taken together, these results demonstrate that signals from epithelial cells influence Mac immunophenotype and that O_3 exposure modifies epithelial cell-Mac homeostasis, leading to O_3-induced changes in innate immune responses that mimic the effects of O_3 exposure observed *in vivo*.

Epithelial cells and Mac compose the majority of resident innate immune cells in the lung, suggesting that they initiate and regulate the initial response to injurious stimuli such as O_3 in tandem. Our results suggest that signals derived from epithelial cells modify Mac response to O_3, resulting in higher cytotoxicity and lower phagocytosis, similar to what has been observed *in*

vivo (33, 34). When co-cultured with 16HBE, Mac had enhanced expression of CD206, increased cytotoxicity, and reduced phagocytic activity following O_3 exposure compared to Mac in monoculture (Figures 5.3-5). However, there was no effect of co-culture on patterns of O_3-induced production of the chemokine IL-8 (Figure 5.8 A). Our result suggest that *in vitro* study of Mac response to O_3 in isolation may mask the important interaction with other cell types, such as airway epithelial cells, that influence the inflammatory response to O_3.

Results shown here suggest that signals from 16HBE promote an alternative Mac phenotype in response to O_3, as indicated by reduced phagocytosis and increased expression of CD206. CD206 (macrophage mannose receptor) is a C-type lectin binding protein that is commonly associated with alternatively activated macrophages (35). A previous study in rats identified both classical/pro-inflammatory Mac and alternative/anti-inflammatory Mac in the lung within 24 hours after exposure to O_3 (36). Interestingly, suppression of classically activated Mac activity using gadolinium chloride has been shown to protect against O_3-induced lung injury (18). While the alternatively activated state of Mac is necessary to resolve airway damage and control inflammation, it may also contribute to the enhanced susceptibility to and severity of respiratory infections that is associated with *in vivo* exposure to oxidant pollutants such as O_3 (34, 37-39). Previous studies have shown that *in vivo* O_3 exposure of healthy human volunteers is associated with reduced phagocytic activity of Mac and several murine studies have shown that reduced Mac phagocytosis following O_3 exposure is associated with enhanced susceptibility to bacterial infections, including *Klebsiella pneumonia* and *Streptococcus zooepidemicus* (30, 40, 41). Additional studies have suggested that reduction in phagocytosis may be attributed to oxidation of surfactant protein (SP)-A, an epithelial cell-derived component of the lung lining fluid and known inducer of Mac phagocytosis (30). Our results indicate that Mac in co-culture

had further reduced phagocytosis at 1 hour after exposure to O_3 compared to Mac in mono-culture (Figure 5.4). Thus, O_3-induced oxidation of 16HBE-derived mediators or epitopes may further impair phagocytosis by Mac, contributing to increased susceptibility to respiratory infections following O_3 exposure.

In the absence of secondary infections, exposure to O_3 causes sterile inflammation, suggested to be mediated by the release of damage-associated signals. The airway damage caused by O_3 exposure generates inflammatory danger signals in the airway, such as short fragments of hyaluronic acid (HA) (7, 11). Elevated levels of HA have been observed in sputum from allergic asthmatic volunteers exposed to O_3 and murine studies have shown that accumulation of HA in the airway following exposure to O_3 is associated with airway hyperresponsiveness and inflammation (7, 10, 11). HA is a nonsulfonated glycosaminoglycan that is produced by airway epithelial cells and submucosal glands as long strands (high molecular weight) that serve anti-inflammatory functions, but with tissue injury short HA fragments (low molecular weight) accumulate and induce inflammation (9, 42). Clearance of degraded extracellular matrix components, such as HA fragments, is necessary to limit inflammation and resolve tissue injury and occurs via CD44-mediated uptake by cells such as Mac, suggesting that the interaction between Mac and epithelial cells regulate HA levels (9, 43). Our results indicate that 16HBE constitutively produce HA, and that co-culture with Mac led to a stark reduction in HA availability (Figure 5.8B). Surprisingly, we did not observe an effect of O_3 on HA production by 16HBE; however, Mac-dependent regulation of 16HBE-derived HA was impaired by O_3 exposure, thus leading to accumulation of HA in the cultures at 24 hours post-exposure. These data were confirmed in BAL fluid from healthy volunteers exposed *in vivo*, demonstrating elevated HA levels 24 hours after exposure to O_3 (albeit not statistically significantly, $p=0.054$,

Figure 4.3 A). Considering the impaired phagocytosis of Mac co-cultured with epithelial cells, we hypothesize that increased availability of HA in the airway after O_3 exposure observed *in vivo* is not due to enhanced production by epithelial cells, but rather caused by a loss in Mac-dependent clearance. Future assessment of Mac CD44 expression is necessary to determine if CD44-mediated uptake of HA by Mac is also impaired by O_3 exposure.

Previous murine studies have found that O_3-induced airway hyperresponsiveness is dependent on low molecular weight HA, whereas high molecular weight HA protects against O_3-induced airway hyperresponsivenss (7). Additionally, oxidative stress has been directly shown to cause fragmentation of HA produced by airway epithelial cells (44). Due to limited sample volumes, we were unable to assess HA size. Future studies will be necessary to determine how O_3 and co-culture affects 16HBE-derived HA size *in vitro* and if low molecular weight HA accumulates in the airway after *in vivo* O_3 exposure in humans.

The immune response in the airways represents a complex interaction between structural, resident, and circulating cells to clear the injurious stimuli and resolve the resulting injury. *In vitro* co-culture models present a controlled system to investigate the role of cell-cell interaction during immune cell activation. Compared to other macrophages in the body, pulmonary Mac have a unique phenotype that is determined by the tissue-microenvironment of the lung (19, 45). Mac are constantly assailed with inhaled stimuli, and must maintain a quiescent state with relatively low phagocytic activity and cytokine production in order to prevent unnecessary inflammation in response to innocuous stimuli (46). The adherence of Mac to airway epithelial cells suggests that Mac are bathed in epithelial cell-derived mediators, including cytokines, growth factors, and surfactants, and interact with epithelial cell receptors and adhesion receptors that may influence Mac phenotype (19, 47). In support of this hypothesis, we demonstrate that

Mac cultured with 16HBE had enhanced expression of the surface receptors CD14 and CD206 and reduced phagocytic activity compared to Mac in monoculture (Figure 5.3). The enhanced expression of CD206, a marker of alternatively active macrophages, and low phagocytic function suggests that Mac interaction with epithelial cells promotes a quiescent state at baseline conditions similar to that which has been previously observed of alveolar macrophages (46).

Interestingly, treatment of Mac with conditioned media from air or O_3 exposed 16HBE had no effect on Mac immunophenotype, phagocytic activity, or viability, suggesting that the effects of co-culture on Mac baseline phenotype or response to O_3 were not due to soluble mediators derived from 16HBE (Figure 5.7). Though it is possible that the conditioned media was too dilute, our data suggest that direct interaction between 16HBE and Mac was necessary to affect Mac response to O_3. These results are in line with previous airway epithelial cell-Mac co-culture models that found contact-dependent effects were necessary to potentiate inflammatory responses to particles or particulate matter (48, 49). These effects could be due to signaling through integrins, which mediate cell-cell and cell-extracellular matrix interactions; yet, blocking I-CAM-1 or β1/β2/β3-integrins in previous studies did not inhibit the effects of epithelial cell-Mac co-culture on response to particles (48, 49). Furthermore, we have previously shown that co-culture of natural killer (NK) cells with O_3-exposed nasal epithelial cells modifies NK cell activity, and that these effects were mediated by direct cell-cell interaction and the expression of the NK cell receptor ligands ULBP3 and MICA/B by nasal epithelial cells (26). Future studies will be necessary to determine the specific receptor-ligand interactions that mediate the effects of co-culture on Mac immunophenotype and response to O_3.

In conclusion, we show here a co-culture model to study the interaction between airway epithelial cells and Mac in the response to O_3 that replicates *in vivo* findings. Our results indicate

that signals from 16HBE influence Mac phenotype and that the interaction between 16HBE and Mac is a determinate of the innate immune response to O_3. As previous studies have shown that mediators from O_3-exposed Mac enhance alveolar epithelial cell chemokine production (50), future studies are necessary to focus on the effect of Mac on epithelial cell phenotype and response to O_3.Our results add to the literature suggesting that monoculture systems do not fully reconstitute the biological response to pollutants such as O_3, and may mask the complex interaction between cell types that is required for these responses (26, 48-50). Furthermore, these results suggest that epithelial cells not only serve as structural barriers against the inhaled environment, but also function as orchestrators of immunity that can significantly influence immune cell phenotype and activity. Improved understanding of the interaction between airway epithelial cells and immune cells such as Mac may reveal new therapeutic targets to modify immune cell function in the lung.

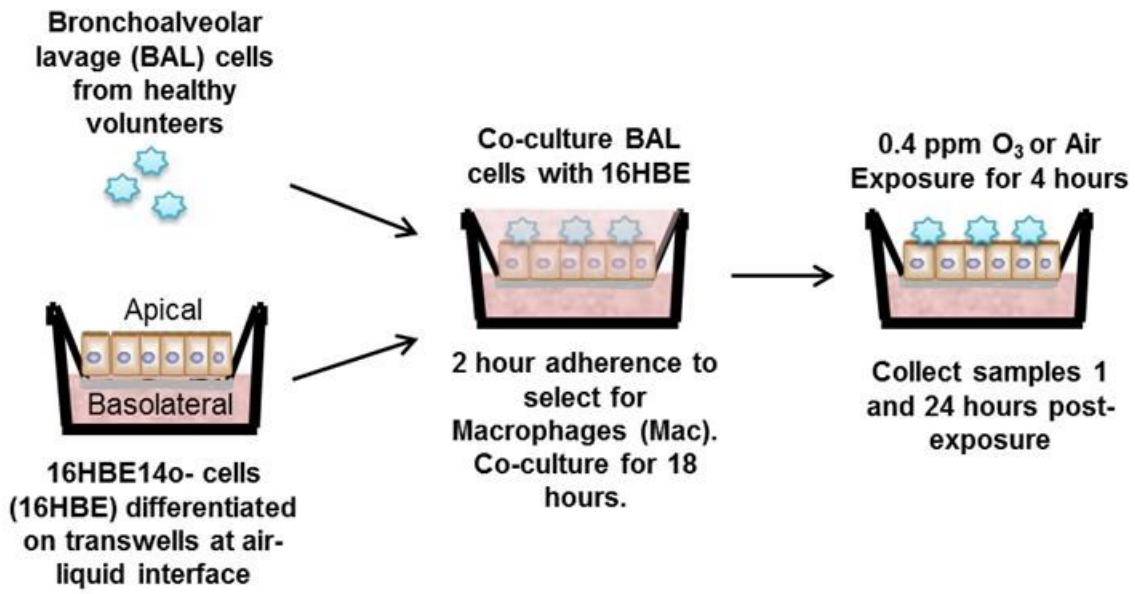

Figure 5.1. Schematic of the human bronchial epithelial cell (16HBE) and airway macrophage (Mac) co-culture model. 16HBE14o- cells (16HBE) were grown to confluency on transwell membrane supports and differentiated at air-liquid interface for 24 hours before co-culture. Bronchoalveolar lavage (BAL) cells from healthy volunteers were added to the apical side of 16HBE and Mac were selected by adherence for 2 hours. 16HBE and Mac were co-cultured for 18 hours before exposure to 0.4ppm O_3 or air for 4 hours. Samples were collected 1 and 24 hours after exposure.

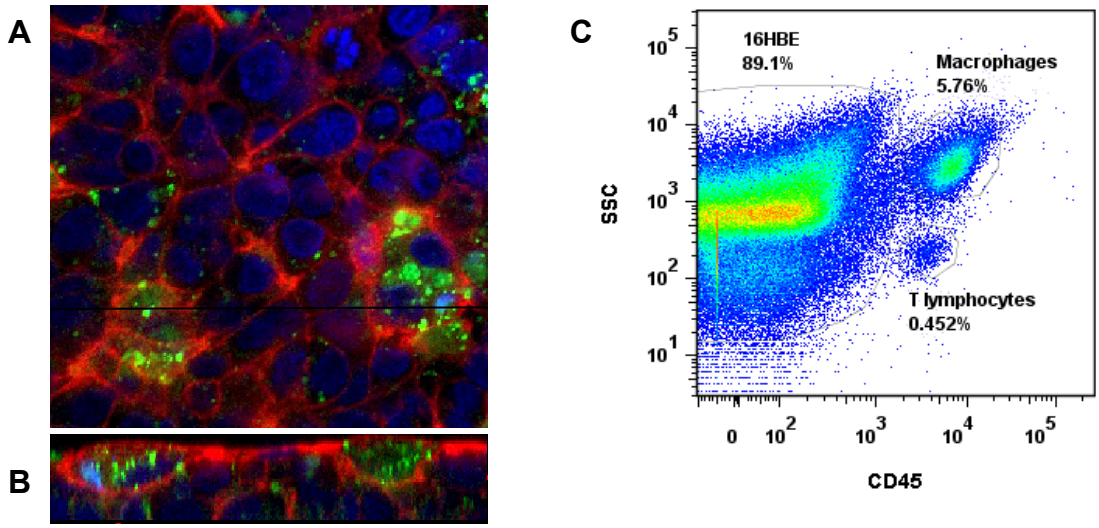

Figure 5.2. Characterization of the human bronchial epithelial cell (16HBE) and airway macrophage (Mac) co-culture model. A-B) Bronchoavleolar lavage cells (green) were stained with a fluorescein-emitting dye before addition to the apical side of the 16HBE. Mac were selected by adherence for 2 hours. The entire co-culture was stained with phalloidin-rhodamine for the F-actin cytoskeleton (red) of the 16HBE and Mac. Cell nuclei (blue) were stained with DAPI. Images were acquired by laser-scanning microscopy. **A)** En face view of the co-culture shows the presence of Mac on the apical side of the 16HBE monolayer. **B)** Cross-sectional view of the co-culture shows the Mac are embedded within the 16HBE monolayer. **C)** Flow cytometric analysis of the co-culture shows a large population of $CD45^+$, SSC^{high} Mac adhere to the 16HBE, as well as a small population of T lymphocytes $CD45^+$, SSC^{low}.

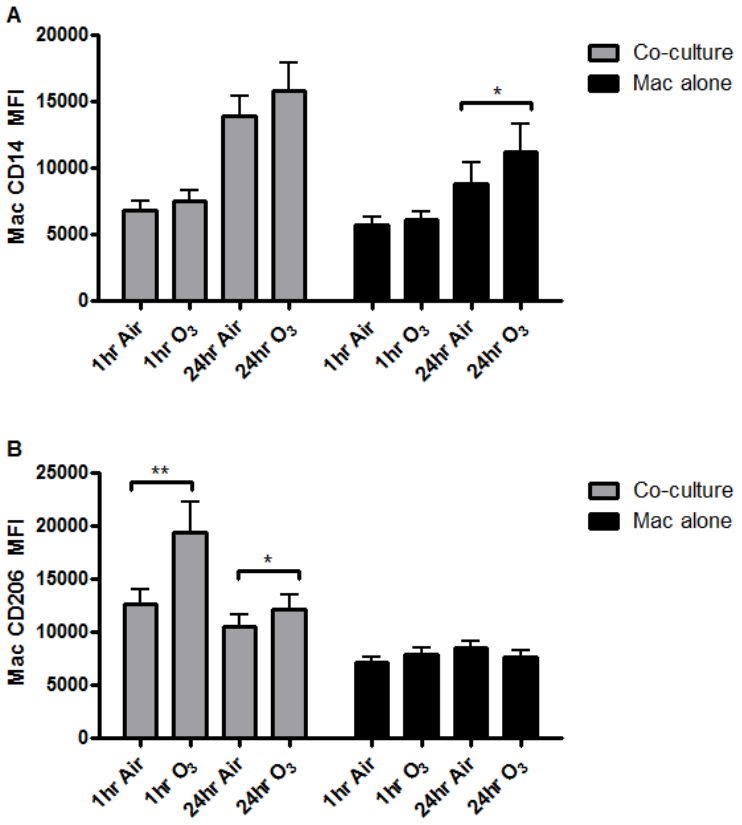

Figure 5.3. Co-culture of Mac with 16HBE modifies Mac immunophenotype in response to O_3 exposure. Surface expression of **A)** CD14 and **B)** CD206 by Mac co-cultured with 16HBE (co-culture) or grown on transwells alone (mac alone) was measured by flow cytometry at 1 and 24 hours after exposure to 0.4ppm O_3 or air for 4 hours. Mac were distinguished from 16HBE by positive CD45 staining. Data shown as mean fluorescent intensity (MFI). n=7-9. P-value determined by Wilcoxin Signed Rank Test. *$p<0.05$, **$p<0.01$.

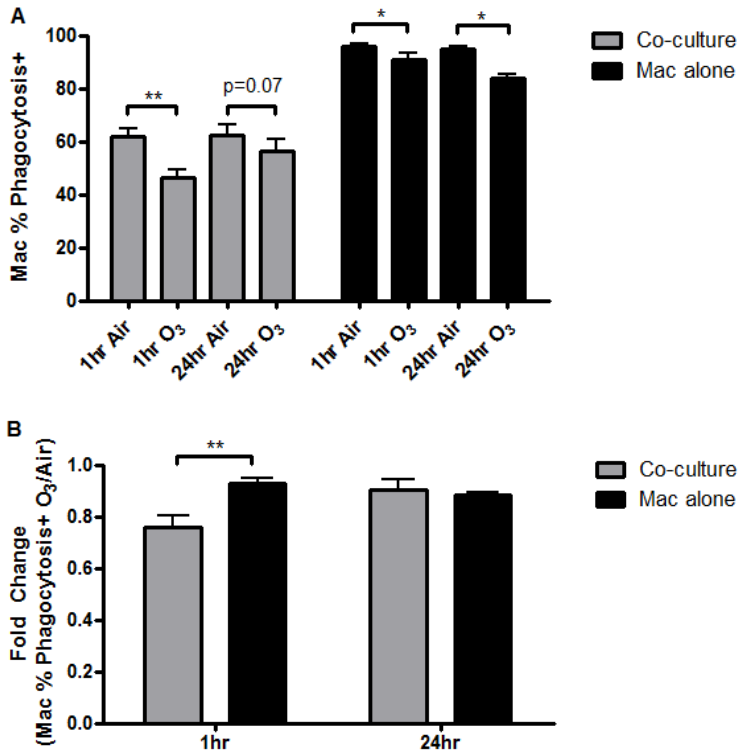

Figure 5.4. Exposure to O_3 reduces Mac phagocytosis of *S. Aureus* bioparticles. 1 or 24 hours after exposure to 0.4ppm O_3 or air for 4 hours, Mac-16HBE co-cultures or Mac monocultures were incubated with fluorescent *S. Aureus* bioparticles for 1 hour and assessed for phagocytosis by flow cytometry. Macs were distinguished from 16HBE by CD45+ staining. Data expressed as **A)** % phagocytosis positive Macs or **B)** fold change (% phagocytosis positive Macs Air/O_3). n=6-8. P-value determined by Wilcoxin Signed Rank Test. *$p<0.05$, **$p<0.01$.

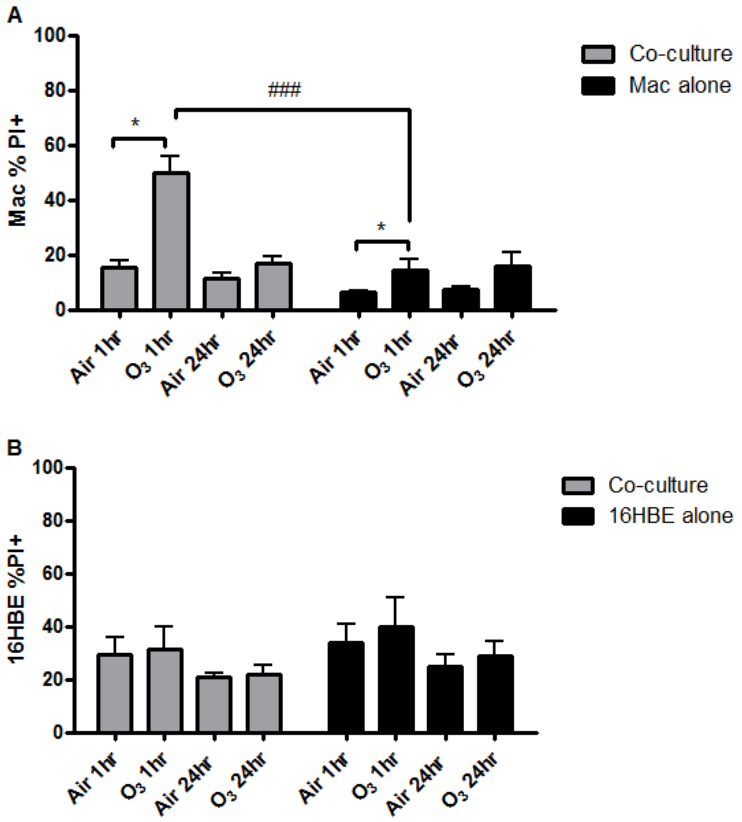

Figure 5.5. Mac in co-culture have enhanced cytotoxicity following exposure to O_3. Cytotoxicity in Mac-16HBE co-cultures or Mac monocultures at 1 or 24 hours after exposure to 0.4ppm O_3 or air for 4 hours was measured by propidium iodide (PI) staining. Macs were distinguished from 16HBE by CD45+ staining. Data expressed as **A)** % PI positive Macs or **B)** % PI positive 16HBE. n=6-8. P-value determined by Wilcoxin Signed Rank Test (*$p<0.05$, **$p<0.01$) or non-parametric one way analysis of variance (Kruskall Wallis Test, ###$p<0.001$).

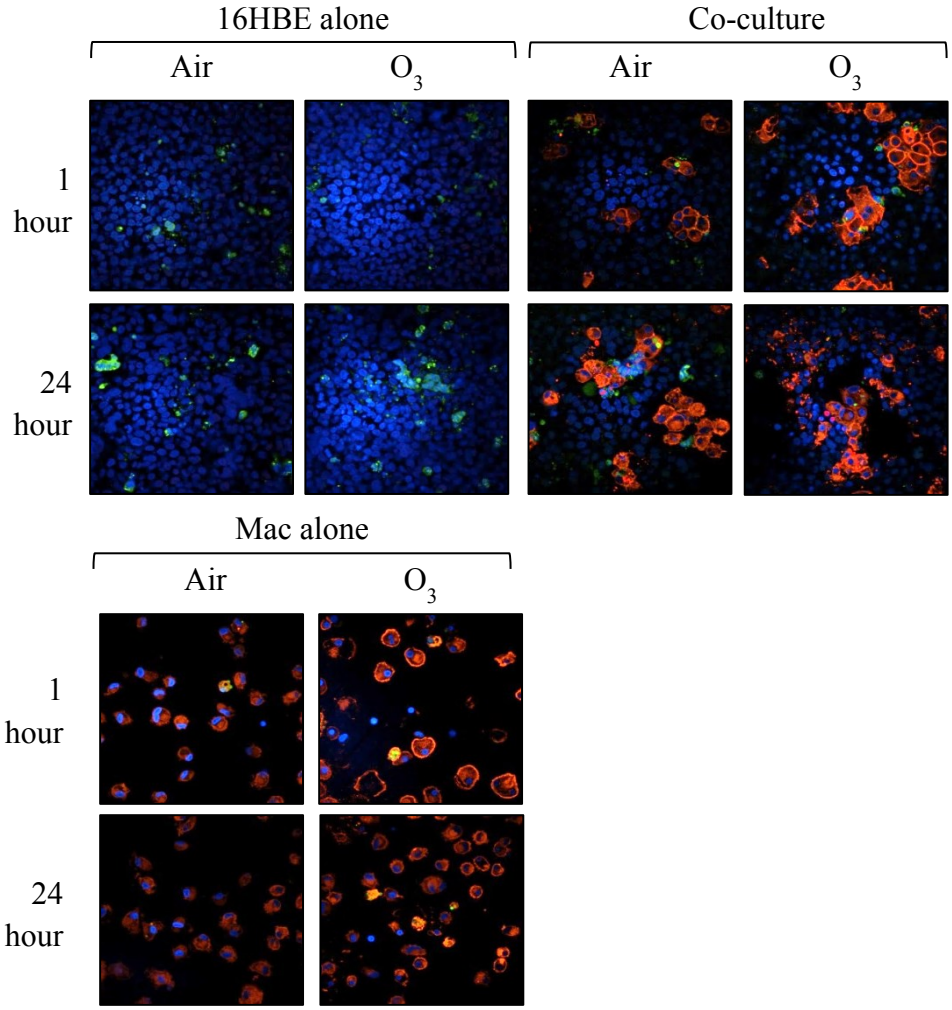

Figure 5.6. No effect of O_3 or co-culture on cleaved caspase-3. 16HBE monoculture, Mac monoculture, and 16HBE-Mac co-culture were fixed 1 or 24 hours after exposure to 0.4ppm O_3 or Air and probed for cleaved caspase-3 (green), HLA-DR (Mac, red), and DAPI (nuclei, blue) with appropriate fluorescent antibodies. Images were acquired by laser-scanning microscopy.

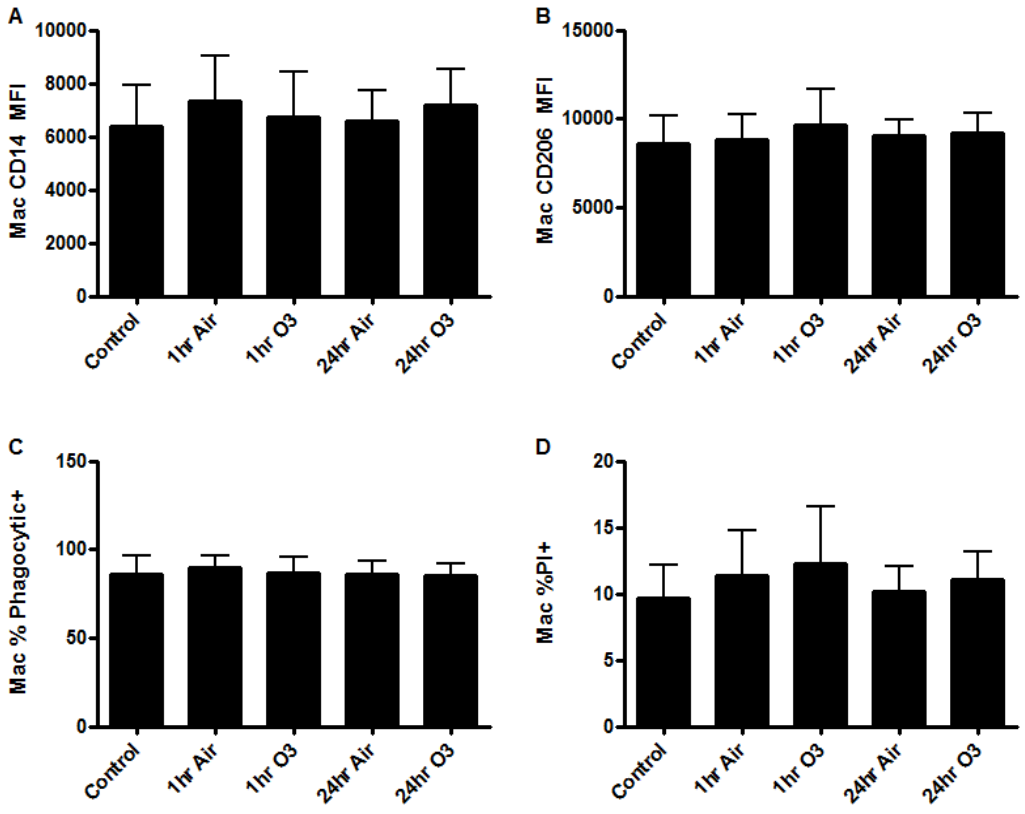

Figure 5.7. Exposure of Mac to conditioned media from 16HBE exposed to Air or O_3 does not mimic the effects of co-culturing. Apical washes from 16HBE exposed to 0.4ppm O_3 or air for 4 hours was collected in RPMI media at 1 or 24 hours after exposure as conditioned media. Mac were incubated with conditioned media or RPMI media control for 4 hours and then assessed for surface expression of **(A)** CD14 and **(B)** CD206, **(C)** phagocytosis of *S. aureus* bioparticles (expressed as %phagocytic+), and **(D)** viability using propidium iodide staining (expressed as %PI+) by flow cytomtery.

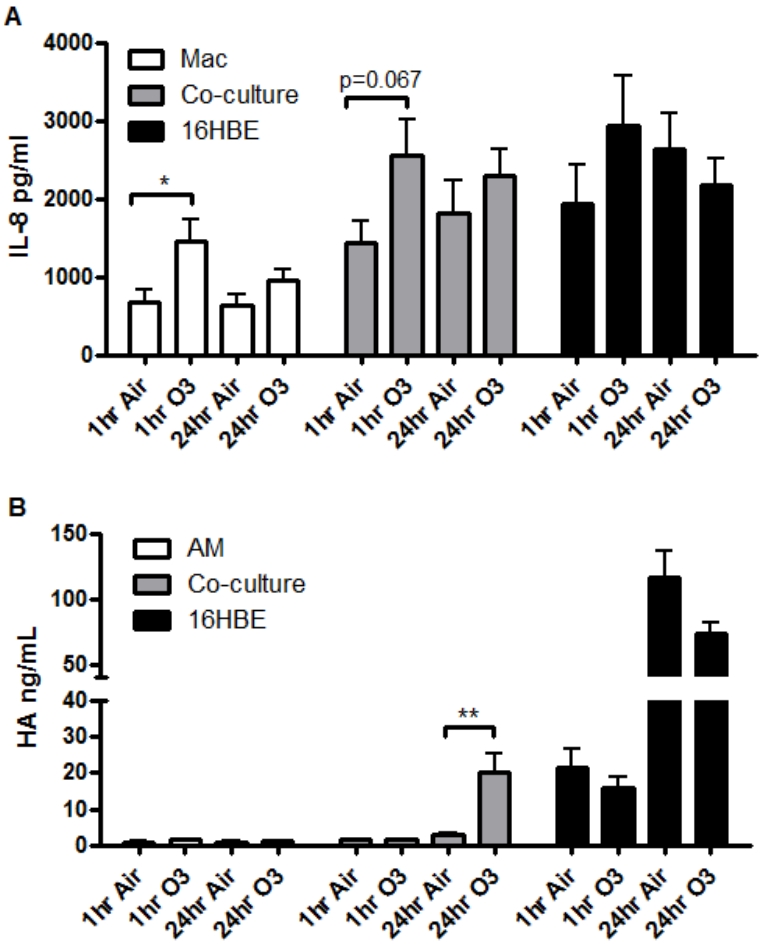

Figure 5.8. Effect of co-culture on O_3-induced IL-8 and hyaluronic acid (HA) production. Concentrations of **A)** IL-8 and **B)** HA were measured in the apical washes from Mac monocultures, 16HBE monocultures, and 16HBE-Mac co-cultures collected 1 and 24 hours after exposure to 0.4ppm O_3 or air for 4 hours. n=7-11. P-value determined by Wilcoxin Signed Rank Test (*$p<0.05$).

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Chapter 6: Discussion

> *"…innate immunity has usually been treated as a minor curiosity, shunted to the beginning or the end of treatises on immunology, and given short shrift in study sections bound and determined to spare no effort first to solve the antibody problem and later the T-cell receptor and MHC-restricted antigen recognition receptor" – Charles Janeway, 1989, from Approaching the Asymptote? Evolution and Revolution in Immunology. Cold Spring Harb Symp Quant Biol. 54:1–13.*

In 1989, Charles Janeway coined the term "pattern recognition receptor" (PRR) in a seminal essay for the Cold Spring Harbor Symposia on Quantitative Biology and predicted that sensing of microbes by these innate immune receptors was the critical link between innate and adaptive immunity (1). Until this time, innate immunity was considered a primitive component of the immune system intended only to initiate more refined adaptive immune responses (2). The discovery of the surface receptor Toll in *Drosophila melanogaster* in the early 1990s facilitated the identification of a number of Toll-like receptors (TLRs) and other innate immune receptors in humans and demonstrated that innate immunity is a highly evolved and necessary mechanism for host defense (3).

The innate immune system serves as the first line of defense against the inhaled environment in the lung, and is composed of a network of both structural cells, such as airway epithelial cells, and immune cells, such as macrophages (4). Interaction between these cell types determines the appropriate inflammatory response to inhaled stimuli, and informs adaptive immune cells of specific pathogens (5). The immune response requires a tight balance between inflammatory events that clear the injury and anti-inflammatory responses that mediate healing.

Exaggerated or prolonged immune responses can lead to chronic inflammation of the airways and the development of diseases such as asthma (6).

Innate immunity is initiated by recognition of microbial pathogen-associated molecular patterns (PAMPs) or endogenously derived damage-associated molecular patterns (DAMPs) from damaged tissue by PRRs (5). Recently, several PRRs, including a number of Nod-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-I), and absent in melanoma 2 (AIM2), have received increased attention for their involvement in an intracellular signaling complex termed the inflammasome (7). The inflammasome is composed of a PRR, the protease caspase-1, and, in some cases, the PYD and CARD domain containing (PYCARD) adaptor protein. Formation of the inflammasome complex induces activation of caspase-1, which can then mediate the processing and release of IL-1β and IL-18. Caspase-1 also performs a number of other functions, including initiation of a pro-inflammatory form of programmed cell death termed pyroptosis, signaling through NF-kB, and cleavage of proteins involved in glycolysis, cell survival, and cytoskeleton function (8). The NLRP3 inflammasome has received special attention for its involvement in the response to a diverse range of pathogens and environmental pollutants, suggesting that the NLRP3 inflammasome may be a general sensor of cellular stress (9, 10).

Asthma is a chronic inflammatory disease of the conducting airways that is associated with airway obstruction and variable airway hyperresponsiveness in response to a challenge, often an allergen, virus, or environmental pollutant (11). Though expansion of type 2 helper (Th2) T lymphocytes and IgE produced by B lymphocytes of the adaptive immune system are considered central mediators of asthma pathogenesis, innate immunity is essential for the initiation and propagation of adaptive immune responses and may be of particular importance

during exacerbation by stimuli such as viruses or pollutants (6). Stimulation of PRRs by an viruses, allergens, pollutants, etc. activates cytokine and chemokine production by innate immune cells and the expression of co-stimulatory molecules by dendritic cells (DC) that drive T lymphocyte differentiation (13). Additionally, inflammatory mediators produced in response to PRR activation can exacerbate asthma symptoms (14).

As NLRP3 is an innate immune receptor broadly implicated in the response to cellular stress, several murine studies have investigated the contribution of NLRP3 to the development of allergic airway inflammation and have found conflicting results (15-18). Despite involvement of inflammasome signaling in the response to a number of stimuli that exacerbate asthma symptoms, a role for the inflammasome in asthma exacerbation has not been investigated. In this dissertation, we sought to determine the contribution of the inflammasome to asthma exacerbation caused by respiratory viral infection or inhalation of the environmental air pollutants and elucidate the function of the inflammasome in the development of allergic asthma. We focused our analysis on caspase-1, which is common component of all inflammasome complexes. Our results yield insight on the innate immune mechanisms involved in the response to viruses and pollutants and how these pathways may contribute to asthma pathogenesis.

6.1 The inflammasome in the airway epithelial cell response to influenza virus

In chapter 2, we investigated inflammasome signaling in response to influenza A virus (IAV) and how these pathways are altered by asthma. Respiratory viral infection is the leading source of asthma exacerbation, and IAV-induced exacerbation is of particular concern during seasonal epidemics (11). Previous studies in mice have shown that NLRP3 and caspase-1 are activated by IAV and protect against IAV-related mortality in mice (19, 20). In bone marrow-derived macrophages, the NLRP3 inflammasome was shown to be activated by changes in

intracellular ion concentration induced by the influenza M2 channel during infection (21). Since airway epithelial cells are the main target of IAV infection and have not been well characterized for inflammasome signaling, we focused our analysis on describing the function of the inflammasome in human bronchial epithelial cells (HBEC) in the response to IAV and how these pathways may be altered by asthma (22). Our results suggest that caspase-1 plays an important role in the airway epithelial cell innate immune response to IAV infection, which may be independent of IL-1β production and is enhanced in epithelial cells from asthmatics.

Though our result suggest that caspase-1 contributes to the HBEC innate immune response to IAV, the PRR that mediates inflammasome complex mediates inflammasome complex formation in HBEC is less clear. We observed co-localization of caspase-1 with the adaptor protein PYCARD during infection suggesting inflammasome complex formation (Figure 2.3), but were unable to detect gene expression of many NOD-like receptors in HBEC, including NLRP3. Since enhanced *NLRP3* gene expression is important priming step for activation of the NLRP3 inflammasome, our results suggest that NLRP3 may have a less pronounced function than caspase-1 and other PRRs in the HBEC response to IAV infection (23). Of the NLRs that we could detect by gene expression (*NLRC4, NLRC5, NLRP1, NLRP6, NLRP9, NLRX1,* and *NOD2*), only NLRC4, NLRP1, and NLRP6 are known to form inflammasome complexes (and only in response to bacteria), whereas the others may be regulators of inflammasome signaling, activate NF-kB, or preform unknown functions (24). Beyond the NLRs, we detected IAV-induced expression of two other receptors that have been shown to form complexes with caspase-1 in response to viral infections: RIG-I, a sensor of single stranded RNA previously implicated in the innate immune response to IAV, and AIM2, a double stranded DNA sensor (Figures 2.2 and 2.7). The detection of enhanced AIM2 was perplexing since IAV is an RNA

virus, but as AIM2 is an interferon-inducible gene, may be a reflection of IAV-induced interferon production (25).

Since publication of these findings, a subsequent report found that RIG-I forms inflammasome complexes with caspase-1 and PYCARD to produce IL-1β and also regulates downstream *NLRP3* gene expression in primary HBEC (26). Thus, in contrast to macrophages, RIG-I may be the pivotal IAV-induced activator of caspase-1 and IL-1β release in airway epithelial cells, and downstream activation of *NLRP3* transcription may be a secondary response to the virus (21, 26). Notably, this study used H1N1 viruses (Influenza A/Puerto Rico/8/1934 and A/USSR/90/77) and found large amounts of IL-1β production from human bronchial epithelial cells (>400 pg/mL), whereas we and a previou study (Allen *et al*.) used H3N2 viruses (Influenza A/Bangkok/1/79 and A/Victoria/3/75, respectively) and found very low levels of IL-1β production (<20 pg/mL), suggesting the capacity to produce IL-1β production may differ across IAV subtypes (Figure 2.1) (20, 26). The influenza non-structural 1 (NS1) protein has been shown to inhibit RIG-I-mediated IFNβ and IL-1β production, and previous studies have shown variation in NS1 function across IAV subtypes, suggesting that diversity in the NS1 protein may affect IAV-induced IL-1β production (26-28).

Since we were unable to detect significant IL-1β (Figure 2.1) or IL-18 (data not shown) produced by HBEC, we sought to further define a role for caspase-1 in the HBEC response to IAV infection. We infected tracheal epithelial cells from caspase-1 deficient (*Casp1*$^{-/-}$*)* and wild type (WT) mice with IAV, and found that caspase-1 deficiency was associated with diminished IAV-induced expression of several inflammatory and antiviral genes (*Ifnα, Ifnβ, Cxcl10, Rig-i,* and *Il6*, Figure 2.10). Likewise, a previous study in mice found reduced quantities of several inflammatory cytokines in the bronchoalveolar lavage fluid of *Casp1-/-* mice infected with IAV

in vivo (19). Many of these inflammatory genes are regulated by the transcription factor NF-kB. We found that expression of *RIPK2*, a kinase that interacts with caspase-1 to mediate NF-kB activation, was also enhanced with IAV infection (Figure 2.2), suggesting that caspase-1 may signal independently of the inflammasome to activate NF-kB (29). In addition, we found that MTEC from *Casp1*$^{-/-}$ mice had reduced IAV replication (Figure 2.11), and that in HBEC *CASP1* expression positively correlated with viral replication (Figure 2.9), suggesting that caspase-1 may be a determinate of viral replication. Previous studies suggest that caspases may be harnessed by viruses, including IAV, to cleave viral-proteins involved in viral replication (31). Several studies indicate that the IAV nuclear protein and matrix protein 2 may be cleaved by caspases leading to enhanced virulence, though caspase-1 cleavage of these viral proteins has not been shown (32). Caspase-1 specifically has been shown to cleave components of Epstein-Barr virus, contributing to more productive infection (33). Thus, caspase-1 may have additional non-inflammsoame functions during a viral infection that may include regulation of antiviral and pro-inflammatory gene expression possibly via activation of NF-kB and/or cleavage of IAV proteins that may enhance viral replication.

In summary, our findings suggest caspase-1 plays an important role in the airway epithelial cell innate immune response to IAV infection. Activation of caspase-1 by IAV may differ between macrophages, where NLRP3 is highly expressed and well characterized, and structural airway epithelial cells, where other immune receptors such as RIG-I may be of greater importance (26). Airway epithelial cells are the primary target of IAV, which hijacks the cell for to produce virus progeny. The key objectives of epithelial cells are to halt viral replication and secrete cytokines and chemokines to attract immune cells for clearance of apoptotic cells and virus particles (34). Comparatively, replication of IAV in macrophages is typically lower than in

epithelial cells and infection is often abortive depending on the strain and type of macrophage (35). Unlike airway epithelial cells, macrophages are specialized phagocytes that function not only to alert other cells of the infection, but also to clear virus particles and virus-infected cells; thus activation of different signaling pathways may be necessary to mediate these functions (36). Furthermore, our results indicate that caspase-1 may have inflammasome-independent functions in airway epithelial cells that include regulation of inflammatory and antiviral gene expression or involvement in viral replication. There is clear evidence that caspase-1 and other inflammasome components such as PYCARD have inflammasome-independent functions, yet current understanding of these activities and their contribution to viral infection are limited (8, 37, 38). Future studies will be necessary to investigate inflammasome-independent functions of caspase-1 in airway epithelial cells, and how these pathways may differ in myeloid versus non-myeloid cells.

6.2 The inflammasome in response to ozone

Inhalation of environmental air pollutants, such as particulate matter, diesel exhaust, and ozone (O_3), is associated with increased hospitalization for asthma exacerbation (39-41). Pollutants may directly induce airway inflammation that contributes to asthma exacerbation or act as an adjuvant whereby the pollutant primes the airway for enhanced response to a subsequent challenge (42, 43). Unlike viruses, which may be directly recognized by PRRs via PAMPs, air pollutants are thought to primarily activate PRRs via DAMPs derived from damaged cells and tissue (44, 45). Interestingly, many of the receptors for DAMPs and PAMPs are shared, such TLR4 and NLRP3, suggesting overlap between non-infectious and pathogen-mediated inflammatory responses (43). The NLRP3 inflammasome has been deemed a general sensor of cellular stress and is implicated in the innate immune response to a number of environmental

pollutants, including asbestos fibers, silica particles, and nanoparticles (37, 38). A recent study found that NLRP3 and the inflammasome components caspase-1 and PYCARD contribute to O_3-induced airway inflammation and hyperresponsiveness in mice via activation of the inflammasome by the DAMP hyaluronic acid (HA); however these findings have not been confirmed in humans (39). Thus, before investigating the involvement of caspase-1 in O_3-induced asthma exacerbation, we sought to determine a role for DAMPs and inflammasome signaling in the innate immune response to *in vivo* O_3 exposure in healthy individuals.

Our results indicate that inhalation of O_3 induces a number of DAMPs in the airway, and that the presence of these DAMPs differs over time after exposure. Of the early DAMPs, we found significantly increased levels of several oxidized cholesterols (oxysterols) in the airway 1 hour after O_3 exposure (Figures 4.4). Oxysterols have been best characterized in the context of atherosclerosis. Lipid loading of blood macrophages enhances activation of TLR4 and intracellular cholesterol crystals are recognized by the NLRP3 inflammasome leading to the release of pro-inflammatory mediators (48, 49). However, activation of the innate immune system by oxidized lipids and cholesterol overload has not been well studied in the lung.

Cholesterol composes a large component of surfactant and antioxidants (e.g. γ-tocopherol) in the lung lining fluid (50). Previous studies have shown that *ex vivo* exposure of bovine surfactant to O_3 or *in vivo* O_3 exposure of rodents is associated with the presence of oxysterols in the airway similar to those we detected after human *in vivo* exposure, particularly cholesterol epoxide β, and that these oxysterols are mediators of inflammation and cytotoxicity in airway epithelial cells and monocytes (51-54, 57). Clearance of oxysterols is primarily mediated by reverse cholesterol transport in macrophages via uptake by scavenger receptors and efflux through cholesterol transports such as ATP binding casette (ABC) A1 and G1, which are

regulated by the oxysterol-responsive transcription factor liver X receptor (LXR) (55-56). Mice deficient in *Abcg1* have enhanced neutrophil and inflammatory response to LPS inhalation, whereas treatment of mice with LXR agonists may reduce neutrophil influx and inflammation in response to LPS inhalation or infection (58-60). These studies suggest that LXR agonists may serve as a useful therapeutic to enhance clearance of oxysterols and reduce inflammation and neutrophil influx in the lung after O_3 exposure. However, it should be noted that *Abcg1*$^{-/-}$ mice had reduced Th2 responses responses in an OVA-allergen sensitization model, suggesting that ABCG1 may promote Th2 immunity (61). Moreover, treatment of mice with an LXR agonist (GW 3965) during OVA sensitization increased airway smooth muscle mass and airway reactivity, but had no effect on airway inflammation (62). Thus, the function of oxysterols and LXR in allergy and immunology is not clear and should be further investigated to determine the utility of LXR agonists as therapeutics.

As the oxysterols subsided by 24 hours after O_3 exposure, we detected enhanced levels of hyaluronic acid (HA) in the BAL fluid and in our *in vitro* co-culture model of airway epithelial cells (16HBE) and pulmonary macrophages (Mac), suggesting that HA may be a late mediator of O_3-induced airway inflammation (Figure 4.3A and 5.8B). HAis an extracellular matrix component produced by epithelial cells that when fragmented by tissue injury or oxidative stress activates PRRs and pro-inflammatory responses (65, 66). HA has been previously shown to contribute to O_3-induced airway hyperresponsiveness (AHR) and inflammation via signaling through TLR4, CD44 (the HA receptor), and the NLRP3 inflammasome in mice (67-69). These studies showed accumulation of HA in the airway beginning 12-24 hours after exposure to O_3 and peaking at 48 post-exposure, which is in agreement with our findings (69). Using the 16HBE-Mac co-culture model, we determined that enhanced HA in the airway after O_3 is not

likely due to increased production by epithelial cells, but rather altered regulation of epithelial cell-derived HA by Mac (Figure 5.8B). Similar to previous studies, we found that Mac have reduced phagocytosis in response to O_3 (Figure 5.4), and propose that reduced Mac phagocytosis may lead to the accumulation HA. Studies in mice have also found that CD44 mediates endocytic clearance of HA and degradation by Mac, suggesting that altered CD44-endocytosis by Mac may contribute to reduced clearance of HA (67, 70). Future studies are necessary to specifically determine the mechanism by which O_3-induced Mac dysfunction induces HA accumulation.

A shortcoming of our study is that our results do not address the downstream effect of HA on the human inflammatory response to O_3. Additionally, due to limited sample volume we were unable to determine the size of HA that accumulates in the BAL fluid or co-cultures with O_3. As size is an important determinate of the inflammatory potential of HA, this will be an important end point for future human studies (65). Surprisingly, treatment of Mac with conditioned media from O_3-exposed 16HBE did not mimic the effects of direct O_3 exposure on Mac phenotype, viability, or phagocytic function, suggesting that soluble mediators from 16HBE, such as HA, may not mediate O_3-induced Mac dysfunction (Figure 5.8). Accumulation of HA may rather affect the function of other cells besides Mac or epithelial cells, such as neutrophils, which are highly present in the airway at 24 hours post-exposure (71, 72).

Despite detection of several DAMPs that have been previously shown to activate the inflammasome, including HA and uric acid, we did not find strong evidence to support activation of the inflammasome in humans after O_3 exposure *in vivo.* IL-1β, a major endpoint of inflammasome activity, was not detectable in any BAL fluid samples at either 1 or 24 hours post-exposure (data not shown). Likewise, we did not observe O_3-induced IL-1β production in

16HBE mono-cultures, Mac mono-cultures, or 16HBE-Mac co-cultures (data not shown). We also did not observe changes in Mac caspase-1 activity 1 hour after O_3 exposure, or a priming effect of O_3 on inflammasome activation (Figure 4.7). These results were surprising because HA-mediated activation of the NLRP3 inflammasome and IL-1β signaling have been previously shown in the response to O_3 in mice (69, 73, 74). We believe that the data we show here in humans is preliminary and should be considered with several qualifications in mind: 1) We only measured caspase-1 activity 1 hour after exposure to O_3, when the immune response to O_3 is just beginning and HA has not accumulated in the lung. The studies in mice found the greatest evidence of inflammasome activity 24 hours after O_3 exposure when HA is elevated (69); 2) the BAL fluid may have been too dilute to detect IL-1β and we did not assess IL-18 levels; and 3) caspase-1 may have alternative functions beyond IL-1β release that we did not measure, such as activation of NF-kB signaling, and some of these functions may occur independent of caspase-1 enzymatic activity (75, 76). Despite these qualifications, we believe our data does not suggest a strong contribution of inflammasome-mediated IL-1β to the early human innate immune response to O_3.

In summary, using a combination of *in vivo* and *in vitro* models of O_3 exposure in humans, we found evidence that O_3-induces the accumulation of DAMPs in the airway that may mediate the innate immune response to O_3. Our results uncovered a potential role for oxysterols as early mediators of O_3-induced lung injury that we speculate could serve as therapeutic targets for pharmacological regulation with LXR agonists. Notably, as inhalation of oxidant air pollutants such as O_3 is associated with increased risk for cardiovascular events, future studies should assess migration of oxysterols from the airway to the blood stream after O_3 exposure and the contribution of these oxysterols to atherosclerosis and cardiovascular disease (63, 64).

Moreover, we provide mechanistic insight on accumulation of HA in the response to O_3 and propose that accumulation of HA is not due to a direct effect of O_3 on HA generation, but is rather a secondary effect of immune cell dysfunction induced by O_3.Though we found a number of DAMPs increased in the airway after exposure to O_3, we did not find evidence of caspase-1 and inflammasome activation. Thus, future studies are necessary to determine the signaling pathways by which O_3-induced DAMPs activate the innate immune response, and how these pathways may contribute to pathogenesis of diseases such as asthma and cardiovascular disease.

6.3 **The inflammasome and asthma**

Asthma is a chronic inflammatory disease characterized by airway obstruction and bronchoconstriction in response to a challenge (12). Though asthma is traditionally considered a disease of adaptive immunity, mediated by T and B lymphocytes, innate immune cells contribute to asthma by secreting mediators that promote inflammation, mucus production, and bronchoconstriction and informing the development of adaptive immune responses (6). Innate cells are of even greater importance during acute asthma exacerbation, where inflammation caused by viral infection or inhalation of air pollutants provokes asthma symptoms (11). Though the role of IL-1 and inflammasome signaling in asthma development has proven controversial, the inflammasome response to PAMPs and DAMPs induces inflammatory mediators that likely contribute to exacerbation of asthma symptoms (15-17, 77). Since the inflammasome is involved in the response to both viruses and air pollutants, two major causes of asthma exacerbation, we hypothesized that inflammasome signaling may be a common pathway that contributes to virus- and pollutant- induced asthma exacerbation.

As described above, epithelial cells are the primary target cell for infection by respiratory viruses, such influenza A virus (IAV). An increasing number of studies have underscored the

important contribution of airway epithelial cells to asthma pathogenesis (22). Epithelial cells release cytokines and chemokines that attract effector cells to the airway and promote Th2 responses in neighboring DC (6, 78-80). In addition, *in vitro* and *in vivo* studies have shown that epithelial cells from asthmatics have deficient barrier functions, possibly due to altered tight junction formation, which increases the likelihood of antigen interaction with underlying DC (78, 81). At the molecular level, epithelial cells from asthmatics may be more susceptible to oxidative stress and have modified innate immune responses to viral infection, which may be due to inherent differences in expression of genes involved in epithelial repair and immune response, including *IL1B* (82-86). As the inflammasome was previously shown to mediate antiviral responses to IAV (20), and virus-induced *IL1B* expression was enhanced in human bronchial epithelial cells (HBEC) from asthmatics (82), we hypothesized that innate immune and inflammasome signaling may be altered in HBEC from asthmatics and contribute to virus-induced asthma exacerbation.

In line with our hypothesis, we found that compared to HBEC from non-asthmatics, HBEC from asthmatics had increased IAV-induced IL-1β production that was associated with increased expression of a number of inflammasome or NLR-related genes, including all of the "inflammatory caspases" (*CASP1*, *CASP4*, and *CASP5*) and two NLRs (*NLRC5* and *NOD2*) (Figure 2.1 and Table 2.6). By immunofluorescence, we observed that caspase-1 and PYCARD co-localization was enhanced in HBEC from asthmatics with IAV infection (Figure 2.3). Additionally, we found increased expression of *RIG-I* and downstream markers of the interferon signaling pathway, including *IFNβ* and *IP10,* in HBEC from asthmatics (Figure 2.7). As RIG-I has been shown to both regulate interferon production and form inflammasome complexes with PYCARD to induce caspase-1-mediated IL-1β release, these results suggest that RIG-I mediated

inflammasome signaling may be enhanced in HBEC from asthmatics (26). Notably, production of IL-1β by HBEC from asthmatics was low, suggesting that caspase-1 may also have inflammasome-independent functions, such as caspase-1 activation of NF-kB signaling through RIPK2 (29). Notably, *RIPK2* expression was also enhanced in HBEC from asthmatics (Table 2.6). Though increased caspase-1 and innate immune gene expression was associated with enhanced IAV replication, we did not detect a significant difference in viral replication between HBEC from asthmatics or non-asthmatics. Likewise, a number of previous studies investigating RV or RSV that found no difference in virus replication between HBEC from asthmatics and non-asthmatics, suggesting that an increased inflammatory response, but not increased virus replication, contributes to virus-induced asthma exacerbation (82, 83, 85). In summary, we found that intrinsic differences in HBEC from asthmatics contribute to enhanced IAV-induced innate immune and inflammasome signaling, and propose that enhanced inflammatory responses to IAV may exacerbate asthma symptoms.

We next sought to confirm a role for caspase-1 in virus-induced asthma exacerbation *in vivo* in mice. Before doing so, we needed to first establish a mouse model of allergic asthma and investigate the role of caspase-1 in asthma development (Chapter 3). Previous studies that have investigated the contribution of IL-1, caspase-1 and NLRP3 signaling to asthma development have yielded conflicint results (15-18, 77, 87, 88). These inconsistent findings may be in part due to differences in allergen sensitization protocols and reagents (e.g. endotoxin contamination can vary significantly across HDM formulations (89)) though a comprehensive study of asthma development in *Nlrp3*$^{-/-}$ mice that compared multiple models of OVA and HDM sensitization could not identify a role for NLRP3 in allergic lung disease (15). To add to this confusing literature, we found that HDM sensitization of *Caspase-1* deficient (*Casp1*$^{-/-}$) and wild type

(WT) mice revealed divergent roles for caspase-1 in the development of allergic inflammation, whereby HDM-sensitized *Casp1-/-* mice had enhanced airway inflammation, but reduced airway hyperresponsiveness (AHR) compared to HDM-sensitized WT mice (Chapter 3). Our results show that increased inflammation in *Casp1*$^{-/-}$ mice was largely localized to the alveolar tissue, but we found no evidence of increased airway resistance in the alveolar tissue (G) compared to WT mice (Figure 3.5B and 3.9B). Conversely, we observed similar inflammation along the large airways in both *Casp1*$^{-/-}$ and WT mice, but increased resistance of the large airways (Raw) in *Casp1*$^{-/-}$ mice (Figure 3.5A and 3.9C). These results were surprising as airway inflammation has been previously shown to contribute to AHR (90-92). However, AHR is also determined by airway smooth muscle (ASM) mechanics and structural changes to the airway, such as increased ASM mass and extracellular matrix deposition (72, 93, 94). Thus, our results indicate that caspase-1 may have different functions in structural cells versus inflammatory cells. These results are in accordance with our previous findings (chapter 2) and those of other that suggest inflammasome signaling pathways may differ in structural versus non-myeloid cells, and suggests alternative functions for caspase-1 beyond IL-1β production (26, 95, 96). Indeed, we surprisingly saw enhanced IL-1β in the airway of HDM-sensitized *Casp1-/-* mice compared to HDM-sensitized WT mice, suggesting non-caspase-1 mediated production of IL-1β, as can occur via neutrophil proteases or release from lytic cells (Table 3.1) (97). Since airway remodeling is not typically observed in acute models of allergen sensitization, a chronic sensitization model will be necessary to better determine the contribution of caspase-1 to structural changes in the lung.

Though we advise that the results of the caspase-1 and asthma development study are preliminary, we believe that these findings indicate a possible role for caspase-1 in the

pathogenesis of allergic asthma and predict that caspase-1 will more strongly contribute to asthma exacerbation as caused by IAV. Based on our findings that caspase-1 signaling is enhanced in HBEC from asthmatics and MTEC from *Casp1*$^{-/-}$ mice (chapter 2), we hypothesize that IAV-induced asthma symptoms will be less severe in *Casp1*$^{-/-}$ mice, and that innate immune responses in epithelial cells may be major determinates of IAV-induced asthma exacerbation. However, the fact that *Casp1*$^{-/-}$ mice had increased airway inflammation after HDM sensitization suggests that the role of caspase-1 in innate immunity and allergy is very complex and may depend on the exact stimulus. In addition, future studies investigating the contribution of caspase-1 to asthma development exacerbation should use new models of *Casp1*$^{-/-}$ mice, as the strain most commonly used for caspase-1 studies (including that shown here in chapters 2 and 3) was recently found to also be deficient in caspase-11 (98). The implications of this double-deficiency will be discussed in the "Further Insights" section below.

We were unable to address the function of the inflammasome in O_3-induced asthma exacerbation in this dissertation, but will speculate on this topic here. As described in chapter 4, we found increased levels of several DAMPs in the airway after exposure to O_3 (uric acid, hyaluronic acid (HA), oxysterols), but no evidence of O_3-induced inflammasome signaling. However, our results in chapter 2 show that the inflammasome response to IAV is enhanced in HBEC from asthmatics, suggesting that inflammasome signaling may be more active in individuals with asthma and contribute to asthma exacerbation. A previous study of O_3 exposure in humans found that atopic asthmatics had enhanced HA and IL-1β in induced sputum collected 6 hours after *in vivo* O_3 exposure compared to healthy volunteers, suggesting that O_3-induced HA may activate inflammasome signaling in asthmatics (69, 99, 100). Notably, the increased HA and IL-1β were also associated with increased neutrophil numbers in the sputum (99). In addition to

IL-1β cleavage by caspase-1, neutrophils contain proteases that can mediate secretion of active IL-1β, such as neutrophil elastase, proteinase-3, and cathepsin G can cleave extracellular IL-1β (97, 101, 102). As neutrophils are the primary cell recruited to the airway after O_3 exposure, neutrophil-derived proteases rather than caspase-1 may mediate the release of IL-1β after exposure to O_3 (103-194). Thus, future studies must carefully distinguish between inflammasome and non-inflammasome mediated- IL-1β production after pollutant exposure.

Our results also indicate that the DAMP uric acid was increased in the airway of healthy volunteers 1 hour after O_3 exposure (Figure 4.3B). High concentrations of uric acid form monosodium urate crystals that have been shown to activate the NLRP3 inflammasome (105, 106). Increased uric acid has been previously shown in BAL fluid from asthmatics after *in vivo* allergen challenge, and in mice uric acid crystals had an adjuvant effect on OVA-induced Th2 inflammation, though this effect was independent of NLRP3, PYCARD, and caspase-1 (17). Previous controlled exposure studies found that allergic asthmatics exposed to O_3 have heightened responses to subsequent allergen challenge, suggesting that DAMPs such uric acid produced after O_3 may prime for enhanced response to subsequent challenge (107). Thus, O_3-induced uric acid in the airway may induce inflammation that exacerbates asthma symptoms or prime the airway for an enhanced response to a subsequent challenge, though whether this occurs through inflammasome signaling is unclear.

In summary, we believe that our results suggest that caspase-1 and inflammasome signaling may contribute to both asthma development and exacerbation, but that the involvement of these pathways in asthma pathogenesis is complex and likely varies with stimuli. In the context of IAV infection, the enhanced innate immune response to IAV by HBEC from asthmatics, which we found to be associated with enhanced caspase-1 expression and activity,

may contribute to virus-induced asthma exacerbation *in vivo*. In addition, we suggest that O_3-induced DAMPs have the potential to signal through the inflammasome to induce airway inflammation that exacerbates asthma symptoms or primes the airway for an adverse response to subsequent challenge.

6.4 Epithelial cells as orchestrators of innate immunity and asthma

The respiratory mucosal surface is lined by a layer of epithelial cells that protects the underlying tissue from the inhaled environment (108). Epithelial cells have been traditionally considered bystander cells of the immune response, serving only as a site of gas exchange and as a structural barrier against inhaled pathogens, antigens, and particulates. An increasing number of studies have demonstrated that epithelial cells are active components of the innate immune response that secrete a number of mediators important for host defense, including cytokines/chemokines, surfactants, mucins, proteases/anti-proteases, and defensins (34, 109, 110). Moreover, epithelial cells are among the first cells to interact with inhaled stimuli, suggesting that epithelial cell response is a determinate of subsequent innate and adaptive immunity. A number of findings in this dissertation demonstrate an active role for epithelial cells in maintaining immunologic homeostasis and activating innate immune responses in the lung, thus supporting the notion that epithelial cells are orchestrators, rather than bystanders, of innate immunity that can significantly contribute to airway disease.

The respiratory surface is constantly exposed to a broad range of stimuli that may activate immune responses in the lung. Resident cells, consisting predominately of epithelial cells, macrophages, and underlying dendritic cells (DC), must discriminate between innocuous and pathogenic stimuli to determine the appropriate immune responses, and also prevent against unnecessary inflammation that may damage the airway (4). Resident immune cells must exist in

a subdued state in order to prevent against excessive inflammatory response to harmless stimuli (111). Resident pulmonary macrophages (Mac) have been previously shown to exist in a quiescent state with relatively lower phagocytic capabilities compared to other tissue resident or peritoneal macrophages (111-115). Our results in chapter 5 show that airway epithelial cells promote a quiescent Mac phenotype, as evidence by increased expression of CD206, a marker of anti-inflammatory "alternatively" activate macrophages, and reduced phagocytic activity (Figure 5.3). In addition, we found that Mac regulate the availability of HA produced by epithelial cells, suggesting a homeostatatic relationship between the two cell types (Figure 5.8).

When the airway is presented with an injurious stimulus, the resident immune cells are quickly activated and recruit inflammatory cells to the airway. In the response to IAV infection, we showed that epithelial cells produce cytokines and chemokines such as IL-6, IL-1β, TNFα, IP-10, IFNβ, and CCL5 to activate local immune cells and promote inflammatory cell influx to the airway and that caspase-1 may be an important regulator of these responses (Chapter 2). In the context of a pollutant exposure, we showed that signals from epitheial cells modify Mac immunophenotype and activity in response to O_3 (Figures 5.3-5.5). Interestingly, we found that treatment of Mac with media from O_3 or Air-exposed epithelial cells had no effect on Mac immunophenotype or activity, suggesting that local or direct cell-cell interaction is necessary to mediate the effects of co-culture on Mac phenotype and response to O_3 (Figure 5.8). These findings are in agreement with previous airway epithelial cell-Mac co-culture studies that demonstrated cell-cell interaction was necessary to mediate the effects of co-culture on the response to particles or particulate matter (116, 117). Moreover, our group has shown that epithelial cells can interact with other inflammatory cells in the airway to influence innate immune responses. In the context of O_3, NK cells co-cultured with O_3-exposed nasal epithelial

cells have altered phenotype and cytotoxic activity, and these effects were mediated by direct cell-cell interaction (118). Using a nasal epithelial cell and dendritic cell co-culture model, our group also showed that nasal epithelial cells from smokers have deficient interferon responses to IAV infection, which promotes altered DC response to IAV (119, 120). Taken together, our results and those of others show that that the response of epithelial cells to inflammatory stimuli, such as viruses or pollutants, influence the activation of Mac, DC, and NK cells, demonstrating that epithelial cells are indeed important orchestrators of the innate immune response.

The important function of epithelial cells as regulators of immunologic homeostasis and as orchestrators of immunity is highlighted by diseases such as asthma, where altered epithelial cell barrier and inflammatory functions contributes to pathogenesis (22, 78, 121). For example, altered ability of epithelial cells from asthmatics to form tight junctions may increase access of antigens to underlying DC and promote allergen sensitization (78, 81). In addition, epithelial cells produce mediators in response to inhaled allergens, pollutants, or viruses that promote DC activation and Th2 lymphocyte differentiation and also induce inflammation that exacerbates asthma symptoms (6, 78-80). Finally, goblet cell metaplasia during asthma is associated with increased mucus production and airway obstruction (22, 122). Our findings suggest that epithelial cells have intrinsic differences that are associated with enhanced inflammatory responses. We found that at baseline conditions, HBEC from asthmatics produced several pro-inflammatory cytokines, IL-1β, IL-6, and TNFα, that were not present in HBEC from non-asthmatics (Figure 2.1) and had increased expression of *CCL5* (Table 2.6). With IAV-infection, HBEC from asthmatics had even further increased cytokine production and enhanced expression of many innate immune and inflammasome genes compared to HBEC from non-asthmatics, including *CASP1*, *RIG-I*, *IP-10*, *IFNβ,* and *CCL5* (Figure 2.1 and Table 2.6). Of particular

interest is elevated expression of the eosinophil and T lymphocyte chemoattractant *CCL5* in HBEC from asthmatics both at baseline and in response to IAV, as enhanced CCL5 protein expression has likewise been shown in bronchial biopsies from asthmatics (125, 126). These findings suggest that HBEC from asthmatics have intrinsic differences in inflammatory status that are maintained after several weeks of culturing *ex vivo* and may contribute to enhanced inflammatory responses to viral infection. Similar modifications in inflammation, repair, and proliferation have been previously shown in studies of cultured epithelial cells from asthmatics, suggesting that the chronic inflammatory environment of the asthmatic airway may cause intrinsic changes in the epithelium (83, 123, 124, 127).

The intrinsic differences in HBEC from asthmatics may be due to inherent genetic or epigenetic changes. Asthma has a clear hereditary component, and a number of studies have identified candidate genes involved in innate immunity and epithelial cell function that are associated with susceptibility to asthma or allergy, including polymorphisms in *CCL5* and *Clara cell 16-kD protein* (*CC16*) (128-130). However, not all individuals with familiar history or genetic susceptibility develop asthma, suggesting that environmental factors, such as early life viral infection or exposure to second hand tobacco smoke, play a major role in asthma development (131-133). Epigenetics, or changes in gene function that are not due to a change in the DNA sequence, may shed light on the interaction between genetic susceptibility and environmental exposures in asthma pathogenesis (134). A number of studies have shown epigenetic alterations are associated with asthma, though relatively few have assessed epigenetic changes specifically in epithelial cells (134, 135). Of these studies, a concordant increase in methylation and decreases in gene expression of *STAT5A*, a transcription factor associated with Th2 cell differentiation and epithelial cell proliferation, was identified in epithelial cells from

asthmatics (136). Additionally, bronchial biopsy samples from asthmatics were shown to have reduced histone deacetylase (HDAC) 1 and 2 expression and function, and increased histone acetyltransferase activity, which are associated with global hyperacetylation and an open chromatin structure that promotes gene transcription (137). Clearly, additional studies are necessary to determine if specific genetic or epigenetic mechanisms in HBEC from asthmatics contribute to altered function and response to inhaled stimuli.

In summary, our results demonstrate epithelial cells play an important role in orchestrating the innate immune responses to viruses and pollutants in the lung and provide evidence that intrinsic differences in epithelial cells from asthmatics contribute to an altered response to respiratory viral infections. Moreover, we identify caspase-1 and the inflammasome pathway as an important innate signaling pathway in airway epithelial cells that is altered in epithelial cells from asthmatics. These results are consistent with recently described epithelial-centric models of asthma pathogenesis, in which unresolved epithelial injury is proposed to be the basis for the chronic inflammation and airway remodeling assocatied with asthma, and clearly demonstrate that airway epithelial cells are central to respiratory immunity and airway disease (22, 123, 127).

6.5 Final Insights

In this dissertation, we investigated the role of the inflammasome in the innate immune response to viruses and air pollutants in the lung, and how these pathways may contribute to asthma pathogenesis. We show that caspase-1 contributes to the airway epithelial cell innate immune response to influenza a virus (IAV) infection, and that this pathway is enhanced in epithelial cells from asthmatics, but that caspase-1 may have divergent functions in the development of allergic asthma in mice. Additionally, we show that many damage associated

molecular patterns (DAMPs) are found in the airway after exposure to O_3, but these DAMPs are not associated with caspase-1-mediated IL-1β production. Using an airway epithelial cell and macrophage co-culture model, we demonstrated how exposure to O_3 alters the homeostatic relationship between airway epithelial cells and macrophages, leading to dysregulation of the DAMP hyaluronic acid (HA). These results provide insight on the human innate immune response to viruses and pollutants and highlight many future directions for inflammasome research.

Studies of the inflammasome signaling, including those presented here, have largely focused on mechanisms of inflammasome-mediated IL-β release. Though IL-1β is a traditional marker of inflammasome and caspase-1 activity, neutrophil and mast cell-derived proteases may also cleave and activate IL-1β (97, 101, 102). As neutrophils are heavily recruited to the airway upon infection or injury as caused by many inflammasome stimuli, the contribution of neutrophil mediated IL-1β release in the airway should be better investigated. In addition, many interesting studies have highlighted alternative activites of many inflammasome components, and data shown here suggest caspase-1 may have alternative functions in the response to IAV and asthma, such as NF-kB activation (8, 38, 138, 139). These alternative pathways may be of even greater importance in non-phagocytic cells, such as epithelial cells, which serve very different functional roles in innate immunity than macrophages or monocytes and vary in the capacity to release IL-1β.

The results presented here are unique in that we studied the inflammasome in human cells. Though animal studies have been successful in revealing inflammasome mechanisms, species differences in inflammasome signaling between human and mice has not been well studied. The human genome encodes 22 NLRs whereas the mouse encodes at least 30 NLRs, and

species differences in the function of the same NLR have been observed (140,141). Likewise, the inflammatory (non-apoptotic) caspases differ between mice and humans. The mouse genome encodes caspase-1, caspase-11, and caspase-12, whereas the human genome encodes caspase-1, caspase-4, and caspase-5 (142). Caspase-4 and 5 are thought to be orthologs of murine caspase-11 based on similar sequences, though the function of these caspases in humans is not clear (143). Our results demonstrate that *CASP4* and *CASP5* expresssion is induced with IAV infection in airway epithelial cells and is further enhanced in epithelial cells from asthmatics, suggesting a yet-to-be-determined contribution to the innate immune response to IAV. Further underscoring the need for increased research on human caspase-4/5 and murine caspase-11 is the recent discoveries that the commonly used C57BL/6 caspase-1 knockout model (*Casp1*$^{-/-}$) is also deficient for caspase-11 (*Casp11*), and that caspase-1 and -11 have non-redundant functions (10, 98). In initial characterization studies using the caspase-1 and caspase-11 double knockout mice concluded that *Casp1*$^{-/-}$ mice were resistant to endotoxic (LPS-induced) shock (144). More recent studies corrected these findings and showed that caspase-11, but not caspase-1, mice are resistant to endotoxic shock, suggesting distinct functions (98-145). Since the mice used in the studies shown here in chapters 2 and 3 are actually caspase-1 and caspase-11 double knockout mice, these results should be interpreted with caution and future studies will be necessary to delineate the roles of caspase-1 and caspase-11 in the innate immune response to IAV or in the development of allergic asthma. More importantly, future studies are needed to better understand the functions of caspase-4 and -5 in the human innate immune response and their similarity to murine caspase-11.

If this discussion of the complexities of inflammasome research is of any indication, Charles Janeway was correct in 1989 when he predicted that innate immunity is a highly evolved

(and still evolving) first line of defense against infection and injury (1). The innate immune system is composed of a network of structural cells and immune cells that have the difficult job of distinguishing between harmless and harmful stimuli and beckoning the appropriate inflammatory reinforcements. Innate immune cells do so by employing pattern recognition receptors that bind to PAMPs and DAMPs to recognize the harmful stimuli and initiate appropriate immune responses, such as formation of the inflammasome complex. The research shown here sheds light on the function of inflammasome signaling in the response to viruses and oxidant air pollutants in the human airway and how these same pathways may contribute to asthma pathogenesis. We specifically highlight the role of the airway epithelium as a key orchestrator of innate immunity, and demonstrate that asthma is associated with dysfunctional epithelial cell innate immune responses. It is our hope that in the future, these findings contribute to the development of improved therapeutics for the treatment of chronic airway diseases such as asthma and implementation of more informed standards for air pollutants such as O_3.

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