I, Mark Webb, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Immunobiology.

It is entitled:
Allergen-Induced Chemokine Release from the Bronchial Epithelium: A Novel Lysosomal Release Mechanism

Student’s name: Mark Webb

This work and its defense approved by:

Committee chair: Marsha Wills-Karp, Ph.D.
Committee member: Simon Hogan, Ph.D.
Committee member: Marshall Montrose, Ph.D.
Committee member: Nives Zimmermann, Ph.D.
Allergen-induced chemokine release from the bronchial epithelium:

A novel lysosomal release mechanism

A dissertation submitted to the University of Cincinnati

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Of the College of Medicine

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Marsha Wills-Karp, Ph.D.
Abstract

Asthma is a chronic inflammatory disease of the lung. In recent years, the airway epithelium has been identified as an important contributor to the initial development of asthma after allergen exposure. The mechanisms by which epithelial cells respond to allergen to help drive downstream inflammation and the initiation of an immune response are poorly understood. In this dissertation we sought to understand the basic mechanisms that control release of chemokines and cytokines in general and CCL20 in particular. To do this, we measured levels of CCL20 and other cell mediators retained in cells and released from cells, and we visualized these mediators’ intracellular localization via immunofluorescence microscopy. We found that a number of chemokines and cytokines are stored within bronchial epithelial cells under homeostatic conditions. In addition, CCL20 was stored in lysosomes, and its release was partially mediated through secretion of these lysosomes. In order to determine the intracellular localization of multiple chemokines and cytokines simultaneously, we again employed fluorescence microscopy. We also developed a novel technique to measure specific proteins in subcellular organelles using modified flow cytometry. We found that lysosomes containing CCL20 are released after exposure to HDM. We also found that the secretory lysosomes containing CCL20 also appear to contain other chemokines and cytokines released in response to HDM. We also found that allergens other than HDM induce release of specific subsets of chemokines and cytokines. These studies demonstrate a complex storage and release mechanism for chemokines and cytokines of the bronchial epithelium. These findings improve our understanding of the initial interactions between allergen and the epithelium, and provide
an interesting potential target for future treatments that will allow them to be both targeted and robust.
Acknowledgments

I would like to thank my advisor, Dr. Marsha Wills-Karp for her time and effort in helping me refine my scientific understanding. In addition, she provided a lab staffed with excellent post-doctoral fellows and graduate students who have also impacted me tremendously as I completed my degree. In particular, Stephane Lajoie, Stacey Burgess, and Ian Lewkowich have always provided a listening ear and sound opinions about specific hypotheses as needed. Their insights proved valuable as I sought to uncover the mechanisms presented herein. I would also like to thank my wife for her support and encouragement throughout the dissertation-writing process.
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<th>Definition</th>
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<tbody>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
</tr>
<tr>
<td>APTI</td>
<td>Airway pressure time index</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUB</td>
<td>Ambient urban Baltimore particulate matter</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance receptor</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HDM</td>
<td>House dust mite</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cells</td>
</tr>
<tr>
<td>i.t.</td>
<td>intra trachea</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosome associated membrane protein</td>
</tr>
<tr>
<td>LD50</td>
<td>Median lethal dose</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysacharrhride</td>
</tr>
<tr>
<td>mTEC</td>
<td>Mouse tracheal epithelial cell</td>
</tr>
<tr>
<td>NHBE</td>
<td>Normal human bronchial epithelial cell</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na-K-Cl cotransporter</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD-like receptor family, pyrin domain containing 3</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen (measure of acidity)</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PNS</td>
<td>Post-nuclear supernatant</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RAB</td>
<td>Ras-related protein</td>
</tr>
</tbody>
</table>
ROS  Reactive oxygen species
SOFA  Single organelle fluorescence analysis
SYK  Spleen tyrosine kinase
TCR  T-cell receptor
TGF-β  Transforming growth factor beta
TH  Helper T-cell
TNF-α  Tumor necrosis factor alpha
TSLP  Thymic stromal lymphopoietin
V-ATPase  Vacuolar-type H⁺ adenosine triphosphatase

**Common Ions**

<table>
<thead>
<tr>
<th>Ion</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>Phosphate ion</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>Sulfate ion</td>
</tr>
</tbody>
</table>
CHAPTER 1 – GENERAL INTRODUCTION

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Chapter 1 – General introduction

Asthma

Asthma is a debilitating chronic disease of the airways characterized by chest tightness, difficulty breathing, coughing, and wheezing. These symptoms are mainly caused by changes in the lungs that result in narrowing of the conducting airways. Breathing becomes more difficult because the constricted airways increase resistance to air flow.

Symptoms and treatment

The changes in the airways that lead to many of the symptoms described above include increased mucous production due to goblet cell hyperplasia, airway inflammation, angiogenesis, and smooth muscle hypertrophy. One of the primary treatments for asthma symptoms is inhaled corticosteroids. Inhaled corticosteroids are an inexpensive treatment that has proven effective at controlling more common, mild cases of asthma since the 1960s. They work by reducing inflammation in the airways. However, patients with more severe asthma show a decreased response to corticosteroids, and increasingly higher doses are given to patients with greater disease severity. As the dose of corticosteroids increases, many negative side effects begin to manifest including bone loss, hyperglycemia, depression, and cataracts among others. The reason why some asthma sufferers develop mild symptoms, while others
develop severe disease have been poorly characterized in the past. Thus, much research has focused on better understanding the molecular mechanisms of asthma pathogenesis, and especially the differences between pathogenesis of mild and severe asthma, in the hopes of developing more effective strategies for treatment and control – particularly for those who do not respond well to current treatment strategies.

**Incidence and costs**

Asthma is among the most common chronic diseases in the world, with an estimated 300 million people affected worldwide. Annual worldwide deaths from asthma are estimated at around 250,000 individuals. In the United States, the asthma prevalence as of 2008 was 8.5% of the population among adults, and 9.0% of children.

In recent decades, incidence of asthma in developed countries has been increasing, with one study in the United States showing asthma rates doubling over a 20 year period from 1964 to 1983. Although both genetic and environmental triggers have been shown to contribute to the development of asthma, evidence suggests that the increased incidence of asthma we have observed over the past few decades is the result of changes in environment and lifestyle in developed societies, which can occur over the course of a few decades; as opposed to genetic changes, which tend to occur over much longer time periods. One striking feature about elevated levels of asthma is that they are localized mainly to economically developed countries, with developing countries experiencing higher rates of asthma as they become more economically
developed.\textsuperscript{17} This can be seen from the heat map of asthma depicted in figure 1. This region-specific elevation in asthma prevalence has led to a number of questions about why some individuals develop asthma, in an attempt to better understand how to prevent further increases in the general development of asthma.

![Figure 1. World map of the global burden of asthma. Adapted from GINA 2004.](image)

**Etiology**

Although the exact etiology of asthma is currently unknown, it is thought to be a heterogeneous disease that is driven by both genetic\textsuperscript{18–22} and environmental influences.\textsuperscript{16,23} Many genetic risk factors have been explored through the use of genome-wide association studies (GWAS), which have identified a number of genes that may play a role in asthma pathogenesis. For some of these, the potential
mechanisms are well characterized, such as with tumor necrosis factor (TNF)-α and interleukin (IL) 4. For others, potential functional implications, and the link to asthma pathogenesis, are only beginning to be elucidated – such as with ORMDL3 and gasdermin B.\textsuperscript{24,25} Although mutations in any one of these genes may increase the risk of asthma marginally, no one gene has been directly identified as causing asthma and as such, asthma has been identified as a polygenic disease. This is in contrast to monogenic diseases – such as cystic fibrosis, which is directly linked to mutations in the cystic fibrosis transmembrane conductance receptor (CFTR).\textsuperscript{26} The genetic cause of cystic fibrosis is much more well-characterized, as it is the primary driver of disease. This is not the case with asthma, where multiple genetic changes contribute to a greater susceptibility to developing the disease, and asthma is not the result of a single aberrant gene. This may be due to the apparent need to activate multiple pathways leading to the development of asthma.

Indeed, in recent years asthma has become increasingly sub-divided into different types, including exercise-induced asthma, atopic asthma, childhood asthma, and so on. Within atopic asthmatics, we also see multiple different types of exacerbations with mild, moderate, and severe asthmatics who are responsive or resistant to interventions that target different molecular pathways.\textsuperscript{27} This is similar to
categorization of different types of cancers, where cancers driven by different molecular pathways respond to different types of treatment.

As mentioned previously, the dramatic elevation in asthma prevalence in developed regions is too rapid to be driven by genetic changes and therefore a number of non-genetic explanations have been explored to better understand this phenomenon.\textsuperscript{28} Thus, in addition to genetic differences, a number of environmental changes have also been shown to contribute to asthma susceptibility. For instance, exposure to tobacco smoke causes increased wheezing in genetically susceptible children.\textsuperscript{29} Socio-economic status is also a risk factor for asthma, with higher asthma incidence and hospitalization rates for individuals from poorer communities.\textsuperscript{30} Increased use of antibiotics in recent decades has led to changes in the gut microbiota that may contribute to allergic sensitization.\textsuperscript{31}

Other major behavioral and environmental changes seen more in developed nations include migration away from farms into cities. In addition, as populations move into urban environments, they are exposed to airborne pollutants such as particulate matter and nitrous acid, both of which may exacerbate their asthma symptoms,\textsuperscript{32-34} and contribute to a change in their microbiota.\textsuperscript{35-37} Living in an urban environment has also lead to increasing amounts of time spent indoors.\textsuperscript{38-41} Even the
altered diets in developed nations may contribute to the development of asthma.\textsuperscript{42} Our understanding of how each of these changes may lead to increased risk of asthma has improved in recent years. Better understanding of the specific mechanisms leading to asthma development may lead to the development of better strategies to prevent further increases in asthma incidence. Although many of these changes affect similar downstream pathways related to the development of asthma, a better understanding of how each change, and indeed each allergen, contributes uniquely to the development of atopy differently is poorly understood.

**Major allergens associated with allergic asthma**

Atopic asthma is defined as the development of an allergen-specific IgE response in association with asthma. Another way to characterize atopic asthma is the development of an aberrant immune response to otherwise innocuous airborne stimuli. Early exposure to specific allergens has been linked to development of atopy to those allergens.\textsuperscript{43} However, not all potential antigens that enter the airways have the potential to become allergens. Instead, a relatively small number of antigens have been identified as capable of driving allergic asthma. For example, one of the most potent allergens is house dust mite (HDM), with HDM-specific IgE responses found in up to 80\% of atopic asthmatics.\textsuperscript{44} Common classes and features have been recognized among the many identified allergens in atopic individuals. Some classes include insect
allergens, such as HDM and cockroach feces (frass), animal allergens, such as the major cat allergen Fel d 1; various species of mold, including Aspergillus fumigatus; and many species of pollen, notably maple and birch pollens. Additionally particulate matter, such as diesel exhaust and urban particulate collections, have been identified as potent irritants that dramatically affect the airways. These additional danger signals may contribute to increased asthma incidence or severity, especially in urban environments.

Specific features of these organisms and chemical mixtures have been identified that lead to their allergenicity. One such feature is the presence of active proteases, which contribute to damage of the airway epithelium. This may contribute to their allergenic behavior. For example, the protease activity in Der p 1, found within HDM, contributes to the degradation of the tight junctions between airway epithelial cells, providing access to other proteins and molecules found in HDM to the sub-epithelial environment. Other allergens; such as pollens from birch, maple, and ragweed; have also been demonstrated to contain active proteases that may play a role in allergic sensitization. Thus, allergens often contain active proteases which may help provide a “danger” signal to the responding airway epithelium.
However, breakdown of the epithelial barrier itself may not be sufficient to induce an immune response, nor is it required. Allergens are often a complex combination of proteins, carbohydrates and other moieties whose combined effect is more potent at eliciting an asthmatic response than individual proteases, or other components, alone. HDM also contains high levels of chitins, which are also found in many insects and molds. These chitins may serve to signal through pattern recognition receptors (PRRs) to promote an asthma response. In addition, HDM contains many species of bacteria in its gut, these bacteria aid in digestion of the mite’s food source (skin cells) and may also play a role in allergic sensitization. Upon exposure of the bacteria, pattern recognition receptors on the surface of the epithelial cells (such as toll-like receptor 4) recognize molecular patterns on bacteria such as lipopolysaccharide (LPS). This provides an initiating signal the promotes the further development of an immune response against the antigen as a whole. HDM is a prime example of the complex composition of many allergens. As such, it is also a good model for studying the complex interactions that contribute to the development of atopy. In this work, HDM was chosen as a model to study the effect of allergens on eliciting multiple different types of responses from bronchial epithelial cells. In addition, the divergent effects of other allergens on the bronchial epithelium are briefly explored.
Physiological changes in asthmatic lungs

Smooth muscle surrounds the airway epithelium, and may contribute to narrowing of the airways when they contract. For example, when the lungs are exposed to cold air, contraction of the airways slows air flow, which allows the conducting airway to warm the incoming air prior to exposure to the blood in the alveoli. Excessive growth of smooth muscle cells, or hypertrophy, in asthmatics contributes to narrowing of the airways under homeostatic conditions. During an asthma attack the airway smooth muscle contracts, further narrowing the airways.63

The cells lining the airways, bronchial epithelial cells, also contribute to lung function by helping to clear the lung of particulate matter and debris. These cells produce a mucous layer that coats the airways. As particulate matter travels along the airway, turbulence causes contact with the mucous layer, which it adheres to. The epithelial cells continuously push the mucous layer up, out of the lungs to the esophagus, where it is swallowed. In this way, the mucous layer is constantly recycled as new mucous is produced.64 In asthmatics, clearance of this mucous layer becomes impaired, and some airways may even become plugged with mucous, obstructing air flow completely.65
Finally, in addition to the features mentioned above, sub-epithelial fibrosis is commonly observed in asthmatics. Fibrosis develops after repeated inflammation in the airways. Fibrotic tissue is deposited below the epithelial layer, which contributes to the thickening of the airway. The combined effect of this process is an overall remodeling of the airway, which results in permanently reduced lung capacity. The gross anatomical manifestation of this is narrowing of the airways, which is the primary cause of the wheezing and shortness of breath that are characteristic of asthma.
Pathophysiological manifestations of disease

Cellular and molecular mechanisms have been identified for each of the events described above that contribute to the thickening of the airways. Thickening of the airways is caused by collagen deposition and by hypertrophy of airway smooth muscle cells.\textsuperscript{70,71} In addition, allergen exposure may induce constriction of smooth muscle cells, further narrowing the airways.\textsuperscript{72}

After repeated allergen exposure, fibrosis begins to occur. Fibrosis is driven by cells called fibroblasts, which proliferate and migrate into sites of tissue injury in response to the cytokine transforming growth factor (TGF)-\(\beta\). Fibroblasts secrete a combination of collagen and fibronectin in the formation of fibrotic scar tissue.\textsuperscript{73}

The pattern of airway inflammation in asthmatics includes the presence of increased numbers of eosinophils, neutrophils, and T lymphocytes,\textsuperscript{74–78} although acute airway inflammation in some severe asthmatics exhibits high levels of neutrophilia and little or no eosinophilia.\textsuperscript{79} This indicates that asthma may be a heterogeneous disease, with differing inflammatory phenotypes. Whatever the inflammatory phenotype, T-lymphocytes play a crucial role in regulating the behavior of inflammatory cells, and thus the type of immune response that develops.
Allergic asthma is mediated by type-2 cells

T cells are central to cell-mediated immunity. T cells each display a unique T cell receptor (TCR). This receptor is generated in the thymus, where multiple TCR genes recombine at random to produce a receptor that can bind to a unique epitope, or recognition site. Millions of these unique cells are generated in the thymus, from which they migrate in a naïve state, circulating through the blood. These naïve cells are activated through interactions with dendritic cells carrying antigen specifically recognized by that cells TCR. Once activated, these cells have tremendous proliferative capacity, and help direct the immune system to respond to specific antigens recognized by their unique TCR. This process of expanding previously generated populations of antigen-specific cells is known as Clonal Selection Theory.\(^{80}\)

After activation, CD 4\(^+\) T cells differentiate into specific subsets, instructed by the specific co-stimulatory molecules and cytokines supplied from the presenting DC.\(^{81}\) Initially, these CD 4\(^+\) helper T cells were thought to differentiate into two distinct subsets, T\(_{H1}\), and T\(_{H2}\) cells. In recent years, many different types of T cell subsets have been identified, including T\(_{H1}\), T\(_{H2}\), regulatory T cells, and T\(_{H17}\) cells with additional CD 4\(^+\) T cell subsets having been proposed.

Each T cell subset plays an important role in a number of immune responses.\(^{82}\) In normal lungs, T\(_{H2}\) cells function to promote humoral defense, and defense against
parasites. In the pathogenesis of asthma, Th2-type T cells play a central role, and are enriched in the lungs of asthmatics. They are characterized by production of many interleukins (IL), including IL-4, IL-5, IL-6, IL-10, and IL-13. Th2 cells are characterized by their activation of the transcription factor GATA-3, which helps preserve its differentiation to the Th2 phenotype.

In asthma, IL-4, IL-5, and IL-13 are particularly important in helping to promote many of the phenotypic changes associated with the disease. These interleukins together mediate B-cell class switching to IgE, eosinophilia, goblet cell metaplasia, and bronchoconstriction. IL-4 is necessary for B cell class switching to IgE. Additionally, IL-4 signals to Th2 cells themselves to drive GATA-3. This positive feedback loop is critical to maintain a Th2 phenotype. IL-5 is necessary for development of eosinophilia in mouse models of asthma. IL-13 promotes mucous production and AHR. Previously, the importance of IL-13 in allergic asthma was highlighted by demonstrating that IL-13 can directly induce experimental allergic asthma in mice. Interestingly, mice deficient in IL-4 can still develop AHR in an OVA-induced asthma mode, however these same mice do not develop AHR when IL-13 neutralizing antibodies are administered. Additionally, IL-13 knockout mice do not develop AHR in an allergen model of asthma. Thus, IL-13 has been considered a central mediator of allergic asthma.
Recent studies have highlighted a new cell type that is also capable of driving a type-2 immune response. These cells are a type of innate lymphoid cells (ILC) that produce type-2 cytokines (ILC2 cells). They respond to cytokines secreted by the bronchial epithelium, IL-25 and IL-33, by secreting large amounts of IL-5 and IL-13. They are distinct from Th2 cells in that they do not express T-cell or B-cell markers, though they are hematopoietic in origin. Although these cells are not very abundant in tissues, their ability to secrete large amounts of cytokines highlights their potential importance in establishing a type-2 response.

In addition to type-2 cells, our lab previously demonstrated a role for Th17 cell responses in asthma. While Th17 responses alone were insufficient to drive an asthmatic phenotype, we found that the Th17 cytokine IL-17 synergizes with the classical Th2 cytokines IL-4, IL-5, and IL-13 to produce exacerbated disease, including increased neutrophilia and increased airway hyperresponsiveness. Interestingly, IL-17 alone was insufficient to drive increased AHR, but required the establishment of a Th2 response to elicit its exacerbating effect on AHR. Thus, asthma dominated by type-2 responses alone, whether driven by Th2 cells, or ILC2 cells, may represent a milder phenotype, while asthmatics that exhibit both a type-2 and a Th17-mediated response may represent a severe asthma phenotype.
Development of a $T_h2$ response

When T cells migrate out of the thymus, they are characterized as naïve T-cells, and require stimulation in the presence of antigen to drive differentiation. Naïve T-cells circulate through secondary lymphoid organs, such as the spleen and lymph nodes, continuously sampling antigen that is presented on the surface of dendritic cells attached to major histocompatibility complex class II molecules. When it encounters the specific antigen-MHCI complex recognized by its TCR, the T-cell is arrested at the site of the DC. This first signal is accompanied by two additional signals that instruct T cell differentiation. The second signal provided by the DC is co-stimulatory molecules. These are receptor/ligand pairs on the surface of both cells and include CD80/86 which binds to CD28, and OX40 which binds to OX40L.\textsuperscript{101,102} In asthmatics, expression of CD80 and CD86 are both up-regulated, and correlate with increased asthma severity.\textsuperscript{103,104} In a murine model of asthma, mice deficient in OX40L were unable to mount a TH2 response after OVA challenge.\textsuperscript{105} In addition to direct co-stimulation, T-cells require a third signal that participates in T-cell skewing, secreted cytokines. In allergic asthma, these signals have been shown to be provided by IL-25, IL-33, and TSLP to drive a TH2 phenotype.\textsuperscript{106–108} For example, T-cells stimulated with anti-CD28 and IL-33 \textit{in vitro} began to produce the TH2 cytokines IL-4, IL-5, and IL-13 whereas anti-CD28 or IL-33 alone did not induce production of these three signals.\textsuperscript{109} T-cells exposed to these three signals mature and begin to proliferate exponentially.
In asthmatics, allergen-specific T-cells differentiate into the Th2 lineage. These T-cells then begin to proliferate over the course of the next several days, and form effector and memory T-cell lineages. Th2 effector T-cells in asthma mediate many of the events described above. Memory T-cells are long-lived cells that reside in secondary lymphoid organs and respond to subsequent antigen stimulation by DCs to produce additional effector T-cells. Thus, the action of dendritic cells is critical to development of a Th2 response, and asthma for atopic individuals.

**Dendritic cells in asthma**

Pulmonary dendritic cells (DCs) are important for T cell differentiation into a Th2 phenotype in allergic asthma. Knockout mice lacking the co-stimulatory molecules found on DCs display impaired development of experimental asthma For example, PD-L1 knockout mice display reduced AHR and development of a Th2 response to an OVA model of asthma, while PD-L2 knockout mice display increased AHR. Thus, different co-stimulatory molecules have varying effects on the development of a Th2 response. Recently, two subsets of dendritic cells, myeloid DC (mDCs) and plasmacytoid DCs (pDCs) have been identified, and have opposing effects on T-cell differentiation. Our lab has shown that mDCs are particularly important in promoting an asthma phenotype.
Under homeostatic conditions, dendritic cells reside in the lung near the basement membrane at a density of around 600 DCs per mm² (in histological examinations) in the upper airways. This decreases toward the lower airway. These DCs reside just below the bronchial epithelium, and continuously sample antigen in the airway lumen through interdigitating processes. These processes travel between epithelial cells to gain access to antigens in the airway. When the airways are exposed to a danger signal, additional DCs are recruited into the airways; this recruitment peaks around 24 hours after initial exposure at nearly 1000 DCs per mm². In addition to recruited DCs, resident DCs may become activated and both begin to take up antigen. Prior to antigen uptake, DCs are referred to as immature DCs. Immature DCs express the chemokine receptor CCR6, which binds to the chemokine CCL20. This binding to CCL20 induces DC recruitment toward the source of chemokine secretion. CCR6 is required for DC recruitment in the lungs in mouse models of asthma.

After taking up antigen, DCs mature. They stop expressing CCR6, and begin expressing CCR7. This signals DCs to migrate out of the lungs or other tissues, and into draining lymph nodes. As they mature, DCs break down the antigen they took up into short peptide sequences that are loaded onto MHC II and transported to the cell surface. They also begin to express co-stimulatory molecules and secrete cytokines induced by signaling from PRRs at the site of antigen uptake. Once in the lymph
nodes, the mature DCs present the antigen they took up on their surface to T cells. The context in which the DC took up antigen contributes to the co-stimulatory molecules expressed by the DC during antigen presentation. These co-stimulatory molecules determine the type of T cell differentiation that results from antigen uptake.\textsuperscript{122}

\textbf{Figure 3.} Development of a Th2 response after allergen exposure.
The bronchial epithelium in asthma

There are two main types of epithelial cells in the conducting airways: goblet cells and ciliated cells. Goblet cells are the main source of production for the mucous layer that coats the airways. They produce various mucin proteins, and secrete them into the airway lumen. Mucins are large, highly glycosylated proteins that form the basic component of mucus. The mucous traps particles and bacteria from the air and prevents them from reaching the lower airways and the alveoli, thereby clearing most airborne debris from the lungs. Ciliated cells continuously move this mucous layer up toward the esophagus, where it is eventually swallowed. This mechanism is called the mucociliary escalator. A thin layer of liquid, called the sol layer, resides between the cilia and the mucous layer. This, much less viscous, layer allows the cilia to move freely. On their forward stroke, cilia strike the mucous layer and propel it forward. On the reverse stroke, the sol layer allows the cilia to release the mucous layer and prepare for another forward stroke. Bronchial epithelial cells play an important role in regulating this periciliary fluid layer.

In asthmatics, several factors contribute to increased mucous, and even mucous plugs, in the airways. The mucous itself is produced by specialized epithelial cells such as goblet cells in the larger airways and Clara cells in the bronchioles. In asthmatics, the
balance between few goblet cells and many ciliated cells shifts as ciliated cells become, or are replaced by, goblet cells. This leads to an increase in mucous production, and puts greater strain on the mucociliary escalator. Additionally, the periciliary fluid layer becomes dysregulated, leading to stalled mucous secretions. This also results in impaired clearance of mucous from the airways. Interestingly, in addition to its role in Th2 cells, IL-13 has also been shown to directly induce mucous production.

**Ion transport in bronchial epithelial cells**

As the cell type covering the surface of the airways, bronchial epithelial cells are responsible for maintaining a balanced luminal environment. They do this by transporting various ions from the basolateral side of the cell membrane to the apical, or luminal, side and vice-versa. By controlling solute transport, the epithelium can also control movement of water, and luminal pH. However, ion transport also affects other, precisely maintained aspects of cellular homeostasis, so a number of mechanisms have evolved to handle this complex balance. These mechanisms involve the coordinated function of multiple different ion transporters, outlined below. The work outlined in later chapters further explores the role of ion transporters of the bronchial epithelium in the development of downstream immune responses and an asthma phenotype. Understanding how these mechanisms function in the normal lung environment is
important to appreciating how dysregulation of these mechanisms by allergen exposure may promote airway epithelial cells to release danger signals.

Under homeostatic conditions, sodium is maintained at a concentration near 140 mM in the interstitial fluid surrounding most cells, and located at the basolateral membrane of the bronchial epithelium. Intracellular sodium in most cell types is maintained at a concentration near 15 mM. This large difference in sodium concentrations generates a gradient across the cell membrane. Like a capacitor in electronic circuits stores energy as a voltage difference across a similar type of gap, this sodium gradient stores free energy that can be used to transport other ions or solutes across the cell membrane against smaller gradients. This same mechanism exists in reverse for potassium, where intracellular potassium concentrations are maintained at around 120 mM, and interstitial fluid contains approximately 5 mM potassium. The potassium gradient is formed in part through the function of the sodium-potassium adenosine triphosphatase (Na⁺/K⁺-ATPase). Na⁺/K⁺-ATPase transports three sodium ions out of the cell for every two potassium ions it transports into the cell, supporting both gradients. The energy to drive this transport is driven by ATP hydrolysis. Another major ion transporter involved in cell homeostasis is the sodium-potassium-chloride cotransporter (NKCC). NKCC is a symporter that transports one sodium, one potassium, and two chloride ions into the cell for each round of transport. This
transport is driven by the electrochemical gradient of sodium. Maintenance of specific cellular concentrations of sodium, potassium, and chloride is important to allow the cell to respond to many different stimuli, and perform homeostatic functions, including trans-epithelial ion transport.

Ion channels transport ions across the cell membrane through passive diffusion. Two major ion channels active in many cell types, and important in bronchial epithelial cells, are the cystic fibrosis transmembrane conductance regulator (CFTR), and the epithelial sodium channel (ENaC). In bronchial epithelial cells, CFTR resides on the apical surface and exports chloride into the lumen. In contrast, ENaC also resides on the apical surface of the epithelium, but it imports sodium from the lumen.

In addition to ion transport directly through cells (across both the apical and basolateral membranes for epithelial cells) passive diffusion between cells can also occur. Epithelial cells maintain a barrier through close association with one another. Tight junctions between cells bring the membranes close together, and are continuous enough to exclude large molecules from diffusing between the cells. These tight junctions are still permeable to ions, allowing some passive movement of ions across the epithelial barrier. As previously discussed, some active proteases in allergens, such as Der p 1, break down these tight junctions, thus disrupting the tightly regulated flow of
both ions and water into and out of the airway lumen. This disruption potentially impacts the electrochemical balance surrounding and within bronchial epithelial cells, and could potentiate a response from these cells.

Another class of cell junction proteins also plays a role in ion transport. Gap junctions form between cells and allow movement of ions and some small molecules between cells. Gap junctions are formed when two heterodimeric pore complexes, called connexons found on each adjoining cell, come together. Each connexon is a hemichannel made up of different connexin proteins. These hemichannels may be gated, allowing them to open or close based on the cell’s condition. This allows multiple cells to share similar electrochemical properties, and even second messengers, providing a more coordinated response to stimuli.

The function of multiple ion transporters working in concert is required for fluid secretion and absorption in the lumen. For example, water absorption begins with movement of sodium from the lumen, through the cell, to the basolateral membrane. This is accomplished through ENaC on the apical surface, and Na+/K+-ATPase on the basolateral surface (the extra intracellular potassium that results escapes through basolateral potassium channels). Sodium transport through the cell induces paracellular transport of chloride through tight junctions. This in turn generates an
osmotic gradient across the epithelium, as more salt exits the airway, and drives water from the lumen into the interstitial space (figure 4).\textsuperscript{131}

Fluid secretion occurs when CFTR exports chloride from the apical membrane into the lumen. This is balanced on the basolateral surface by NKCC1 importing additional chloride into the cell. Again, this is accompanied by passive diffusion of sodium from the interstitial space through paracellular transport, followed by water.\textsuperscript{132,133} All these mechanisms work in concert to promote a balanced electrochemical environment in the airways under homeostatic conditions. However, many of these mechanisms are dysregulated in asthmatic airways.

The airways of acute asthmatics are markedly more acidic than non-asthmatic airways.\textsuperscript{134} One mechanism by which luminal pH can be controlled is by transporting bicarbonate (\(\text{HCO}_3^-\)) into the airway lumen. Chloride-bicarbonate exchangers have been proposed to regulate luminal pH.\textsuperscript{135,136} Note that chloride movement is used to power bicarbonate transport. This is yet another way that careful control of ion transport and ion gradients can affect physiological changes in the airways.
The bronchial epithelium and immunity

Airway epithelial cells have long been recognized as aiding in immunity by maintaining the mucociliary escalator, and by their barrier function. In recent years, there has been greater appreciation for a more direct interaction between the airway epithelium and dedicated immune cells. In response to pathogen-associated molecular patterns (PAMPs), they release multiple chemokines and cytokines that help drive inflammation and clear infection. For example, double-stranded RNA, an agonist for TLR3, induced expression of IL-8 and CCL20 in the human lung epithelial cell line
BEAS-2B; while zymosan, an agonist for TLR2 and dectin-1, induced expression of GM-CSF and CCL20 in these same cells.\textsuperscript{138} In asthmatics, epithelial cells release these mediators in response to molecular patterns found within allergens, helping to drive an inappropriate immune response.\textsuperscript{139} As mentioned previously, airway epithelial cells release IL-33 and IL-25. These activate ILC2 cells and help drive a type-2 response, and stimulate DCs as well.\textsuperscript{106,107} In addition, the epithelium responds to signals from T\textsubscript{H}2 T-cells. For example, the bronchial epithelial cells of asthmatics have been shown to exhibit phosphorylated STAT-6, and they respond to IL-4 exposure by secreting IL-8.\textsuperscript{140} Thus, in addition to their role in regulating the airway environment, bronchial epithelial cells provide many initial signals that promote down-stream immune responses.

**Role of chemokines/cytokines in allergic asthma**

Cytokines are proteins released from cells that serve as intercellular signaling molecules. They differ from hormones in that they normally function at shorter distances where the target cells are generally within the microenvironment of the cell releasing the cytokine, as opposed to hormones which generally circulate systemically. Cytokines that induce chemotaxis of leukocytes toward the chemokine source are called chemokines, because they act as *chemotactic cytokines*. Many chemokines and cytokines are critical in the development and pathogenesis of allergic asthma. Some important examples include cytokines such as IL-4, IL-5 and IL-13; and chemokines such as CCL20
and IL-8. The bronchial epithelium participates in the release of IL-8 and CCL20; but not IL-4, IL-5, and IL-13.

**Inflammatory chemokines**

Bronchoalveolar lavage of asthmatic lungs reveals significant increases in cellularity compared to non-asthmatics. This same lavage fluid also contains elevated levels of a number of chemokines and cytokines that have inflammatory effects. In recent years, this has led to work on a number of cytokine-directed therapies that are currently under development. Some specific chemokines and cytokines that are important in the development of asthma are discussed below.

**CCL20**

One chemokine that is elevated in the BAL of asthmatics is CCL20. The only known receptor for CCL20 is the chemokine receptor CCR6, which also binds beta-defensins, although with much lower affinity. CCL20 was originally discovered in liver tissue and named liver and activation-regulated chemokine, or LARC. It is constitutively expressed in multiple cell types, including epithelial cells of the skin, intestine, and airways. In addition to this constitutive expression, production and release of CCL20 may also be induced by various stimuli. These include cytokines such as TNF-α or IL-1α, as well as bacterial pathogens or other danger signals. We have previously shown that bronchial epithelial cells release CCL20 when exposed to the allergen house dust mite (HDM). Indeed, this release can happen rapidly after
HDM exposure (Figure 5). CCL20 has also been shown to be released from bronchial epithelial cells in response to particulate matter.\(^\text{157}\)

![Figure 5. CCL20 release within 30 minutes of HDM exposure. (Nathan et al. 2009)](image)

A number of studies have demonstrated that signaling of CCL20 through CCR6 contributes to the development of allergic asthma. Studies with CCR6 knockout mice showed that it is required for pulmonary inflammation after allergen challenge, and have linked it to recruitment of DCs and CD-4\(^+\) T-cells in asthmatic airways.\(^\text{158}-\text{161}\) CCL20 has also been linked to the recruitment of T\(_{H}17\) cells,\(^\text{162}\) which also express CCR6.\(^\text{163}\) As mentioned previously, T\(_{H}17\) cells may promote more severe, steroid resistant asthma. Signaling through CCL20 may explain why we see elevated levels of T\(_{H}17\) cells in asthmatic airways.\(^\text{164}\) Indeed, patients with steroid resistant asthma had ten times as much CCL20 in their bronchoalveolar lavage fluid as patients with asthma that responded to steroid treatment.\(^\text{165}\)
In addition to its role in inflammation, CCL20, like many chemokines, is also a potent antimicrobial peptide, with an LD$_{50}$ of 400 ng/mL for *Escherichia coli*.\(^{166}\) This antimicrobial activity is thought to be particularly active in acidic environments\(^ {167}\) where dimeric CCL20 separates into monomers, thus exposing its carboxy-terminal end, which is thought to directly mediate the protein’s antimicrobial activity.\(^{168}\) This cell-free antimicrobial activity varies greatly depending on the microbial target (table 1) and is not limited to the chemokine CCL20 (table 2).

**Table 1.** Adapted from Yang, et al. 2003.
### The antimicrobial activity of selected chemokines

<table>
<thead>
<tr>
<th>Chemokine Family</th>
<th>Chemokine Member</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC</td>
<td>CXCL1/Gro-α</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CXCL2/Gro-β</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CXCL3/Gro-γ</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CXCL6/GCP-2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CXCL8/IL-8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CXCL9/MIG</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>CXCL10/IP-10</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>CXCL11/I-TAC</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>CXCL12/SDF-1</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CXCL13/BCA-1</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CXCL14/BRAK</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>CX3C</td>
<td>CX3CL1/fractalkine</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
<td>XCL1/lymphotactin</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>CC</td>
<td>CCL1/I-309</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CCL2/ MCP-1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL3/ MIP-1α</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL5/RANTES</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL7/ MCP-3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL8/ MCP-2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL11/eotaxin</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>CCL13/MCP-4</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL16/LEC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL17/TARC</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>CCL18/PARC</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>CCL19/ MIP-3β</td>
<td>++++</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL20/MIP-3α</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>CCL21/SLC</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>CCL22/MDC</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>CCL25/TECK</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>CCL27/CTAK</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Table 2.* Adapted from Yang, et al. 2003.
Other inflammatory chemokines and cytokines

Many other chemokines have been shown to be released in the BAL of asthmatics in addition to CCL20. Among others, these chemokines include Gro-α, IL-8, and to a lesser extent IP-10. These chemokines help to recruit many of the cell types seen in asthmatics. Gro-α and IL-8 recruit neutrophils by signaling through the chemokine receptors CXCR2 (for both Gro-α and IL-8) and CXCR1 (for IL-8). IP-10 is responsible for the recruitment of a variety of cell types, including T-cells and DCs, through its receptor CXCR3.

Cytokines reported in the BAL of asthmatics include granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-2, IL-5, IL-6, IL-13, and TNF-α. Additionally, other cytokines that have been reported to be released by the bronchial epithelium, such as thymic stromal lymphopoietin (TSLP) and IL-33, have also been reported to play a significant role in asthma pathogenesis. Major roles for each of these cytokines in asthma are listed in table 3. In addition to de novo production after allergic stimuli, production of many chemokines and cytokines by the bronchial epithelium has been reported under homeostatic conditions. Thus a number of
proteins work in concert to promote a T\textsubscript{H}2 response, with many redundant signals leading to some important phenotypic changes, such as neutrophilia.

The role of cytokines active in the pathogenesis of asthma

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Role in asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>Promotes DC maturation and differentiation.\textsuperscript{179}</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Processed from proIL-1β through inflammasome activation. Leads to production of other inflammatory cytokines, such as IL-6.\textsuperscript{180}</td>
</tr>
<tr>
<td>IL-4</td>
<td>Promotes the development and maintenance of T\textsubscript{H}2 cells.\textsuperscript{80,88}</td>
</tr>
<tr>
<td>IL-5</td>
<td>Promotes eosinophilia.\textsuperscript{91}</td>
</tr>
<tr>
<td>IL-6</td>
<td>Prevents T\textsubscript{reg} development, promotes T\textsubscript{H}17 development.\textsuperscript{181}</td>
</tr>
<tr>
<td>IL-13</td>
<td>Necessary for goblet cell metaplasia,\textsuperscript{182} and the development of asthma.\textsuperscript{93,95}</td>
</tr>
<tr>
<td>IL-25</td>
<td>Produced by BECs. Promotes T\textsubscript{H}2 differentiation.\textsuperscript{183}</td>
</tr>
<tr>
<td>IL-33</td>
<td>Produced by BECs. Promotes T\textsubscript{H}2 differentiation.\textsuperscript{184}</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Airway remodeling,\textsuperscript{185} T-cell activation,\textsuperscript{186} neutrophil and eosinophil recruitment.\textsuperscript{187}</td>
</tr>
<tr>
<td>TSLP</td>
<td>Produced by BECs. Helps drive DC activation and development of a T\textsubscript{H}2 response.\textsuperscript{188,189}</td>
</tr>
</tbody>
</table>

Table 3.

\textit{Secretory lysosomes}

One cellular compartment that is identified in this thesis as a major storage location for chemokines and cytokines is in lysosomes. Lysosomes are acidic organelles within the cell that serve as a primary site of digestion for various cell components.\textsuperscript{190} The structure of lysosomes is surprisingly complex, and reflects the multiple roles they play beyond recycling of cellular components traditionally thought to be their main function. Lysosomes are highly acidic, with a pH of around 4.3.\textsuperscript{191} This acidification is driven by the vacuolar-type H\textsuperscript{+} ATPase, or V-ATPase.\textsuperscript{192} V-ATPase is a proton pump
that uses the energy from ATP hydrolysis to transport protons from the cytosol into lysosomes. V-ATPase function can be inhibited by a number of molecules, including bafilomycin A.\textsuperscript{193} Since many of the enzymes within lysosomes require low pH to function, inhibition of V-ATPase may affect both the development of new lysosomes, and the function of existing lysosomes.

The action of V-ATPase presents an interesting physiological problem. As protons are pumped from the cytosol into the lysosome, they are moving from an environment that holds a net negative charge into an environment whose positive charge increases with each additional proton. If this mechanism acted alone, acidification would soon cease as the energy from ATP hydrolysis soon became insufficient to drive new protons into the lysosome, and therefore the low lysosomal pH could not be achieved. In recent years, a number of papers have been published showing that the charge balance problem can be overcome in lysosomes by allowing negatively charge chloride ions into the organelle. Proton-chloride exchangers, such as CLC-7 and CLC-5, are found on lysosomes and developing endosomes, respectively.\textsuperscript{194,195} These exchangers use the free energy from allowing the protons to escape through the transporter to drive chloride movement into the lysosome (figure 6). The net result of the action of both V-ATPase and Cl/H\textsuperscript{+} exchanger action is an acidic lysosome with a high chloride concentration.\textsuperscript{196} This high chloride concentration
further contributes to cytotoxic killing by lysosomes. These structural components of lysosomes will be explored in later chapters as the localization of intracellular chemokines and cytokines is investigated.

**Figure 6.** (A) Diagram of lysosome, showing the electron-dense protein core, ion transporters involved in acidification, the membrane protein lamp-1, and membrane voltage (V) that is balanced by chloride ions. (B) Double labeling of Lamp-1 (10 nm gold) and granzyme B (15 nm gold) in cytotoxic T lymphocytes. Granzyme B labeling is mainly present within the electron-dense core. Lamp-1 is predominantly present at the outer membrane of the granules. The plasma membrane is labeled (p). Adapted from Peters et al 1991.

Many proteins are found within lysosomes in various cell types, including some chemokines and cytokines in cells of the hematopoietic lineage. These proteins are not solely associated with lysosomal localization, however. Some lysosome-specific
proteins include proteases such as cathepsins, and membrane-associated proteins such as LAMP-1. The aggressive proteases found in lysosomes are inactive at the higher pH within the cell, or at lower chloride concentrations, which is a protective mechanism against inappropriate digestion during enzyme transport. Not all proteins found in lysosomes are immune to the caustic environment there. These proteins are targeted to a protective structure in lysosomes that, when visualized by electron microscopy, appears as an electron dense core within the lysosome. This core is made up of proteins bound to sulfated proteoglycans that help to protect the proteins from degradation. In secretory lysosomes these complexes of proteins and proteoglycans are released upon exocytosis.

Secretory lysosomes are a specific class of Lamp-1—containing lysosomal vesicles that are capable of fusing with the plasma membrane and releasing their content into the extracellular milieu. Secretory lysosomes have been studied predominantly in cells of the hematopoietic lineage, but they have been identified in other cell types as well – including epithelial cells. In these various cell types, secretory lysosomes have been shown to perform a diverse variety of functions. For example, skin pigment-producing cells, called melanocytes, produce lysosome-like organelles, called melanosomes, through which they transfer pigment to the surrounding epithelial cells. A specialized type of macrophage, called osteoclasts,
use enzymes stored in secretory lysosomes to break down and recycle bone matrix. And in many specialized immune cell types, cytotoxic and antimicrobial peptides and mediators are stored in secretory lysosomes. These can then be released in a context-dependent manner. For example, upon cross-linking of IgE mast cells release histamine and other mediators that can attack invading pathogens. Additionally, natural killer cells and cytotoxic T cells store granzyme and perforin, which they use to kill specific target cells that may be cancerous or virally infected. Thus, secretory lysosomes operate in a variety of different cell types, and their release allows for context-specific responses to a variety of stimuli.

Finally, in some cell types, such as neutrophils, multiple types of secretory lysosomes have been identified within a single cell. This allows the cell to respond to different stimuli by releasing the contents of different lysosomes. Many different chemokines and cytokines have been shown to reside within these vesicles. For example, the chemokine IL-8 is stored and secreted from Weibel-palade vesicles in endothelial cells. Although much research has contributed to a better understanding of the complex loading and release mechanisms of different types of secretory lysosomes in cells of the hematopoietic lineage, identification of potential secretory lysosomes and their contents in epithelial cell types, such as the bronchial epithelium, has yet to be explored.
**Syndecans and heparin sulfate proteoglycans**

Another potential storage site for chemokines and cytokines is on structural molecules attached to the cell membrane on the extracellular side. On the baso-lateral surface of the cell, chemokines and other proteins can be stored on syndecans.\(^{215}\) Syndecans are proteoglycans, where the trans-membrane protein stalk is modified with chains of polysaccharides called glycans. The syndecan protein consists of a small intracellular domain, a trans-membrane domain, and a larger extracellular domain where the glycan modifications are attached. The glycan linkages on syndecans contain of heparin sulfate (HS). These side chains bind to other elements of the extracellular matrix and help anchor the cell.\(^{216}\)

In addition to their structural role, syndecans also participate in cell signaling. Many chemokines, cytokines, and growth factors bind to HS, like that found on syndecan, including: interferon-\(\gamma\), epidermal growth factor, hepatocyte growth factor, vascular endothelial growth factor, fibroblast growth factor family members, IL-1\(\alpha\), IL-1\(\beta\), IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, GM-CSF, and Gro-\(\alpha\).\(^{217-227}\) When the side chains are released, the bound chemokines and cytokines are released as well, providing a rapid storage and release mechanism on the cell surface.\(^{228}\) Near the base of the extracellular domain of syndecans are cleavage sites where syndecans may be cut by matrix
metalloproteinases. In addition to cleavage of the protein stalk, the bonds holding side chains themselves together may be broken by a variety of mechanisms, including hydrolysis by nitric oxide, and degradation by release of heparanase which is secreted from lysosomes. Through cleavage of the protein stalk or by direct cleavage of HS the proteins associated with the side chains of syndecan may be released from the cell surface. In the case of chemokines, like IL-8, this release forms a gradient that can help recruit inflammatory cells, such as neutrophils.

In asthma, HS in general and syndecans in particular play an important role in the development of an allergic response. Mice deficient in HS develop less severe airway responses in an ova-induced model of asthma. In contrast, mice deficient in syndecan-1 displayed increased airway inflammation in a mold model of asthma. Thus it appears that the specific agonist used to induce an allergic response determines the role of syndecans and HS in either promoting or attenuating the response.
Summary

Asthma is a chronic inflammatory disease affecting millions of people worldwide. In affected individuals, it causes narrowing of the airways through airway smooth muscle hypertrophy and contraction, increased airway mucous, inflammation, and eventually permanent changes in airway structure brought about by fibrosis. These features are primarily mediated through type-2 responses, whether through adaptive Th2 cell responses or through the newly identified ILCs. The Th2 response in asthmatics develops as DCs in the lungs take up allergens in the lungs and
subsequently drive the development of TH2 cells. Dendritic cells receive signals to migrate into the sub-epithelial space and take up antigen by chemokines and cytokines that may be secreted by bronchial epithelial cells. Many allergens have been shown to induce the secretion of these chemokines and cytokines into the lavage fluid of asthmatics.

Airway epithelial cells manage the mucociliary escalator, airway pH, and fluid in the airways. Much of this regulation is maintained by balancing ion transport across the epithelium. Additionally, airway epithelial cells release chemokines and cytokines in response to allergens and other agonists.

Previously, we and others found that CCL20, an important chemokine for immature dendritic cell recruitment, is strongly released directly from bronchial epithelial cells after HDM stimulation. However, the mechanisms driving production and release of protein mediators from the bronchial epithelium are poorly understood. Many mechanisms have been identified in other cell types for the storage and secretion of chemokines and cytokines. These include storage and secretion from intracellular compartments such as secretory vesicles and secretory lysosomes. In addition, chemokines and cytokines may be stored on the cell surface on syndecans. They are then released by enzymes that degrade the heparin sulfate side chains, or protein stalks...
found on syndecans. Finally, chemokines and cytokines may be produced and secreted
*de novo* after cell stimulation.

Understanding the mechanism and kinetics of chemokine release in general, and
CCL20 release specifically, from bronchial epithelial cells will help in our
understanding of the initial processes driving sensitization. In addition, this may lead
to better methods of intervention in the treatment of asthmatics. *We hypothesize that
rapid CCL20 release from bronchial epithelial cells is driven through the release of pre-formed
extracellular and intracellular stores.*

The specific aims of this dissertation are:

1. Determine the kinetics and localization of CCL20 chemokine release from BECs
   from both intracellular and extracellular stores.
2. Determine the signaling pathways that lead to mobilization and inhibition of
   CCL20 stores.
3. Identify the allergen selectivity of CCL20 release.
4. Identify other chemokines and cytokines that are stored in BECs and rapidly
   released after allergen stimulation.
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Chapter 2. Mechanism of CCL20 rapid release from allergen exposed epithelial cells

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Abstract

Rapid chemokine release by the bronchial epithelium is an important initial step in the development of an adaptive response against allergen. Previous studies demonstrated CCL20 release within 30 minutes of HDM exposure. We asked whether this rapid release of CCL20 is from pre-formed stores. We found that after allergen exposure, CCL20 is quickly secreted via degranulation from preformed intracellular lysosomal stores. Targeting the lysosome-specific V-ATPase and CLC-7 ion channels decreased HDM-induced CCL20 secretion. Lysosomal CCL20 release was dependent on signaling through syk and raf1 kinases. Our findings reveal a novel pathway by which the bronchial epithelium releases pre-formed intracellular chemokine stores.
Introduction

Allergic asthma is a chronic inflammatory disease of the airways whose prevalence continues to rise in developed nations. Although the origins of allergic asthma are complex, excessive activation of T helper type 2 (Th2) cells specific for normally innocuous environmental allergens drives disease pathology.\(^1\) However, the initiating events following allergen exposure leading to the development of a Th2 response remain to be elucidated.

After exposure to allergen, the development of a Th2 response is dependent on the recruitment of immature dendritic cells (iDCs) to the lungs. This recruitment is primarily mediated by the chemokine CCL20,\(^2\) a strong chemoattractant for iDCs. CCL20 binds uniquely to CCR6, which is strongly expressed by iDCs, and is vital for their recruitment into the lungs following allergen exposure.\(^3,4\) The epithelium appears to be the major source of CCL20 in the airways,\(^5\) and we and others have shown that its secretion is strongly induced by allergen.\(^6\) Interestingly, chemokine release from cells can happen within a few minutes after contact with pattern recognition receptors or other agonists,\(^7\) suggesting they are contained in pre-formed stores. Rapid secretion of cytokines from preformed stores has typically been studied in granulocytes – in which the mechanisms governing release have been examined extensively – and this release often involves secretory lysosomes.\(^8,9\) Rapid release of chemokines from structural cells has also been reported,\(^10\) but the mechanisms driving this release – including whether they are released from extracellular stores, intracellular stores, or both – remain to be explored.
Dendritic cell recruitment to the lungs has been shown to occur within a few hours after allergen exposure in both mice and humans. As iDCs are recruited by the chemoattractant CCL20, this suggests that CCL20 is released within this time period. We thus hypothesized that bronchial epithelial cells contain preformed stores of CCL20 for quick release in response to HDM exposure and this release leads to the initial DC recruitment. We demonstrate that pools of CCL20 are stored within lysosomes, and these pools rapidly degranulate upon exposure to HDM. These data point to a previously unknown mechanism of chemokine/cytokine release by AECs from secretory lysosomes, and suggest that the epithelium may be a source of pre-formed cytokines designed for rapid release. This newly identified mechanism could contribute to the initiating events that lead to allergic inflammation.
Materials and Methods

**Bronchial Epithelial Cells:** 16HBE14o- cells were seeded at 12,500 cells/cm² in 24-well collagen-coated culture plates in DMEM including 10% FBS. At 75% confluence, cells were serum starved in DMEM plus 0.1% FBS overnight prior to cell stimulation. All inhibitors were added one hour prior to HDM addition. HDM was added to cell supernatants at a concentration of 100 μg/ml.

**Inhibitors:** Inhibitors were titrated to determine maximal efficacy without affecting cell viability. Concentrations used are: bafilomycin A (Sigma, 0.5 pM), piceatannol (4 uM), Bay 61-3606 (Sigma, 2 uM), Raf 1 kinase inhibitor I (EMD Millipore, 10 uM), R0 31-8220 (Cayman Chemical, 10 uM).

**Separation of cellular components:** Supernatants were removed directly from cells after treatment and stored at -20°C to -80°C. Cell lysates were obtained by rinsing treated cells in PBS followed by 1-2 rapid freeze-thaw cycles. RNA was obtained using TRizol reagent (Invitrogen) and isolated according to manufacturer’s protocol. First-strand cDNA was synthesized using the Superscript II First-Strand Synthesis kit (Invitrogen).

**ELISA:** Human CCL20 (DuoSet, R&D Systems) and human IL-8 (OptEIA, BD) ELISAs were performed according to their respective protocol specifications.

**Degranulation:** Alexa-fluor647-conjugated Lamp-1 antibody (eBioscience, clone# eBioH4A3) was added to cell culture concurrently with allergen. After 30 minutes, cells were
rinsed with PBS, and removed from culture plate with trypsin. Antibody uptake was then analyzed by flow cytometry.

*Tissue and cell slide preparations:* Mouse lungs were fixed in formaldehyde, and paraffin embedded. 2 μM sections were cut, then deparaffinized in xylene, and rehydrated in a graded ethanol series followed by water. Antigen retrieval was done in citrate buffer (10 mM sodium citrate, 0.05% Tween-20) at 90°C for 20 min. Human 16HBE14o- cells were grown and treated in 8-well chamber slides. After treatment, slides were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes and rinsed with PBS.

*Immunofluorescence staining:* Slides were permeabilized with 0.1% Triton-X 100, and blocked in 10% species serum matching the host of the secondary antibody. Slides were incubated overnight at 4°C in primary mouse CCL20 antibody (Abcam, ab9829) or human CCL20 antibody (R&D Systems, AF360). Fluorescently labeled secondary antibodies (Invitrogen) were incubated for one hour at RT; slides were then rinsed with PBS-Tween 0.1% and mounted with VectaShield (Vector Labs).

*Live immunofluorescence:* To visualize CCL20, we generated a CCL20-mCherry fusion protein by inserting the human CCL20 open reading frame (minus the endogenous stop codon) into a mammalian expression plasmid (BD Biosciences), upstream of mCherry. Lamp1-YFP was obtained from AddGene. Rab3a-GFP, and Rab7-GFP were obtained from Origene. 16HBE14o- cells were grown in Fluorodish (WPI) confocal dishes, and plasmids were transfected with FuGENE HD (Roche) overnight. Cells were then serum-starved (0.1% FBS) for 24h, before stimulation with HDM. LysoTracker (Invitrogen), ERTracker (Invitrogen), and NBD-
C6ceramide (Invitrogen) dyes were individually loaded into cells 30 minutes prior to imaging according to manufacturer protocol. Images were taken with a Zeiss LSM 710 confocal microscope.

*Immunofluorescence quenching:* Cells transfected with CCL20-mCherry were fixed, but not permeabilized. Cells were then stained with anti-RFP polyclonal antibody (Invitrogen, R10367), or control antibody prior to immunofluorescence imaging.
Results

*Kinetics suggests release of CCL20 is derived from preformed stores.*

To better understand the mechanisms controlling CCL20 release, we first sought to assess the kinetics of CCL20 release after stimulation with HDM. We treated the human bronchial epithelial cell line, 16HBE14o-, with HDM and measured CCL20 and IL-8 release, as well as mRNA at various times. We found that HDM induced significant CCL20 release into the supernatant in as little as five minutes (Fig. 1A), which continued to increase throughout the first hour. We saw little further increase in CCL20 release from two hours to six hours. This suggests that early release of CCL20 came from preformed stores, and that these stores were depleted during the first one to two hours after HDM exposure. In support of this hypothesis, the early burst of CCL20 protein was followed by an increase in CCL20 message 6h after HDM exposure (Fig. 1C), after which we saw a further increase of CCL20 in the supernatant from 6 to 24 hours after HDM exposure (Fig. 1A). Treatment with cycloheximide, which inhibits translation of new protein from mRNA, for one hour prior to HDM exposure had no effect on CCL20 release after 2 hours, suggesting early CCL20 release is independent of *de novo* synthesis (Fig. 1E).

In addition to HDM-induced release at 24 hours, significant accumulation of baseline release also appears to contribute to the overall level of CCL20 in the supernatants of these cells. This suggests that low levels of CCL20 are released from bronchial epithelial cells at baseline, with an increase induced after HDM exposure. The difference between CCL20 released from HDM-exposed cells and CCL20 released from control-treated cells appears to represent the
contribution of HDM to the CCL20 released from bronchial epithelial cells. Interestingly, this difference (HDM minus control) at 24 hours of HDM exposure represents a doubling of cumulative release over the difference of HDM minus control after 2 hours. This suggests that the first two hours of CCL20 release that can be attributed to HDM exposure represents half of the total release in the first 24 hours, and that HDM-induced release slows after the first two hours of HDM exposure.

In contrast to the kinetics seen for CCL20, significant increases of IL-8 protein release into the supernatant only began to appear after 2h of HDM treatment (Fig. 1B). This suggests IL-8 may be released through a different mechanism than that governing CCL20. Furthermore, IL8 expression levels did not appear to increase before 24h of HDM exposure (Fig. 1D). Given that IL-8 is released prior to this time, these data suggest that the increase in IL-8 release is derived from preformed stores of IL-8 protein, or IL-8 mRNA that is later transcribed. To determine whether transcription of IL-8 mRNA is required for IL-8 protein release in response to HDM, we pre-treated cells with cycloheximide one hour prior to exposing them to HDM for 2 hours. Again in contrast to results seen with CCL20, IL-8 release was dramatically reduced in the presence of cycloheximide, suggesting secretion of IL-8 requires translation of previously formed mRNA. Also, the ratio of HDM-induced IL-8 release to control IL-8 release also remained the same at 2 and 24 hours, suggesting the rate of release did not decrease over time, as was observed in HDM-induced CCL20 release. Taken together, our findings indicate that CCL20 and IL-8 are regulated by two different mechanisms.
**CCL20 is stored in intracellular and extracellular pools.**

To determine whether CCL20 is stored in distinct cellular pools, we transfected 16HBE14o- cells with CCL20 that was conjugated with the RFP-derived fluorophore mCherry (CCL20-mCherry). The CCL20 gene was inserted at the N-terminus, with mCherry at the C-terminus, in order to ensure that any signal peptide remained intact. Epithelial cells transfected with a CCL20-mCherry fusion construct show both diffuse cellular staining (compatible with the hypothesis that stores of chemokine are retained on the surface of the cell) and punctate, granule-like stores (consistent with the hypothesis that stores of chemokines are retained in intracellular vesicles); these results support previous reports indicating that chemokines can be stored both in intracellular and extracellular locations.\textsuperscript{13,14} To test whether intracellular CCL20-mCherry fluorescence could be distinguished from extracellular fluorescence, cells were left not permeabilized and incubated with an anti-RFP antibody, which has been shown to quench RFP fluorescence upon binding.\textsuperscript{15} We observed that the diffuse staining seen in the control panel was lost in cells treated with the quenching antibody (Fig. 2A), suggesting that fluorescence from cell surface stores of CCL20-mCherry were quenched by the antibody, whereas the punctate CCL20 stores were unaffected by the quenching antibody, as we expect that these intracellular stores are inaccessible to the quenching antibody.

We next sought to distinguish the cellular pools of CCL20 that are specifically released in response to HDM, and whether they are derived from intracellular or extracellular sources. To do this, we removed surface CCL20 in order to isolate the effects of HDM on intracellular CCL20 only. We digested surface CCL20 by treating cells (that had already been exposed to PBS or HDM for two hours) with trypsin until cell adherence was lost. As a control for the
effects of a sudden loss of adherence, cells were also dissociated using a non-enzymatic method. The trypsin was then neutralized with 10% FBS (which was also applied to dissociation buffer controls), and the concentration of CCL20 remaining in cell lysates was analyzed (Fig. 2B). As expected, we observed a reduction in preformed CCL20 in the lysates of HDM-treated cells, presumably because it is being released from the cells into the supernatant. Under control conditions, lysates from non-enzymatically lifted cells (CD) contained more CCL20 than lysates from trypsin-treated cells (trypsin), suggesting that significant stores of CCL20 are stored on the exterior of the cells, where they were exposed to the trypsin. Although this extracellular CCL20 may also contribute to HDM-induced release of CCL20 into the supernatant, our measure of intracellular CCL20 (the trypsinyzed cells) showed that remaining stores of CCL20 significantly decreased after HDM treatment, demonstrating that intracellular stores of CCL20 are an important source of HDM-induced release into the supernatant after allergen exposure (Fig. 2B).

To further demonstrate that the early burst of CCL20 is from ready-to-be-secreted stores and does not necessitate trafficking through the endoplasmic reticulum and the Golgi apparatus, we inhibited vesicular trafficking with brefeldin A (which inhibits ER trafficking) or monensin (which inhibits golgi trafficking). These two molecules inhibit vesicular trafficking, from transcription and translation, through different mechanisms. We found that pretreatment of cells with these inhibitors did not prevent early (2h) CCL20 release after HDM exposure (Fig. 2C). In contrast, early IL-8 release was completely sensitive to brefeldin A for both baseline release (control) and HDM induced release; whereas for monensin baseline
release remained unaffected and HDM-induced release was decreased by one third (Fig. 2D).
This suggests that the majority of IL-8 export is not routed through the golgi apparatus in a
monensin-sensitive manner, but that it is processed through the ER. After 24 hours of HDM
exposure, CCL20 was decreased by both brefeldin and monensin, suggesting de novo production
and trafficking through the Golgi apparatus are necessary to replenish CCL20 stores that are
released in response to the initial stimulus. Interestingly, brefeldin A again had a stronger
impact on reducing both baseline chemokine release, as well as the ratio of HDM-induced
release to baseline. This suggests that after 24 hours, substantial stores of CCL20 de novo
production are trafficked through similar pathways to those of IL-8. The IL-8 release
mechanism appeared to remain constant from 2 to 24 hours, affected solely by brefeldin A (Fig.
2E, F). A similar distribution of cellular CCL20 was observed in mouse lung tissues stained
with CCL20, suggesting this pattern applies in vivo, as well as in vitro (Fig. 2G).

We next sought to determine the specific intracellular organelle to which CCL20 is
targeted for storage in 16HBE14o- cells. To do so, we input the human CCL20 peptide sequence
into the SignalP 4.0 server hosted by the Center for Biological Sequence Analysis to identify
putative signal peptide cleavage sites in the CCL20 protein sequence. We found one putative
cleavage site at SEA-AS, between peptides 26 and 27. When we BLASTed the first 26 peptides,
MCCTKSLLAALMSVLLLHLCGESEA, we found a number of proteins with a high degree of
sequence homology within their predicted signal peptide regions. One such protein that
contained a highly similar sequence to that of CCL20 within its own predicted signal peptide
was cathepsin C, a lysosomal enzyme (Table 1). This correlation suggested a similar cellular
trafficking of both CCL20 and cathepsin C to lysosomes. To test whether CCL20 is stored in
lysosomes, we co-transfected cells with CCL20-mCherry and markers for various types of
intracellular organelles, including traditionally classified secretory vesicles (Rab3a-GFP), late
endosomes/lysosomes (Rab7-GFP), and lysosomes (Lamp-1-YFP). No overlay was observed
between CCL20-mCherry fluorescence and that of rab3a-GFP (Fig. 3A), suggesting that CCL20
does not traffic through secretory vesicles distinguished by the marker rab3a. Partial co-
localization was observed with rab7-GFP, suggesting that CCL20 can traffic through the late
endosomal compartment (Fig. 3B). However, CCL20 fluorescence was strongly correlated with
lamp-1-YFP fluorescence, suggesting that the majority of intracellular CCL20 is contained
within lysosomes (Fig. 3C). Our previous observation that at least half of the cellular CCL20
resides on the external cell membrane suggests that some external CCL20 should be expected.
However, this is likely to be distributed throughout the extracellular membrane, and may
therefore be too diffuse to observe juxtaposed to more highly concentrated lysosomal pools of
CCL20. Given that these cells are not grown in a trans-well environment, they are not polarized
and we would not expect them to specifically localize the extracellular CCL20 to the baso-lateral
surface. Accordingly, during imaging, no such polarized surface concentration of CCL20 was
observed.

To test whether lysosomal CCL20 is released in response to HDM, cells were transfected
with CCL20-mCherry and stained with LysoTracker dye, which inserts into acidic vesicles, such
as lysosomes. In un-stimulated cells, we observed that most CCL20-mCherry was localized to
LysoTracker-containing acidic organelles; but after HDM treatment, lysosomal CCL20 was dramatically decreased (Fig. 4A).

We next wanted to examine whether epithelial cells undergo degranulation after HDM treatment. During degranulation, lamp-1 (which is normally found on the lysosomal membrane) relocates to the cell surface and levels of surface lamp-1 can be measured by flow cytometry. We observed that HDM induced a significant increase in degranulation, as measured by lamp-1 uptake, compared to control (Fig. 4B) treatment.

To test whether functioning lysosomes are required for CCL20 release, we treated cells with bafilomycin A, which prevents formation of lysosomes by blocking their acidification via the V-type H^+-ATPase (V-ATPase).^{18,19} We observed that cells treated with bafilomycin displayed reduced CCL20 release in response to HDM (Fig. 4C), suggesting functional lysosomes are necessary for CCL20 release. These data suggest CCL20 is stored and released from secretory lysosomes. V-ATPase has been shown to localize to the cell surface, as well as to lysosomes. To distinguish between the roles of lysosomal and cell surface V-ATPases in contributing to CCL20 release, we specifically blocked extracellular V-ATPase using concanamycin A, which does not penetrate the cell membrane and cannot inhibit lysosomal V-ATPases. Concanamycin had no effect on HDM-induced CCL20 (Fig. 4D), demonstrating that extracellular V-ATPase does not contribute to CCL20 release. To further confirm whether intracellular stores of CCL20 are sensitive to lysosome inactivation, we looked at CCL20 retention in cells treated with the lysosome inhibitor bafilomycin A. To to this, we treated cells with bafilomycin A prior to HDM treatment; we then trypsinized cells (to remove extracellular
CCL20 stores) and measured CCL20 in cell lysates. We observed that in bafilomycin A-treated cells intracellular CCL20 was retained after HDM exposure, suggesting inhibition of lysosomes with bafilomycin A specifically inhibited release of intracellular (lysosomal) CCL20. In contrast, cells containing both intracellular and extracellular stores of CCL20 displayed decreased levels of CCL20 retained in the cell lysates after HDM exposure (Fig. 4E), suggesting that this difference was derived from extracellular CCL20 stores.

In addition to V-ATPase, recent studies have shown that function of the chloride-proton cotransporter CLCN7 is also necessary for lysosome acidification. To further verify that lysosome function is necessary for CCL20 release, Clcn7 was knocked down via siRNA. We observed a reduction of CCL20 release after HDM exposure in Clcn7 knockdown cells compared to scrambled controls (Fig. 4F). These data further support our hypothesis that functional lysosomes are necessary for CCL20 release. Taken together, these data suggest that CCL20 is stored in lysosomes within the cell, and that HDM induces degranulation of these lysosomes.

**CCL20 release is mediated through beta glucan and syk/raf1 kinase signaling.**

Previously we reported that beta-glucan signaling is necessary for HDM-induced CCL20 release after 24 hours. To test whether beta-glucans are necessary for CCL20 release at the 2 hour time point we have explored in this study, we digested HDM with beta-glucanase prior to treatment of 16HBE14o-cells and added either the digested HDM or the untreated HDM with vehicle control to the cells for 2 or 24 hours. We observed reduced CCL20 release in cells treated with HDM where beta-glucans had been enzymatically removed in comparison to cells treated
with HDM plus vehicle control. This observation was consistent after both 2 hour (Fig. 5A) and 24 hour (Fig. 5B) exposures. This finding suggests that HDM-induced CCL20 release after 2 hours is mediated by a similar beta-glucan dependent mechanism to what has been explored previously.20

As shown in this previous study, signaling through spleen tyrosine kinase (syk) is associated with CCL20 release, which has been shown by others to lead to airway inflammation in asthmatics.21 In addition to syk, other pathways that have been shown to be associated with beta-glucan signaling include raf 1 and protein kinase C (PKC). To further determine which intracellular signaling pathways contribute to CCL20 release, we treated cells with inhibitors to spleen tyrosine kinase (Bay 61-3606 and piceatannol), raf 1 kinase (raf 1 kinase inhibitor I), and PKC (RO 31-8220). We found that inhibition of syk or raf 1 kinase reduced CCL20 release, but that PKC had no effect on CCL20 release. For both syk and raf 1 kinase inhibitors this effect more pronounced on baseline release, with some reduction of HDM-induced release after piceatannol exposure.

We next sought to visualize the effect of inhibiting syk/raf 1 kinase signaling on lysosomal stores. Therefore, we transfected cells with CCL20-mCherry and loaded them with LysoTracker dye. Cells were incubated with the inhibitors listed above for 1 hour, and HDM was then added for 2 hours prior to imaging. We observed a dramatic reduction in colocalization of CCL20-mCherry with LysoTracker dye in the presence of syk inhibitors (Fig. 4D), suggesting that syk signaling is important for maintaining CCL20 within lysosomes under homeostatic conditions. Additionally, while cells treated in the presence of raf 1 kinase inhibitor
showed no change in colocalization of CCL20-mCherry and LysoTracker, these cells also failed to mobilize their lysosomal stores in response to HDM, suggesting that raf 1 signaling is a necessary component for signaling the release of CCL20 from secretory lysosomes in response to HDM exposure. Thus, our data suggest that CCL20 release from secretory lysosomes requires both syk and raf 1 kinase signaling, but that these pathways mediate separate cellular events in regards to CCL20 storage and release. Another potential interpretation of these results is that syk inhibition reduces shedding of extracellular CCL20. Thus, as intracellular stores of CCL20 are released, some release may be captured by the extracellular stores that remain intact. This would account for the reduction in baseline release of CCL20 observed in figure 5C.
Discussion

The rapid recruitment of iDCs to the lungs after allergen exposure is a critical initiating event in the development of the allergic response. The necessary signals for this recruitment involve key epithelial-derived mediators like CCL20 and GM-CSF.

We found that preformed stores of CCL20 in bronchial epithelial cells were released within a short (2 hr.) period after exposure to HDM. We also found that early CCL20 release from bronchial epithelial cells in response to HDM is derived from intracellular stores within secretory lysosomes. Lysosomal release required beta-glucan signaling in a syk/raf 1 kinase-dependent manner. Syk signaling was required to maintain CCL20 within lysosomes, while raf 1 kinase was required for release of lysosomal stores.

Interestingly, syk inhibition not only reduced release of CCL20 into the extracellular environment, but also reduced visible pools of pre-formed CCL20, even in the absence of HDM. This suggests that syk signaling may be necessary to prevent CCL20 degradation, perhaps by maintaining a lysosomal structure that allows CCL20 to remain bound within the electron-dense core of the lysosome and thereby avoid degradation. Further biochemical analysis is required to determine the precise mechanism by which syk prevents loss of CCL20 within bronchial epithelial cells. In other cell types, a direct role for syk in lysosome formation and function has been suggested.22,23 Our findings support these previous observations, and suggest that the loss of lysosomal structure in the absence of syk results in a loss of CCL20 stores within the cell that is not associated with chemokine release.
In contrast to the immediate release seen with CCL20, IL-8 was not released until two hours of HDM exposure. At this time point, HDM-induced IL-8 release was completely dependent on protein synthesis and required trafficking through the Golgi apparatus, suggesting the cells increased *de novo* production of IL-8 in response to HDM exposure. Interestingly, increased transcription of IL-8 was not detectable within the first 24 hours of HDM exposure, long after *de novo* production of IL-8, which was observed within 2 hours of HDM exposure. This suggests that prior to HDM exposure, although IL-8 mRNA is present in the cells, it likely undergoes translational repression. One major mechanism for translational repression that has been in recent years is through microRNAs, which bind to mRNA, and depending on the binding energy of the microRNA, either repress transcription or induce mRNA degradation. Future studies will determine whether a similar mechanism may control IL-8 translation. However IL-8 translation is controlled, our data suggest that the early release of IL-8 we observed within the first 24 hours of HDM exposure, similar to early release of CCL20, is not regulated by *de novo* transcription.

Previously, the mechanisms controlling chemokine release in bronchial epithelial cells have been poorly defined. This release is often thought to be controlled by direct secretory trafficking through the golgi. As such, chemokine release is often measured after 12 or 24 hours, and increased mRNA is used as a measure of increased chemokine release. However, our studies suggest that by the time an increase in chemokine mRNA is observed, much initial CCL20 and IL-8 release has already occurred, with *de novo* synthesis functioning to replenish already-released cellular stores, not to produce the initially released stores themselves. We
noted that stores of CCL20 begin to replenish about six hours after HDM exposure. This suggests that measurements of CCL20 release at later time points measure a delayed mechanism of release, which may also be mediated through secretory lysosomes. However, our studies also suggest that lack of message at a 24 hour time point does not suggest a lack of release, or even a lack of production, as message levels that were high at 6 hours decreased to baseline by 24 hours.

Indeed, given that IL-8 release was similarly via an increase in mRNA within this time frame, we might hypothesize that a number of chemokines that display no significant increase in transcription within the first 24 hours could nonetheless be released within this time frame. This also makes attribution of chemokine release to a specific cell type more difficult, as elevated levels of any specific chemokine in the BAL cannot necessarily be attributed to cells in which increased transcription of that chemokine are observed. Much of the observed rapidly released chemokines and chemokines released in the BAL of asthmatics in response to allergen could come from release of either preformed stores of these proteins (as is the case with CCL20), or from preformed mRNA that is subsequently translated (as we observed with IL-8).

In the present study, we focused primarily on CCL20 release from intracellular stores. However, our evidence suggests the existence of extracellular stores of CCL20 whose release may be controlled by separate mechanisms from those controlling release of intracellular stores. For example, when cell lysates were measured after trypsin degradation, some CCL20 was lost suggesting this pool of CCL20 that was exposed to trypsin resides outside the cell. We would not expect this pool of CCL20 to be affected by disruptions to lysosomal stores. Indeed, when
we treated cells with bafilomycin we saw partial inhibition of CCL20 release, suggesting non-
lysosomal stores, such as those positioned on the cell surface, were unaffected by inhibition of
lysosomes.

Kinetics of CCL20 release showed that low levels of CCL20 are released at baseline, in
the absence of HDM stimulation. This constitutive, low-level release of CCL20 may play a role
in the maintenance of resident dendritic cells in the lungs. Interestingly, baseline release of
CCL20 was unaffected by bafilomycin treatment, suggesting this low-level, constitutive release
of CCL20 is not derived from lysosomal stores.

In contrast to bafilomycin A inhibition, when we inhibited production of CLC-7 via
siRNA to CLCN7, we observed that both baseline and HDM-induced CCL20 release were
inhibited. Additionally, ratio of HDM to control CCL20 in knockdown cells was similar to the
ratio in control-treated cells. This suggests that release of all CCL20 (including extracellular
CCL20) is impacted by CLCN7 knockdown, whereas for bafilomycin A knockdown only
lysosomal stores were impacted. One major difference between these two approaches to
lysosome inhibition is that bafilomycin A was applied to cells one hour prior to HDM exposure,
while knockdown of cells began three days prior to HDM exposure. As we have previously
observed that extracellular CCL20 is constitutively shed at a low level, this pool would need to
be replenished through de novo production. Given that long-term inhibition of lysosome
function (CLCN7 knockdown) impacted both intracellular and extracellular stores, this may
suggest that CCL20 is trafficked through lysosomes in order to replenish extracellular stores.
When we measured the effect of signaling pathway inhibition, we again saw incomplete inhibition of CCL20 release after HDM treatment. However, we did not observe residual CCL20 stores within lysosomes as would be expected if lysosomal stores were the sole contributor to HDM-induced CCL20 release, suggesting the CCL20 released in the presence of lysosome inhibition is derived from a separate, possibly extracellular, pool. In contrast to bafilomycin treatment, inhibitors of syk and raf1 reduced baseline CCL20 release, which suggests that the impact of these inhibitors on CCL20 release may not be restricted to lysosomal storage and release, but that these inhibitors may also interfere with constitutive release from extracellular stores discussed previously.

The finding that some preformed stores of CCL20 localize to lysosomes reveals novel insights about its function in response to antigen. CCL20 has been shown to be a potent antibacterial protein (with an LD$_{50}$ of 400 ng/mL against $E.\ coli$) in addition to its signaling role.$^{26}$ That CCL20 is not inactivated in the acidic lysosomal environment is unsurprising, given that previous studies have shown CCL20 exhibits stronger antimicrobial properties at acidic pH, which exposes its antimicrobial C-terminal domain.$^{27}$ Thus, rapid release of preformed CCL20 stores from secretory lysosomes may contribute to a first-line defense against microbes at the bronchial epithelium, with CCL20 release subsequently serving to help recruit DCs and $T_{H17}$ cells. Many other chemokines exhibit similar antimicrobial properties, and some, such as IL-18, have been shown to be rapidly released from the bronchial epithelium in response to allergen exposure, similar to CCL20.$^{28}$ Future experiments will determine whether these two proteins are stored and released in a similar manner. For CCL20, release from secretory lysosomes may
suggest that its antimicrobial role is the more ancient mechanism, as airway epithelial cells
protect themselves by rapidly releasing antimicrobial CCL20, allowing subsequent adaptations
to later link this release of antimicrobial peptides to inflammation.

In the context of HDM exposure, this mechanism may provide a useful target for
intervention. Our finding that CCL20 release requires syk and raf 1 kinase signaling suggests
that blocking these targets may affect early sensitization, and possibly airway inflammation in
general.
References


### Downstream signaling inhibitors used in figure 2

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<tr>
<td>MS-275</td>
<td>HDAC 1-3 (inhibitor)</td>
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</tr>
<tr>
<td>Trichostatin</td>
<td>HDAC 1, 3, 4, 6, 10 (inhibitor)</td>
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</tr>
<tr>
<td>SAHA</td>
<td>HDAC (inhibitor)</td>
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<td>EX-527</td>
<td>SIRT1 (inhibitor)</td>
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<tr>
<td>CAY10603</td>
<td>HDAC 6 (inhibitor)</td>
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</tr>
<tr>
<td>IKK2 inhibitor</td>
<td>NFkappaB (inhibitor)</td>
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</tr>
<tr>
<td>GSbxxx</td>
<td>Gamma secretase/Notch (inhibitor)</td>
<td>1, 10, 100 nM</td>
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**Table 1**
### Signal peptide homology for selected chemokines and cytokines

<table>
<thead>
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<th>Chemokine</th>
<th>Signal peptide homologue</th>
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<tr>
<td>CCL20</td>
<td>CR1L, cathepsin C, β4 integrin, CD46, urocortin</td>
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<tr>
<td>IL-8</td>
<td>CXCL1</td>
</tr>
<tr>
<td>β-defensin 2</td>
<td>CD9</td>
</tr>
<tr>
<td>IFN-α</td>
<td>–</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Cadherin 7</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>–</td>
</tr>
<tr>
<td>IL-10</td>
<td>–</td>
</tr>
<tr>
<td>TNF-α</td>
<td>–</td>
</tr>
<tr>
<td>IL-17a</td>
<td>–</td>
</tr>
<tr>
<td>IL-6</td>
<td>–</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>–</td>
</tr>
<tr>
<td>TSLP</td>
<td>–</td>
</tr>
<tr>
<td>CCL22</td>
<td>Cortexin-1</td>
</tr>
<tr>
<td>CCL18</td>
<td>MIP-1α, LY6H, CYP7B1</td>
</tr>
<tr>
<td>CXCL10</td>
<td>No matches</td>
</tr>
<tr>
<td>XCL1</td>
<td>FGFR4, NMDAR, PLOD1</td>
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Table 2
### Actin inhibitors and their functions

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Function</th>
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<tbody>
<tr>
<td>Cytochalasin D</td>
<td>Binds G-actin and prevents incorporation into filaments</td>
</tr>
<tr>
<td>Jasplakinolide</td>
<td>Prevents F-actin extension and polymerization</td>
</tr>
<tr>
<td>Latrunculin B</td>
<td>Prevents G-actin polymerization</td>
</tr>
<tr>
<td>Swinholide A</td>
<td>Inhibits actin polymerization, and severs F-actin</td>
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</tbody>
</table>

**Table 3**
**Figure 1** – Rapid CCL20 release from bronchial epithelial cells is dependent on preformed stores. 16HBE cells were treated with HDM, and cells were harvested at different time points for determination of (A) CCL20 and (B) IL-8 secretion by ELISA. RNA was extracted, and (C), Ccl20 mRNA and (D), Cxcl1 mRNA were measured by real-time PCR. To determine the involvement
of *de novo* protein synthesis, 16HBE cells were treated with cycloheximide for 1 hour prior to HDM exposure for 2 hours, and (E), CCL20 and (F), IL-8 were measured in supernatants by ELISA. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control. n=3.
Figure 2 – CCL20 is stored in both intracellular and extracellular compartments. (A) Fixed, but not permeablized 16HBE14o- cells transfected with CCL20-mCherry, and exposed to either an anti-RFP antibody, or isotype control antibody. (B) HDM-treated 16HBE14o- cells were lifted with either an enzymatic (trypsin) or non-enzymatic (cell dissociation buffer; CD) method, lysed by freeze-thaw, then CCL20 content in lysates was analyzed by ELISA. Cells were treated with HDM in the presence of brefeldin A, monensin, or vehicle control; supernatants were collected at 2h (C, D) and 24h (E, F) and tested for CCL20 and IL-8 via ELISA. (G) Lung sections from naïve BALB/c mice stained with anti-CCL20 antibody and DAPI nuclear stain. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001.
anti-RFP antibody, or isotype control antibody. (B) HDM-treated 16HBE14o- cells were lifted with either an enzymatic (trypsin) or non-enzymatic (cell dissociation buffer; CD) method, lysed by freeze-thaw, then CCL20 content in lysates was analyzed by ELISA. Cells were treated with HDM in the presence of brefeldin A, monensin, or vehicle control; supernatants were collected at 2h (C, D) and 24h (E, F) and tested for CCL20 and IL-8 via ELISA. (G) Lung sections from naïve BALB/c mice stained with anti-CCL20 antibody and DAPI nuclear stain. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001. n=3.
Figure 3 – Intracellular CCL20 is stored within lysosomes. Live cell confocal microscopy images of cells co-transfected with CCL20-mCherry and either (A) rab 3a-GFP, (B) rab 7-GFP, or (C) lamp-1-YFP.
Figure 4 – HDM induces release of CCL20 from secretory lysosomes. (A) Cells transfected with CCL20-mCherry and loaded with LysoTracker were treated for 2 hours with PBS, or HDM. (B) Cells were incubated with Lamp-1—AF647 antibody and HDM for 30 minutes. Antibody uptake was then assessed via flow cytometry. (C, D) Cells were pretreated with bafilomycin A or concanamycin A for 1h, prior to adding media or HDM for 2h, and measuring CCL20 release into the supernatant. (E) Cells were treated with vehicle control or bafilomycin A for one hour prior to addition of HDM for 2 hours; CCL20 retention was measured as described in fig. 2B.
(F) Cells were electroporated with scramble control or CLCN7-targeting siRNAs and treated with media or HDM for 2h, and CCL20 release into the supernatant was measured. (G) mRNA expression levels of F. *p<0.05, **p<0.01, ***p<0.001. Scale bar = 10 uM. B-E n=3, F-G n=6.
**Figure 5** – *Beta-glucan-dependent release of lysosomal CCL20 is, through a syk/Raf 1 kinase-dependent pathway.* A and B, Cells were treated with HDM that was pre-treated with either beta-glucanase (HDM+b-gluc) or vehicle control (HDM+veh) for 2 hours A or 24 hours B. C, Cells were treated with various signaling pathway inhibitors, including piceatannol (syk), raf 1 kinase inhibitor I (raf 1 kinase), and Ro 31-8220 (PKC) and CCL20 release into the supernatant was measured. D, Live confocal microscopy of cells transfected with CCL20-mCherry, loaded with LysoTracker dye, and treated with the inhibitors mentioned above, or bafilomycin A, then treated with PBS, or HDM for 2 hours. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 vs. PBS control PBS; †p<0.05, ††p<0.01, †††p<0.001 vs. HDM control. Scale bar = 5 uM  n=3.
Chapter 3. Signaling pathways leading to HDM-induced CCL20 release from the bronchial epithelium


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Abstract

A number of intracellular signaling pathways have been identified as important in the generation of allergic asthma. General inhibition of many of these – such as reactive oxygen species generation, ion transport, and the NLRP3 inflammasome – have been shown to reduce airway hyperresponsiveness in animal models of asthma. We hypothesized that these same signaling pathways may contribute to HDM-induced CCL20 release from bronchial epithelial cells. We treated bronchial epithelial cells with inhibitors to a number of intracellular signaling pathways prior to treatment with HDM and measured CCL20 release. We found that CCL20 release does not require glucocorticoid signaling, as do many other chemokines and cytokines. Additionally, we found that while chloride transport is necessary for HDM-induced CCL20 release, excess potassium was sufficient to reverse this effect. Other signaling contributors to CCL20 release included gap junctions, reactive oxygen species, and cyclooxygenase. These findings demonstrate that CCL20 release is downstream of a number of pathways that are active in asthma, suggesting that inhibition of these pathways in asthmatics may have broader effects on inflammation and airway hyperresponsiveness.
Introduction

Chemokines and cytokines have been shown to be stored within a number of different cell types; including cells of the hematopoietic lineage, as well as non-hematopoietic cells, such as endothelial cells. These chemokines and cytokines mediate a diverse set of responses. For example, IL-8 and gro-α (found in endothelial cells) promote neutrophil infiltration,\textsuperscript{3,4} while IL-4 (found in Th2 cells) promotes type-2 immune responses.\textsuperscript{5} The release of these different molecules is controlled by a diverse set of signaling pathways.

Different signaling pathways have been shown to play important roles in either the development or treatment of allergic asthma, such as signaling through the inflammasome, magnesium ion transport, reactive oxygen species (ROS) generation, and prostaglandin synthesis through cyclooxygenases. For example, activation of the NLRP3 inflammasome has been shown to be critical to the development of Th2 and Th17 responses in murine asthma models.\textsuperscript{6,7} Magnesium (Mg\textsuperscript{2+}) treatment may help reduce asthma symptoms by relaxing the airway smooth muscle or by attenuating respiratory burst.\textsuperscript{8,9} For this reason, magnesium is used as a treatment for patients suffering acute asthma attacks.\textsuperscript{10} In severe asthmatics, increased neutrophil inflammation results in release of reactive oxygen species and prostaglandins.\textsuperscript{11–13} The bronchial epithelium has been shown to directly produce both reactive oxygen species, and prostaglandins.\textsuperscript{11,14,15} Each of these pathways has been shown to play a distinct role in inflammation.

One of the primary drivers of inflammation is the release of chemokines, which are critical for the recruitment of migrating cells.\textsuperscript{16} We hypothesized that signaling pathways
already shown to actively contribute to airway hyperresponsiveness in asthmatics may also control release of CCL20 from bronchial epithelial cells. Many of these same mechanisms have already been shown to be active in bronchial epithelial cells following allergen exposure. Others have been shown to contribute to airway inflammation, which suggests they may play a role in chemokine release from airway cells.

One source of inflammatory cytokines in allergic asthma is the inflammasome. Activation of caspase 1 through NALP3 induces the release of IL-1β and IL-18. This signaling cascade can be initiated by a number of factors, including potassium export through pannexin-1, external ATP, uric acid crystals, and bacterial toxins such as nigericin (a potassium ionophore). Interestingly, potassium export has also been shown to be necessary for secretion of TNF-α, and IL-8. Inhibition of potassium export reduced immediate IL-8 and TNF-a release, but this effect began to diminish after 2 hours.

Signaling through other ions has also been shown to be involved in cytokine release. For example, magnesium deficiency leads to early increases in IL-6 and TNF-α release. Indeed, intracellular magnesium levels have been shown to regulate lysosomal pH, possibly through their effect on lysosomal chloride transport. This may be one route by which magnesium regulates release of intracellular chemokines and cytokines.

If individual ions contribute to signaling of CCL20 release, intercellular transport of these ions may serve to enhance this signaling as changes in ion concentration spread from cell to cell. One mechanism by which ions are transported between adjacent cells is by diffusing through gap junctions. Gap junction proteins form gated pores between cells. These pores are
large enough to allow transport of ions and small molecules, but not larger proteins.\textsuperscript{28} In this way, signaling that is initiated in one cell may propagate to neighboring cells. For example, cells infected with limiting numbers of intracellular bacteria promoted NF-κB nuclear translocation and IL-8 release in neighboring, uninfected cells through a mechanism dependent on gap junction function.\textsuperscript{29} A similar mechanism may help promote CCL20 release from bronchial epithelial cells that are not directly contacted by allergen.

Another signaling pathway that has been shown to induce chemokine release in some cell types is induction of reactive oxygen species. ROS are highly reactive oxygen-containing molecules (such as H\textsubscript{2}O\textsubscript{2}) that help protect against foreign microorganisms, and participate in cell signaling. They are often generated in times of cell stress through a process referred to as respiratory burst.\textsuperscript{30,31} During respiratory burst, NADPH oxidase helps to convert oxygen to H\textsubscript{2}O\textsubscript{2}.\textsuperscript{32} Although this pathway has traditionally been linked to metabolic processes within the cell, recent studies have demonstrated that ROS participate in downstream cell signaling events as well.\textsuperscript{33} This is accomplished through the oxidation, mainly of cysteine residues, of target proteins. A variety of different protein oxidation targets have been identified, including calcium and potassium ion channels,\textsuperscript{34,35} MMPs and ADAMs,\textsuperscript{36} JNK and ERK,\textsuperscript{37-39} among others. We have previously identified many of these signaling pathways as important in mediating CCL20 release through either intracellular or extracellular mechanisms. Generation of reactive oxygen species has also been associated with release of the proinflammatory cytokines IL-1β and IL-18 in macrophages.\textsuperscript{40} Given the many roles of ROS in mediating signaling pathways
previously identified as important in CCL20 release, as well as their influence in asthma, we hypothesized that ROS generation may play a role in HDM-induced CCL20 release.

Prostaglandins are lipophilic, paracrine signaling molecules produced from arachadonic acid.\textsuperscript{41} Cyclooxygenase enzymes mediate the synthesis of prostaglandin H\textsubscript{2} from arachadonic acid.\textsuperscript{42} From this point, other prostaglandins are synthesized. Three isoforms make up the cyclooxygenase family: COX-1, COX-2, and COX-3.\textsuperscript{43,44} Inhibition of COX-1 and COX-2 is a major function of aspirin, a common non-steroidal anti-inflammatory drug (NSAID).\textsuperscript{45} Although prostaglandin release in asthmatic airways has been reported,\textsuperscript{13} in some asthmatics aspirin treatment exacerbates airway hyperresponsiveness (AHR), suggesting prostaglandin synthesis may mitigate AHR in these individuals.\textsuperscript{46} Specificity of cyclooxygenase inhibition also seems to be important, as inhibitors of COX-2, but not COX-1 do not affect AHR, suggesting aspirin’s role in exacerbating AHR is due to its role in inhibiting COX-1, not COX-2.\textsuperscript{47} Prostaglandin signaling has also been linked to cytokine secretion. Prostaglandin E2 (PGE2) in T cells suppressed IFN-g, but increased production of IL-5.\textsuperscript{48} In addition, inhibition of JNK or ERK signaling reduced PGE2 production and IL-8 release.\textsuperscript{49} Thus, the function of prostaglandins in asthmatic airways is complex, with some prostaglandins promoting a Th\textsubscript{2} response, and others antagonizing it.

Many of the pathways we have reviewed have been demonstrated to be important in both airway hyperresponsiveness, and intracellular signaling events. We hypothesize that some of these pathways may promote CCL20 release, which would stimulate inflammation and
AHR. To investigate which pathways are important for CCL20 release, we will block various pathways and analyze HDM-induced CCL20 release into the supernatant.
Materials and Methods

Cell culture and treatment: The 16HBE14o- human bronchial epithelial cell line was cultured in 10-15 cm round culture dishes that had been collagen coated. Cells were passaged by applying trypsin (0.05% with EDTA) for 10 min. Treatments were performed in 24-well collagen-coated culture plates in DMEM including 10% FBS, penicillin/streptomycin, and L-glutamine. Upon reaching 70-80% confluence, media was replaced and FBS reduced to 0.1% overnight. All inhibitors or balanced culture solutions were added one hour prior to HDM or allergen addition. Primary normal human bronchial epithelial cells (NHBEs) were thawed directly from storage in liquid nitrogen into 24-well collagen-coated culture plates. NHBEs were grown in Bronchial Epithelial Cell Growth Medium (BEGM) from Lonza (CC-3170) with supplied supplements, including bovine pituitary extract (BPE), insulin, hydrocortisone (HC), GA-1000, retinoic acid, transferrin, triiodothyronine, epinephrine, and human epidermal growth factor (hEGF). Upon reaching 70-80% confluence, media was replaced with BEGM deficient in BPE, HC, and hEGF.

Inhibitors: Reagents used as inhibitors were obtained from SigmaAldrich, unless specified, and were used at the following concentrations: forskolin, gadolinium, carbenoxolone (50 uM), phloretin, 1-octanol, valinomycin (50 uM), SB203580 (10 uM), SP600125 (JNK inh.) (10 uM), Bafilomycin (500 pM), Ly294002 (10 uM), R031-8220 (PKC inh.) (10 uM), Raf1 kinase inh. (10 uM), DIDS (10 uM), Pertussus toxin (100 ng/mL), ZM 336372 (10 uM), U0126 (10 uM), SB216763 (10 uM), CPG 57380 (10 uM), Wormannin (1 uM), PP242 (1 uM), Pimelic Diphenylamide 106 (2 uM), Dexamethasone (1 uM), Rapamycin (0.1 uM), MS-275 (0.5 uM), EX-527 (0.1 uM), SAHA (1 uM), and others.
uM), CAY10603 (0.1 uM), IKK2 inh. (1 uM), Trichostatin (TSA) (0.1 uM), Monensin, brefeldin A, cytochalasin D (10 uM), Jasplakinolide (10 uM), Latrunculin (10 uM), Swinholide (10 uM).

**HDM treatment:** Whole HDM extract was obtained from Greer (B70). 100-200 ug/mL HDM was added to cell supernatant for two hours prior to harvesting cells.

**Solutions:** Chloride sufficient media is a Krebs-Ringer solution consisting of the following (in mM): 120 NaCl, 25 NaHCO$_3$, 11 Glucose, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, and 1.2 KH$_2$PO$_4$. In anion replacement solutions, 100 mM of NaCl was replaced with 100 mM sodium gluconate. For sulfate-free solutions, MgSO$_4$ was replaced with Na$_2$SO$_4$. For magnesium-free solutions, MgSO$_4$ was replaced with MgCl$_2$. For potassium-free solutions, KCl was replaced with NaCl, KH$_2$PO$_4$ and was replaced with NaH$_2$PO$_4$.

**ELISA:** Mouse and human ELISAs were performed in half-well plates using DuoSet ELISA kits from R&D Systems according to their specifications. Multiplex quantification was performed via Luminex using proprietary kits and 50 uL sample volume. Samples were from BAL or cell supernatants that had been frozen at -80°C.

**Intracellular chloride measurement:** Intracellular chloride measurements are modified from the protocol reported in (Coskun T, Baumgartner HK, Chu S & Montrose MH (2002). Coordinated regulation of gastric chloride secretion with both acid and alkali secretion. Am J Physiol Gastrointest Liver Physiol 283, G1147–G1155). After HDM treatment for 30 min, cells were rapidly washed three times in ice cold 140 mM Sodium gluconate solution during continuous aspiration. Samples were allowed to dry before reconstitution in 2N sulfuric acid and 1%
Tween 20. Cells were subsequently diluted to 0.67 N sulfuric acid before chloride concentrations were measured using a Labconco Chloridometer, and compared with a standard curve of known chloride concentrations.

Mouse asthma model: Sensitization to HDM was achieved by intra tracheal (I.T.) administration of 100 ug/mL HDM. Twenty-one days later a second administration of alexafluor 405-labelled HDM was administered I.T. 72-hours after the last HDM challenge mice were anesthetized with sodium pentathol and airway measurements (outlined below) were taken. The mice were then sacrificed, and broncho-alveolar lavage was performed using 1 mL Hanks balanced salt solution without calcium or magnesium. BAL fluid was then centrifuged to remove cells. Lungs were removed and fixed in formaldehyde, then sent to core personnel at CCHMC who embedded them in paraffin, sectioned them, and mounted them on slides.

Airway measurements: 72 hours after the final allergen exposure, mice were anesthetized, intubated, and ventilated at 120 breaths/min. After airway pressure stabilized, mice were given 25 ug/kg weight of acetylcholine intra venous and airway pressure changes were followed for five minutes. Airway measurements were quantified as airway pressure time index (APTI), which is a quantitative measure of airway constriction, such that APTI increases with increasing airway resistance.

Isolation and culture of mouse tracheal epithelial cells (mTECs): Mice were anesthetized with sodium pentathol and the inferior vena cava was severed to prevent recovery. Trachea were then removed and incubated overnight at 4 °C in 1% pronase (Roche) in DMEM/F12 (50/50) mix (Fisher). Cells were then removed from trachea via sheer force in the presence of 10% FBS and
DNase. Cells were then incubated on Primaria plates for four hours to allow fibroblasts to adhere. Non-adherent cells were then removed and grown on rat-tail collagen-coated trans-well plates in DMEM/F12 supplemented with penicillin/streptomycin, L-glutamine, retinoic acid, and 5% FBS. After reaching 1000 ohms trans-epithelial resistance, media was replaced with 0.1% FBS media overnight prior to experimental treatments.

*Statistical analysis:* Statistical analysis was performed using GraphPad Prism 5 software. As the data were normally distributed, statistical significance was determined using an unpaired student’s t-test for comparisons between two treatments, or two-way ANOVA combined with Bonferroni post-test analysis for comparison between multiple groups. Values of p<0.05 were considered statistically significant.
Results

A chloride cotransporter is required for rapid, HDM-induced CCL20 release

We sought to identify the cellular signaling mechanisms that lead to the rapid release of intracellular stores of CCL20 after HDM stimulation. Previously, we showed that CCL20 is released from secretory lysosomes in a V-ATPase and chloride-proton co-transporter—dependent manner. We therefore hypothesized that the intracellular signaling pathways leading to this secretory lysosome release may also be ion transporter-dependent. To test this, we treated HBE cells with a variety of transporter inhibitors prior to HDM stimulation and assayed for early CCL20 release into the supernatant. While common cation transport inhibitors, such as amiloride (epithelial sodium channel) and bumetanide (NKCC1) had no effect on CCL20 release (data not shown), broad chloride transporter inhibitors such as DIDS and niflumic acid dramatically reduced CCL20 release (Fig 1A). This suggests a vital role for anion transport in CCL20 release, as no detectable CCL20 was released in cells where anion transport was inhibited.

To further confirm the role of chloride in CCL20 release, we depleted cells of chloride by treating them in a modified Ringer solution. We replaced 100 mM of sodium chloride with sodium gluconate, thereby creating a reduced chloride solution containing 25 mM chloride (Fig 1B). This solution was applied to the cells one hour before HDM stimulation. Cells under reduced chloride conditions released substantially less CCL20 than did cells treated under normal chloride conditions. Although we retained about 25 mM chloride in reduced chloride conditions (to minimize shock to the cells) we wanted to confirm that the cells were still viable
after the two-hour stimulation with HDM. Therefore, subsequent to collecting the supernatant from the cells in figure 1B (2 hr), we restored normal chloride solution to these cells, while continuing to stimulate with HDM for an additional two hours. In cells that were initially chloride deficient, restoration of chloride was sufficient to fully restore CCL20 release in response to HDM. Interestingly, in cells that were initially chloride sufficient, and had therefore showed strong release of CCL20 after 2 hours, we noted that CCL20 release after the second stimulation was significantly reduced. As the initial burst of CCL20 from these cells had been removed prior to restoring chloride-sufficient solution plus HDM, this low secondary release represents HDM-induced CCL20 release from 2 to 4 hours after exposure. This low release from 2 to 4 hours is consistent with our previous observation that preformed stores of CCL20 are not replenished within the first few hours after HDM stimulation.

To further define the halogen anion selectivity of this CCL20 release mechanism, we replaced chloride in our modified Ringer solution with anions known to exhibit increased permeability through most chloride transporters: namely nitrate, iodide, and fluoride (data not shown). We found that when chloride was replaced with nitrate or iodide CCL20 release increased both at baseline and under HDM-stimulated conditions. (Fluoride-containing solution was highly cytotoxic.) This increase was also susceptible to suppression by DIDS (Fig 1C), strongly supporting our hypothesis that the effect of chloride on CCL20 release is through anion transporter activity. From this we concluded that the anion transporter required for CCL20 release has an anion selectivity of gluconate<<Cl<<NO₃<<I. Interestingly, we saw both baseline and HDM-induced rises in CCL20 release in the nitrate-treated cells. This suggests that
the impact of nitrate on CCL20 release is independent of allergen exposure. Additionally, the difference between PBS and HDM in cells treated in chloride was about 75% less than the difference between PBS and HDM in cells treated in nitrate. However, the percentage increase of HDM over PBS treatment (about 66%) was the same for both treatment groups. This suggests that the impact of nitrate is on the overall rate of CCL20 release.

To determine whether chloride dependence requires active or passive chloride transport, we eliminated any existing or potential chloride gradients by treating cells with TPPMn(III), which is a chloride ionophore. TPPMn(III) had no effect on either HDM-induced CCL20 release or DIDS inhibition (Fig 1D). This suggests the chloride transporter functions via an active mechanism, possibly a co-transporter. To confirm the ionophore was sufficient to equalize chloride concentrations across the epithelium, we measured intracellular chloride concentrations prior to and after HDM treatment in bronchial epithelial cells in the presence or absence of DIDS and TPPMn(III) (Fig 1E). We found that in cells treated with PBS or HDM alone, intracellular chloride decreased after treatment with HDM from baseline levels. Interestingly, cells treated with either DIDS or TPPMn(III) displayed intracellular levels of chloride at baseline that were equivalent to the HDM-treated cells. This evidence suggests that homeostatic levels of chloride are elevated above equilibrium in bronchial epithelial cells, and that they return to equilibrium in response to HDM.

We next asked whether this chloride dependence of CCL20 release affects mobilization of chemokine from lysosomal stores. Cells transfected with CCL20-mCherry and loaded with LysoTracker were visualized in situ over the course of one hour of HDM exposure in various
anion replacement conditions (Fig 1F). Cells in media alone demonstrated CCL20 release from intracellular vesicles, while cells treated in the presence of DIDS did not mobilize intracellular CCL20 in response to HDM. This supports our hypothesis that the observed chloride-dependence of CCL20 release affects lysosomal CCL20 stores.

**Ion depletion modulates CCL20 release**

In addition to chloride, many other ions are physiologically important in bronchial epithelial cells. To determine which of these might play a role in modulating CCL20 release, we created media deficient in, or supplemented with, major ions. When we removed either magnesium or sulfate from bronchial epithelial cells, we found that intracellular chloride concentrations no longer decreased after HDM exposure (Fig 2A). Additionally, adding twice the normal level of magnesium sulfate induced intracellular chloride depletion even in the absence of HDM (Fig 2B). Removal of sulfate only marginally reduced HDM-induced CCL20 release, and removal of magnesium dramatically increased CCL20 release, while excess magnesium sulfate paradoxically had a similar effect as removal of magnesium (Fig 2C). The impact of magnesium appears to be restricted to baseline release, as the increased CCL20 release above baseline (HDM treatment minus PBS treatment) in control cells compared to magnesium deficient cells was identical, suggesting that the increase can be entirely attributed to increased constitutive CCL20 release. These effects persisted after 24 hours (Fig 2D). These data suggest that magnesium and sulfate have opposing roles in modulating CCL20 release.

A major ion transporter family responsible for passage of both chloride and sulfate across the cell membrane is the SLC26A family of solute carrier proteins. We chose two
SLC26A family members that are expressed in bronchial epithelial cells, namely Pendrin (SLC26A4) and SLC26A9, as targets for siRNA knockdown (Fig 3 A, B). This knockdown had no effect on CCL20 release for either Pendrin or SLC26A9, suggesting magnesium and sulfate elicit their effects on intracellular chloride concentrations and CCL20 release through some other mechanism.

**Potassium is sufficient but not necessary to drive rapid CCL20 release**

In addition to reduction of magnesium, sulfate, and chloride, we tested whether removal of potassium from cell media would affect HDM-induced CCL20 release. We replaced the normal 5 mM potassium with sodium, and measured levels of intracellular chloride after HDM treatment. Potassium removal decreased intracellular chloride under homeostatic conditions (Fig 4C). However, this had no effect on CCL20 release (Fig 4B). This suggests that the effect of potassium on intracellular chloride concentration is independent of chemokine release, and that the presence of potassium in solution is not required for CCL20 release. We next tested whether addition of excess potassium would affect HDM-induced CCL20 release. Addition of potassium chloride also causes cell shrinkage due to osmotic changes. To control for this, we compared the effect of added potassium with excess sodium, a similar cation (Fig 4A). We observed that hyperosmolarity, from either potassium chloride or sodium chloride, increased CCL20 release, and that it particularly affected baseline release (PBS treatment). However, for baseline release, excess potassium allowed further increase of CCL20 release in comparison to excess sodium. Additionally, we treated cells with mannitol to induce cell shrinkage without changing the sodium or potassium concentration (data not shown). We observed no change in cells treated with mannitol compared with controls. Taken together, we found that while the
presence of extracellular potassium was not necessary for HDM-induced CCL20 release, the presence of additional potassium was sufficient to induce CCL20 release.

The surprising absence of an effect from potassium removal may be explained by the fact that extracellular potassium concentrations are low relative to intracellular potassium concentrations. In our defined solutions, potassium concentrations were about 5 mM, compared to an estimated 120-140 mM intracellular potassium concentration for most cells. Thus, any gradient that was established under low potassium conditions may not be disrupted under null potassium conditions if the cell were able to retain high intracellular potassium.

To further understand the mechanism of potassium-induced CCL20 release, we treated cells with the potassium ionophore, valinomycin. Valinomycin inserts into the cell membrane and selectively facilitates free diffusion of potassium ions, thereby eliminating any established potassium gradients. Valinomycin treatment alone had little or no effect on HDM-induced CCL20 release. However, when we added excess potassium to cells treated with valinomycin, we observed increased CCL20 release. This release was greater even when compared to the increased release seen with excess potassium alone (Fig 4D). Indeed, although addition of potassium alone increased baseline CCL20 release (with no apparent further increase in HDM-induced CCL20 release), after addition of valinomycin potassium addition increased both baseline release (PBS-treated cells) and HDM-induced CCL20 release. Thus the impact of potassium on release appears to differ depending on which pool of CCL20 is targeted.

To determine whether there was an interaction between the effect of chloride and the effect of potassium on CCL20 release, we treated cells in gluconate replacement media with the
potassium ionophore, valinomycin. Strikingly, when we treated cells with chloride-to-glucconate replacement media concurrently with valinomycin, we saw that CCL20 release returned to normal levels for both baseline release, and for HDM-induced release (Fig 4E). Indeed, this effect was mirrored somewhat when we paired valinomycin with the ion transporter DIDS. However, DIDS plus valinomycin induced additional CCL20 release under control conditions as well as after HDM exposure. These data suggest a strong interaction between chloride and potassium in HDM-induced CCL20 release.

**Gap junctions are necessary for rapid CCL20 release**

Many ions and other small solutes are exchanged between cells via gap junctions. Changes in ion concentration that begin in one cell of the epithelium may affect the ion concentration of adjacent cells, thereby enhancing the initial signal. To test whether gap junctions affect the signaling that leads to rapid CCL20 release, we treated 16HBE14o- cells with different inhibitors of gap junctions, including 1-octanol (Fig 5A), phloretin (Fig 5B), or carbenoxolone (Fig 5C) for one hour prior to HDM treatment for two hours. We then measured CCL20 release into the supernatant. At this concentration, we observed that 1-octanol was highly cytotoxic, but that neither phloretin nor carbenoxolone displayed any appreciable cytotoxicity at the concentrations used (data not shown). We found that each inhibitor partially reduced HDM-induced CCL20 release, suggesting that gap junctions play a role in enhancing HDM-induced CCL20 release.
The JNK and Raf1 signaling pathways contribute to rapid CCL20 release

We next sought to determine whether common signaling pathways implicated in initiating gene transcription and other downstream signaling pathways were required for rapid CCL20 release. To do so, we tested a panel of inhibitors of downstream signaling pathways. Table 1 outlines each of these inhibitors and their targets. Each inhibitor was compared with the appropriate solvent concentration as control [Ctrl (1)=1:500; Ctrl (2)=1:2,000; Ctrl (3)=1:25,000]. HDM was added one hour after incubation with inhibitor. Cells were allowed to incubate for a further 2 hours after HDM addition, after which supernatants were harvested for CCL20 ELISA.

We found that most downstream signaling pathway inhibitors we tested had no effect on HDM-induced CCL20 release. However, the JNK inhibitor SP600126, and the Raf1 kinase inhibitor both significantly inhibited rapid CCL20 release, suggesting these pathways may be involved in HDM-induced CCL20 release. Interestingly, an activator of Raf1, ZM 336372, had no effect on HDM-induced CCL20 release, suggesting Raf1 is necessary but not sufficient to drive HDM-induced CCL20 release.

We previously reported our finding that Raf1 inhibition reduces HDM-induced CCL20 release. Other groups have demonstrated phosphorylation of ERK within 15 minutes of agonist exposure, concurrent with CCL20 release\(^5\). Interestingly, we saw no decrease in HDM-induced CCL20 release after treatment with an inhibitor of MEK, a protein downstream of Raf1, and upstream of ERK,\(^5\) suggesting that MEK (and potentially its downstream target ERK) is not
necessary for HDM-induced CCL20 release, although it may still be activated in response to HDM exposure.

To individually test these panel data, we repeated this experiment with the inhibitors R031-8820, SB216763, (data not shown) and SP600126 (Fig 6A, B). SP600126 remained the only inhibitor to significantly reduce rapid CCL20 release. In addition, SP600126 treatment in the absence of HDM was sufficient to reduce intracellular chloride levels (Fig 6B), similar to DIDS treatment. These data suggest JNK signaling may play a role in HDM-induced CCL20 release upstream of chloride transporter involvement.

**Actin remodeling suppresses rapid CCL20 release**

The actin cytoskeleton is active in the priming and docking of vesicular stores. In addition, many extracellular structural proteins associate with intracellular actin either directly or indirectly. We tested a panel of actin inhibitors to determine their effect on HDM-induced CCL20 release after 2 hours (Fig 7). The inhibitors we used, and their targets/functions, are listed in table 2. Although we hypothesized that inhibition of actin polymerization would decrease HDM-induced CCL20 release, due to the presence of intracellular stores of CCL20 within secretory lysosomes, we observed that inhibition of actin polymerization by any one of a variety of mechanisms was sufficient to increase rapid CCL20 release in response to HDM. These data suggest that under homeostatic conditions, functional actin polymerization contributes to the suppression of HDM-induced CCL20 release.
**Mixed effects of reactive oxygen species on rapid CCL20 release**

To determine the role of reactive oxygen species (ROS) in rapid, HDM-induced release of CCL20, we treated cells with inhibitors targeting different pathways for generating ROS. Diphenylene iodinium (DPI) inhibits nitric oxide synthase and NADPH oxidase, while apocynin is a selective inhibitor of NADPH oxidase alone. When we treated cells with DPI and measured rapid CCL20 release, we observed that HDM-induced CCL20 release was diminished compared to control cells (Fig 8A). However, when we treated cells with apocynin we observed a dose-dependent increase in CCL20 release (Fig 8B). This suggests that nitric oxide synthase contributes to HDM-induced CCL20 release, while NADPH oxidase may antagonize HDM-induced CCL20 release.

We next looked at the effect of inhibiting cyclooxygenase (COX) enzymes. There are two major COX enzymes, designated COX-1, and COX-2 – with COX-3 also having been described in recent years. Celecoxib selectively inhibits COX-2, while aspirin inhibits COX-1 and modifies COX-2 away from production of prostanoids and toward lipoxins. Treatment with either celecoxib or aspirin significantly reduced HDM-induced CCL20 release, suggesting that cyclooxygenase function is required for CCL20 release (Fig 9A, B).

We next supplied the cells directly with the ROS precursor peroxide to determine if excess availability to this reactant would directly affect CCL20 release (Fig 9C). Peroxide (H₂O₂) is a precursor to the generation of superoxide free radicals. Direct addition of peroxide to cell culture had no effect on CCL20 release. Thus, the effect of artificially increased peroxide in
solution may not reflect the function of increased generation of free radicals within the cells, which may have additional signaling roles.

**CCL20 release is not inhibited by corticosteroids**

Inhaled corticosteroids are an established treatment for long-term control of asthma symptoms. We asked whether corticosteroid treatment is capable of reducing CCL20 release from bronchial epithelial cells after exposure to HDM. We measured rapid CCL20, IL-6, IL-8, and GM-CSF release after exposure to HDM in cells that were treated with dexamethasone, a potent corticosteroid (Fig 10A). We found that dexamethasone treatment had no effect on rapid HDM-induced CCL20 release, even as it reduced release of IL-6, IL-8, and GM-CSF. When we looked at longer-term CCL20 release, after 24 hours HDM exposure (Fig 10B), we observed a small increase in CCL20 release, and again saw decreases in IL-6, IL-8, and GM-CSF. We saw this same trend in CCL20 release *in vivo*. We treated mice with HDM *intra trachea*, and looked at chemokine released into the broncho-alveolar lavage fluid (BAL) after four hours. We selected a four hour time point because this was when we saw maximal early release of CCL20. Similar to *in vitro* cultured cells, CCL20 was increased in the BAL of mice treated with HDM, as compared to PBS control (Fig 10C). Additionally, we observed a trend toward increased CCL20 release in mice concurrently treated with both HDM and dexamethasone, although this increase was not considered significant. Taken together, these data suggest that CCL20 release is controlled via a mechanism that is not subject to corticosteroid inhibition, as is the release of other chemokines and cytokines from bronchial epithelial cells.
Discussion

In this chapter we identified a number of signaling pathways that affect CCL20 release, both under homeostatic conditions and after treatment with HDM. Pathways that are necessary for CCL20 release include raf1, cyclooxygenase, and gap junctions. These pathways were often necessary, but not sufficient, to drive CCL20 release; as in each case the presence of HDM was necessary to induce CCL20 release. For example, inhibition of Raf1 reduced CCL20 release, and decreased intracellular chloride. However, the Raf1 activator ZM 336372 had no effect on CCL20 release even after treatment with HDM, suggesting Raf1 is necessary, but not sufficient for CCL20 release.

In contrast, excess potassium was sufficient, but not necessary to drive increased CCL20 release, even under homeostatic conditions. This effect was further enhanced in cells treated with valinomycin. Although the mechanisms driving this effect remain unclear, a role for potassium in countering the effect of chloride removal was suggested when we treated cells with valinomycin and either DIDS or gluconate-replacement media. Previously, we have seen no CCL20 release in the absence of chloride (gluconate replacement media). However, valinomycin overcame this effect and restored normal levels of CCL20 release. Free diffusion of potassium (valinomycin treatment) restored not only HDM-induced CCL20 release, but also baseline CCL20 release – which was almost entirely eliminated in gluconate-treated cells. This suggests that the interaction between potassium and chloride fundamentally affects all mechanisms of CCL20 release (intracellular and extracellular) and not just HDM-induced mechanisms. These processes remain to be further elucidated.
Although lysosome acidification has been shown to be aided by Cl/H\(^+\) co-transporters, some evidence also suggests potassium transport may play a similar role in lysosome acidification.\(^{22}\) This may explain why altering potassium transport with valinomycin is able to compensate for the absence of chloride, and why removal of potassium had no effect on CCL20 release in the presence of chloride.

We observed that baseline release of CCL20 was also eliminated in the absence of the anion chloride, but was enhanced by the cations potassium and magnesium. Previously we demonstrated that lysosome inhibition had no impact on baseline release of CCL20 (chapter 2), suggesting that these changes in ion concentration mainly impact release of extracellular CCL20. Other data from our lab (not shown) suggest that CCL20 binds to the heparin sulfate proteoglycan syndecan-1 on the cell surface. The syndecan binding motif is dependent on electrostatic interactions between heparin-sulfate and CCL20. Our observation that the addition of cations – such as potassium, magnesium, and sodium – into solution has the general effect of increasing CCL20 release may suggest the increased cation concentration interferes with these electrostatic interactions, and passively induces CCL20 dissociation from these heparin-sulfate stores at the cell surface. Additional experiments are required to confirm whether the increased CCL20 release observed in the presence of excess cations is derived from extracellular stores in general, and syndecan-1 in particular. Given that cation addition to the extracellular environment potentiated increased baseline release while leaving additional HDM-induced CCL20 release largely unaffected, our results suggest a model in which constitutive (not
allergen-induced) CCL20 release is derived from extracellular stores attached to syndecan-1, while the main source for HDM-induced CCL20 release is derived from intracellular stores.

Although we observed a number of different treatments that were sufficient to induce reduction of intracellular chloride levels, this reduction alone was insufficient to induce CCL20 release in some instances. However, our observations suggest inhibitors that reduce CCL20 release correspondingly reduce intracellular chloride levels even in the absence of HDM. For example, both DIDS and SP600125 reduced intracellular chloride and CCL20 release. However, potassium depletion and TPPMn(III) also reduced intracellular chloride (potentially by disrupting homeostatic mechanisms driving increased intracellular chloride in these cells) but had no effect on CCL20 release. Thus a reduction in intracellular chloride appears to be necessary but not sufficient to induce release of CCL20. One likely mechanism driving this dependence is the membrane depolarization needed to induce secretory vesicle fusion with the cell membrane, similar to release of synaptic vesicles in neurons.\textsuperscript{55} In neurons, synaptic vesicles are first docked to the cell membrane prior to fusion in a ‘priming’ step.\textsuperscript{56} After this docking takes place, a depolarization event is sufficient to induce fusion with the cell membrane. A similar mechanism in bronchial epithelial cells could explain why reduced intracellular chloride does not always lead to CCL20 release. If CCL20-containing vesicles are not first primed, or if modulation of intracellular chloride does not represent a depolarization event, we would not expect reduced intracellular chloride to potentiate CCL20 release. Further experiments are necessary to determine whether changes in intracellular chloride concentration contribute to CCL20 release through depolarization or through another mechanism.
Interestingly, although we previously showed that impacting chloride transport into the lysosome (by knocking down Clcn7) partially reduced HDM-induced CCL20 release, when we inhibited chloride transport using a more global method (DIDS and NFA) we were unable to detect any CCL20 release. We previously hypothesized that knocking down Clcn7 partially inhibits CCL20 release because it does not affect extracellular chemokine stores. Given that general inhibition of chloride completely eliminates CCL20 release, it is likely that one or more additional chloride-dependent mechanisms impact other aspects of HDM-induced CCL20 release. Additional studies will be needed to outline these mechanisms in more detail.

Increased levels of CCL20 are often observed in the BAL of severe asthmatics, a group that typically displays a weak response to inhaled corticosteroids. Our data suggest a possible explanation for this, as even a potent corticosteroid like dexamethasone had no effect on HDM-induced CCL20 release. Indeed, at higher doses we saw a trend toward increased CCL20 release. These observations were mirrored in vivo. This is in contrast to other signaling molecules like GM-CSF, IL-6, and IL-8; which were all reduced after exposure to dexamethasone. It is therefore not surprising that steroid-resistant asthmatics have elevated levels of CCL20 in their BAL.

Inhibition of reactive oxygen species generation via DPI reduced CCL20 release, but apocynin did not. Other groups have reported a link between the Raf1 pathway and ROS generation. Thus, CCL20 release may be downstream of a pathway that includes Raf1 signaling that promotes ROS generation. Given that increased ROS generation has been observed in
asthmatic airways, this suggests the production of ROS may serve as a danger signal to the cells to enhance release of preformed inflammatory mediators.58

Contrary to our expectations, inhibition of actin polymerization resulted in increased CCL20 release, not decreased release. Additional experiments need to be done to further explain this observation, however based on our current understanding of vesicular release some hypotheses present themselves. For example, actin polymerization is required for endocytic reuptake of vesicles from the plasma membrane, including clathrin-coated endocytosis.59 This reuptake could cycle syndecan-bound CCL20 from the cell surface under normal conditions. In this instance, inhibition of actin could result in increased cell-surface CCL20 and increased release due to HDM-induced syndecan shedding. Further experiments would be needed to demonstrate whether this is the case, or increased CCL20 release due to actin inhibition is due to another mechanism.

We demonstrated here that gap junctions promote HDM-induced CCL20 release. Although the specific mechanism for this remains unclear, one attractive possibility is that the movement of ions through gap junctions allows signals that originate in one cell to propagate to adjacent cells. In one study, epithelial cells infected with intracellular bacteria induced intracellular signaling in uninfected neighboring cells, which responded to the infection of juxtaposed cells by releasing the chemokine IL-8. This mechanism was shown to be gap junction-dependent. A similar mechanism may work to enhance HDM-induced CCL20, leading to our observation of reduced release after gap junction inhibition.29 In addition to this specific mechanism, gap junctions have been shown to allow the passage of small molecules that often
function as second messengers in intracellular signaling pathways. In this way, gap junctions could also enhance intracellular signaling to nearby cells.

We showed that a number of diverse signaling pathways contribute to the release of CCL20 from bronchial epithelial cells. This is unsurprising, as we observed changes in CCL20 release while handling and passaging the cells. Thus, it is likely that the cell responds to many different danger signals by releasing CCL20, thereby helping to recruit iDCs to the site of danger. Additional research will be necessary to show what other chemokines and cytokines released by the bronchial epithelium are similarly affected by these signaling pathways, and whether each pathway is active after exposure to different allergens. As discussed previously, many of the mechanisms we have explored were previously known to promote AHR via separate mechanisms. In addition to these known mechanisms, we demonstrated that they may also promote the release of CCL20 from the bronchial epithelium, suggesting that targeting these pathways could beneficial outcomes in regards to reduction of airway inflammation, in addition to these previously established effects.
References


### Downstream signaling inhibitors used in figure 2

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<th>Inhibitor</th>
<th>Target</th>
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<td>GSIXxx</td>
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**Table 1**
### Actin inhibitors and their functions

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<tr>
<td>Cytochalasin D</td>
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<tr>
<td>Jasplakinolide</td>
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<tr>
<td>Latrunculin B</td>
<td>Prevents G-actin polymerization</td>
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<tr>
<td>Swinholide A</td>
<td>Inhibits actin polymerization, and severs F-actin</td>
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Table 2
Figure 1 – A chloride co-transporter is necessary for HDM-induced CCL20 release. (A) After incubation with DIDS and niflumic acid (NFA) cells were treated with HDM for 2 hours prior to harvesting supernatants for ELISA. (B) Cells incubated in media containing either normal chloride or gluconate nitrate were treated with HDM for 2 hrs. Supernatants were then harvested and cells were further incubated with normal chloride for another two hours prior to harvesting supernatants again (add back). (C) Cells treated in either normal chloride (Cl-) or nitrate-containing media (NO₃⁻) with or without DIDS were treated with HDM for 2 hours prior to harvesting supernatants for ELISA. (D, E) Cells treated with and without TPPMn(III) and DIDS were exposed to HDM for 2 hours prior to harvesting supernatants for ELISA (D) or for 30 minutes prior to measurement of intracellular chloride. (F) Cells transfected with CCL20-mCherry (red) and loaded with LysoTracker dye (green) were treated with DIDS or vehicle control. Cells were then imaged for 60 minutes after treatment with HDM. Data are represented as mean +/- SEM. *p<0.05 compared to control PBS treatment.
nitrate-containing media (NO$_3^-$) with or without DIDS were treated with HDM for 2 hours prior to harvesting supernatants for ELISA. (D, E) Cells treated with and without TPPMn(III) and DIDS were exposed to HDM for 2 hours prior to harvesting supernatants for ELISA (D) or for 30 minutes prior to measurement of intracellular chloride. (F) Cells transfected with CCL20-mCherry (red) and loaded with LysoTracker dye (green) were treated with DIDS or vehicle control. Cells were then imaged for 60 minutes after treatment with HDM. Data are represented as mean +/- SEM. *p<0.05 compared to control PBS treatment. A-D n=3, E n=6
**Figure 2** – Magnesium and sulfate are required for chloride depletion after HDM treatment. (A) HBE cells were incubated in media containing (ctrl) or deficient in magnesium (Mg$^{2+}$) or sulfate (SO$_4^{2-}$) ions prior to HDM treatment for 30 minutes and measured for intracellular chloride concentration. (B) Cells were incubated with balanced ions (ctrl) or in the presence of excess magnesium sulfate (+3.6mM MgSO$_4$) prior to HDM treatment for 30 minutes and measured for intracellular chloride concentration. (C and D) Cells were treated as above with either sufficient ion concentrations (ctrl), sulfate deficient media (no SO$_4$), magnesium deficient media (no Mg), or excess magnesium sulfate (Ctrl + MgSO$_4$); cells were then treated with HDM for either 2 hours (C) or 24 hours (D) prior to harvest of supernatants. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control HDM treatment. A-B n=6, C-D n=3.
**Figure 3** – *siRNA against the Cl-/SO42- exchangers pendrin and SLC26A9 have no effect on HDM-induced CCL20 release.* HBE cells were electroporated with 100 pM (A) or 500 pM (B) of siRNA targeted against pendrin (Pdr) or SLC26A9 (A9). 72 hours later, cells were treated with HDM for 2 hours and supernatants were harvested for ELISA. n=6.
Figure 4 – Potassium is sufficient, but not necessary for HDM-induced CCL20 release. CCL20 release from HBE cells treated with HDM for 2 hours (A) in the presence of excess sodium (NaCl) or potassium (KCl), (B) in the absence of potassium (-K+), (D) in the presence of excess potassium (K+) and/or valinomycin, or (E) in anion inhibition conditions (gluconate or DIDS-treated) with valinomycin. (C) Intracellular chloride concentration after HDM treatment in HBE cells treated in potassium-free conditions. Data are represented as mean +/- SEM. 

*p<0.05, **p<0.01, ***p<0.001 compared to control HDM treatment. tp<0.05, ttp<0.01, tttp<0.001. A-B and D-E n=3, C n=6.
Figure 5 – Gap junction inhibition reduces HDM-induced CCL20 release. Cells were treated with gap junction inhibitors 1-octanol (A), phloretin (B), or carbenoxolone (C) one hour prior to HDM treatment. Two hours after HDM treatment, CCL20 release into the supernatant was measured by ELISA. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001. n=3.
Figure 6 – *SP600126 inhibits CCL20 release and baseline chloride import.* HDM-induced CCL20 release after 2 hours in HBE cells treated with selected inhibitors from figure 1, (A) SP600126. (B) Intracellular chloride concentration after HDM treatment in HBE cells treated with SP600126. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, compared to control HDM treatment. A n=3, B n=6.
Figure 7 – Inhibition of structural proteins alters HDM-induced CCL20 release. HDM-induced CCL20 release after 2 hours in HBE cells treated with various inhibitors. Ctrl=control, Cyto=cytochalasin D, Jasp=jasplakinolide, Lat=latrunculin, Swin=swinholide A, Carb=carbenoxolone. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control HDM treatment. n=3.
**Figure 8** – Inhibition of different ROS generation pathways differentially modulated CCL20 release. HBE cells were treated with the ROS inhibitors DPI (A) or apocynin (B) one hour prior to HDM treatment. Two hours after HDM treatment, CCL20 release into the supernatant was measured by ELISA. Data are represented as mean +/- SEM. **p<0.01, ***p<0.001 compared to control HDM treatment.  n=3.
Figure 9 – Inhibition of different cyclooxygenase pathways differentially modulated CCL20 release. HBE cells were treated with inhibitors of different COX proteins; including celecoxib (A), and aspirin (B); one hour prior to HDM treatment. (C) Alternately, peroxide was added to the culture one hour prior to HDM treatment. Two hours after HDM treatment, CCL20 release into the supernatant was measured by ELISA. Data are represented as mean +/- SEM.

*p<0.05, **p<0.01, ***p<0.001. n=3.
**Figure 10** – *The glucocorticoid dexamethasone has no effect on CCL20 release.* HBE cells were treated with the glucocorticoid dexamethasone one hour prior to HDM treatment for (A) two hours, or (B) for 24 hours. Supernatants were then measured for CCL20, GM-CSF, IL-6, or IL-8 via ELISA. Additionally, (C) mice were treated i.t. with dexamethasone or vehicle control with PBS or HDM for 4 hours prior to harvesting BAL for CCL20 measurement. Data are represented as mean +/- SEM. *p<0.05, ***p<0.001. A-B n=3, C n=6.
Chapter 4. Allergen-induced release of many preformed chemokines and cytokines is through secretory lysosomes

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Abstract

A variety of chemokines and cytokines have been shown to be released into the broncho-alveolar lavage fluid in asthmatics. These act as inflammatory mediators that provide many of the signals that drive asthma pathogenesis. However, the mechanisms controlling their release are poorly characterized. Using a multiplex assay, we measured levels of preformed stores of a broad panel of chemokines and cytokines in bronchial epithelial cells, and found preformed stores of IL-6, IL-8, gro-α, TNF-α, IL-1α, GM-CSF, VEGF, and CCL20 among others. Of these, a subset including IL-6, IL-8, gro-α, TNF-α, IL-1α and CCL20 were released in response to HDM exposure. Using multicolor confocal microscopy and flow cytometry of subcellular organelles, we investigated whether multiple mediators were stored in the same subcellular location. Our data suggest that some mediators, such as gro-α and CCL20, localize to the same subcellular compartments. We also found that release of some, but not all, of these subcellular stores was induced by HDM. We then measured rapid release of various chemokines and cytokines in response to a panel of allergens, including particulate matter, cockroach frass, LPS, Fel d 1, and various pollens; we then compared these allergens to control and HDM stimulation. We found that HDM induced greater overall chemokine and cytokine release. We also found that different allergens induced release of different sets of chemokines from bronchial epithelial cells. These findings identify a mechanism of preformed lysosomal stores for multiple chemokines and
cytokines. These are rapidly released from bronchial epithelial cells in response to different allergens.
Introduction

Multiple inflammatory mediators are released into asthmatic airways in response to allergen exposure. These include CCL20, Gro-a, IL-1a, IL-6, and IL-8, among others. As discussed previously, these chemokines and cytokines mediate many of the phenotypic responses observed in asthmatics, including inflammation and airway hyperresponsiveness. The mechanisms for release of each of these various chemokines and cytokines have previously been poorly defined. Although they are often thought to be released through direct vesicular export from the golgi, storage and rapid release have been observed for other chemokines and cytokines in addition to CCL20, suggesting their release may be mediated by a similar mechanism of secretory lysosome release to what we have seen previously for CCL20.

In some cell types, a variety of different secretory lysosomes may reside within an individual cell. Cells from the hematopoietic lineage are among the most studied examples of this granular release, where cytotoxic T lymphocytes, neutrophils, and natural killer cells all contain these granules. Three major types of granules have been identified in neutrophils. Primary granules, or azurophils, contain antimicrobial peptides called defensins, neutrophil elastase, and cathepsin G; secondary granules, also called specific granules, contain lactoferrin, collagenase, and lysozyme; and tertiary granules, or gelatinase granules, contain gelatinase, lysozyme, and leukolysin. Thus, the cell can respond to different stimuli by selective secretion of
different types of subcellular granules.\textsuperscript{15–18} Although secretory granules are normally thought to reside in hematopoietic cells, recent studies have shown similar granules in other cell types as well.\textsuperscript{19} Previously, we identified CCL20 within secretory lysosomes in bronchial epithelial cells, noting that the signal peptide for CCL20 is most closely related to Cathepsin C. This CCL20 was released after HDM exposure in a similar manner observed in release of secretory granules of other cell types. This suggests that cells of the bronchial epithelium may also contain secretory granules that may be selectively released.

We hypothesized that multiple chemokines and cytokines that are released in response to HDM exposure are contained in the same lysosomal granules. A major challenge in subcellular tracking of multiple chemokines and cytokines simultaneously lies in the limitations of techniques currently developed to identify subcellular localization. Currently, analysis of the contents of subcellular organelles is accomplished by a number of methods, including immuno-transmission electron microscopy (TEM), confocal microscopy, density-dependent centrifugation, and single organelle fluorescence analysis (SOFA). These methods each present practical limitations.\textsuperscript{20}

In immuno-TEM, samples are stained with antibodies containing gold-conjugated particles of different sizes.\textsuperscript{21} The antibodies appear as dark (electron-dense)
dots in the TEM image. Visual distinction between antibodies is accomplished through subjective size comparison. Because of this, effectively distinguishing between more than two antibodies becomes increasingly subjective.\textsuperscript{22} Additionally, colocalization studies using TEM remain qualitative analyses, as images with sufficient resolution to detect immune-stained particles represent a smaller area of the cell, making quantification difficult.

With density-dependent centrifugation, cell lysates are centrifuged in a density gradient to separate specific subcellular components.\textsuperscript{23} This method is useful for quantifying protein differences between fractions of different densities, but it is incapable of distinguishing the contents of subcellular organelles of similar densities.\textsuperscript{24}

Confocal microscopy has sufficient resolving power to identify individual lysosomes, and many proteins may be targeted with fluorescently labeled antibodies within a cell.\textsuperscript{25} However, superposition of fluorophores observed by confocal microscopy does not necessarily imply colocalization, as two proteins may appear together in a 2D image, but be in different cell compartments when viewed on the z-axis.\textsuperscript{26} This effect can be minimized with confocal microscopy, but when visualizing smaller vesicles, apparent colocalization should still be verified using alternate methods.
A relatively newer method that has shown some promise in identification of subcellular organelles has been named SOFA for single organelle fluorescence analysis. However, a major limitation to SOFA is the necessity of transfecting the target cells with fluorescently-tagged proteins for visualization. Previously, fluorescent labeling of subcellular components for SOFA has been limited to cellular dyes, which preferentially insert into specific organelles, or to transfection of the target cells with a plasmid containing a fluorescently-tagged protein of interest.\textsuperscript{20,27} In the present study, we use density-dependent centrifugation, confocal microscopy, and a modified application of SOFA to assess whether multiple chemokines and cytokines reside in the same subcellular organelles.

As discussed previously, atopic asthma is associated with sensitivity to a number of different allergens. These allergens have many attributes in common that contribute to their allergenicity, such as enzyme activity, LPS, beta-glucans, and chitins.\textsuperscript{28–32} In addition to these individual factors, many allergens are a complex mix of multiple factors. Previously, we have focused on HDM for our model allergen, as it is a very potent allergen, and a majority of atopic asthmatics display sensitivity toward HDM.\textsuperscript{33} Given that many of these allergens share similar features that have been linked to their allergenicity, we hypothesized that exposure to each allergen may induce release of many of the same chemokines as HDM.
Although we are interested in the effect of various allergens on secretory granule release, allergen challenge induces the release of a broad array of chemokines and cytokines. This release may be mediated by a variety of mechanisms. We previously showed that CCL20 release is governed by secretory lysosome release. This suggests a mechanism of granular storage that is found in other cell types, and is a major mechanism for the simultaneous release of a number of inflammatory mediators. Therefore we hypothesized that multiple chemokines and cytokines are stored in the same granules within bronchial epithelial cells; that these granules may be selectively released by allergen exposure; and that different allergens induce release of different subsets of chemokines and cytokines. To determine whether multiple types of secretory lysosome are secreted from 16HBE14o- cells in response to HDM, we combined confocal microscopy with SOFA. In order to stain multiple targets with SOFA, we developed a direct antibody staining technique similar to traditional flow cytometry. We found multiple chemokines localized within individual subcellular organelles, with some evidence that multiple combinations of chemokines were contained within different granules. Additionally, we found that chemokine and cytokine release was specific to allergen exposure.
Materials and Methods

Cell culture and treatment: The 16HBE14o- human bronchial epithelial cell line was cultured in 10-15 cm round culture dishes that had been collagen coated. Cells were passaged by applying trypsin (0.05% with EDTA) for 10 min. Treatments were performed in 24-well collagen-coated culture plates in DMEM including 10% FBS, penicillin/streptomycin, and L-glutamine. Upon reaching 70-80% confluence, media was replaced and FBS reduced to 0.1% overnight. Where listed, the inhibitor DIDS (10 μM) was added one hour prior to allergen addition.

Inhibitors: Reagents used as inhibitors were obtained from SigmaAldrich unless specified and were used at the following concentrations: DIDS (10 μM).

HDM treatment: Whole HDM extract was obtained from Greer (B70). 100 μg/mL HDM was added to cell supernatant for two hours prior to harvesting cells.

ELISA: Mouse and human ELISAs were performed in half-well plates using DuoSet ELISA kits from R&D Systems according to their specifications. Multiplex quantification was performed via Luminex using proprietary kits and 50 μL sample volume. Samples were from BAL or cell supernatants that had been frozen at -80°C.

Mouse asthma model: Sensitization to HDM was achieved by intra tracheal (I.T.) administration of 100 μg/mL HDM. Twenty-one days later a second administration of alexafluor 405-labelled HDM was administered I.T. 72-hours after the last HDM
challenge mice were anesthetized with sodium pentathol and airway measurements were taken. The mice were then sacrificed, and broncho-alvelar lavage was performed using 1 mL Hanks balanced salt solution without calcium or magnesium. BAL fluid was the centrifuged to remove cells. Lungs were removed and fixed in formaldehyde, then sent to core personnel at CCHMC who embedded in paraffin, sectioned, and mounted them on slides.

**Airway measurements:** 72 hours after the final allergen exposure, mice were anesthetized, intubated, and ventilated at 120 breaths/min. After airway pressure stabilized, mice given 25 ug/kg weight of acetylcholine *intra venous* and airway pressure changes were follow for five minutes. Airway measurements were quantified as airway pressure time index (APTI).

**Isolation and culture of mouse tracheal epithelial cells (mTECs):** Mice were anesthetized with sodium pentathol and the inferior vena cava was severed to prevent recovery. Trachea were then removed and incubated overnight at 4 °C in 1% pronase (Roche) in DMEM/F12 (50/50) mix (Fisher). Cells were then removed from trachea via sheer force in the presence of 10% FBS and DNase. Cells were then incubated on Primaria plates for four hours to allow fibroblasts to adhere. Non-adherent cells were then removed and grown on rat-tail collagen-coated trans-well plates in DMEM/F12 supplemented with penicillin/streptomycin, L-glutamine, retinoic acid, and 5% FBS. After reaching
1000 ohms trans-epithelial resistance, media was replaced with 0.1% FBS media overnight prior to experimental treatments.

Allergen treatments: Allergens were added to cell supernatant at the following concentrations: HDM (100 ug/mL), cockroach frass, LPS, ragweed pollen (100 ug/mL), ambient urban Baltimore particulate matter (AUB) (50 ug/mL), Fel D 1, alder pollen (50 ug/mL), birch pollen (50 ug/mL), maple pollen (50 ug/mL).

Degranulation assay: Lamp-1 antibody conjugated to Af647 was added to cell culture concurrently with allergen. After 30 minutes, cells were rinsed with PBS, and removed from culture plate with trypsin. Antibody uptake was then analyzed on a BD LSR Fortessa flow cytometer.

Immunofluorescence staining: Cells were grown and treated in 8-well chamber slides. After treatment, slides were fixed in 4% PFA for 15 minutes and rinsed with PBS. Cells were then permeablized with 0.1% triton-x 100, and blocked in 10% rabbit serum. Primary antibody was incubated overnight, followed by rinse with PBS-tween 0.1%. Secondary antibodies obtained from Invitrogen were incubated for one hour, then cells were rinsed with PBS-tween and mounted with VectaShield (Vector Labs) in preparation for imaging.

Immunofluorescence staining on fixed lungs: Paraffin was removed from tissues on slides by immersing them in xylene. Slides were subsequently immersed in 100% Ethanol and
rehydrated by transferring them to 90%, 80%, 70% ethanol, and finally water. Antigen retrieval was accomplished by incubating slides in sodium citrate buffer. Slides were blocked in 10% serum matching the host species of the secondary antibody diluted in PBS-tween 0.1%, followed by application of primary and secondary antibodies to the slides. Slides were then mounted with VectaShield in preparation for imaging.

*Sucrose gradient fractionation:* Cells were cultured as described above, removed from culture via trypsin, and transferred to 370 mM sucrose solution to prevent aggregation of subcellular organelles. Cells were then lysed via Misonix sonicator for 15 cycles. Nuclei were removed by spinning down cells at 3000 x g for 10 minutes. Supernatants were then run on a Percoll gradient of 1.05 g/mL. Fractions were removed serially from the top of the tube, and lysed with RIPA buffer that contained protease inhibitors. Final lysed fractions were then analyzed by ELISA.

*Single organelle fluorescence analysis:* Cells were cultured and treated as described above, and placed in suspension by trypsin removal. Cells were then stained with antibodies as described above under *flow cytometry*. Cells were rinsed in 370 mM sucrose that had been filtered through a 0.2 uM membrane, and lysed using a Misonix sonicator for 10 cycles. Finally, cells were centrifuged at 5000 x g for 10 minutes to remove nuclei, and analyzed via flow cytometry. Voltages for photo-multiplier tubes were set by
comparison of 0.2 to 1.2 uM beads, and threshold values were reduced to accommodate the smaller event sizes.

*Statistical analysis:* Statistical analysis was performed using GraphPad Prism 5 software. Statistical significance was determined using an unpaired student’s t-test for comparisons between two treatments, or two-way ANOVA combined with Bonferroni post-test analysis for comparison between multiple groups. Values of p<0.05 were considered statistically significant.
Results

**Blockade of CCL20 in vivo does not reduce airway responsiveness**

As mentioned previously, knockout mice for the only known receptor of CCL20, CCR6, displayed decreased airway hyperactivity *in vivo*, as demonstrated by other groups.\(^{35}\) We wanted to determine whether directly blocking CCL20 itself *in vivo* would also lead to decreased airway responsiveness, and a decrease in airway inflammation. To do this, we treated mice concurrently with HDM and CCL20 neutralizing antibodies to suppress the signal of CCL20 after HDM release. We did this at either sensitization (Fig 1A,C the first day of HDM treatment), or at challenge (Fig 1B,D two weeks after the first HDM treatment); then measured airway responses, and BAL cellularity 72 hours after the last challenge. Eight mice were used in the treatment groups, and four were used in the PBS control groups. We observed a small decrease in airway responses under both conditions; however this decrease was statistically significant. A small, though insignificant, decrease was also observed in BAL cellularity. A high degree of variability in the treatment groups suggests this experiment does not have sufficient power to resolve whether blocking CCL20 has a substantive effect on airway inflammation and AHR. Although increasing the power of the experiment may allow us to resolve whether there is a statistically significant decrease in AHR and airway inflammation after blocking CCL20, given our current results this decrease is likely to be small compared to the overall effect of HDM on the mouse airways. As such, it is
unlikely that elimination of CCL20 signaling itself is sufficient to eliminate HDM-induced AHR and inflammation.

**Sub-cellular stores of other mediators are found in bronchial epithelial cells**

The observation that CCL20 blockade only partially decreases airway responsiveness and inflammation suggests that additional chemokines and cytokines also contribute to the early recruitment and activation of DCs and the initiation of inflammation and AHR in response to HDM. Indeed, DCs have been shown to reside in the lungs under homeostatic conditions, although not in the numbers seen after allergen challenge. Additionally, many other cytokines and chemokines released into the BAL of asthmatics have been shown to induce inflammation of other cell types in addition to DCs. In chapter 2, we outlined an intracellular storage and release mechanism for CCL20. We hypothesized that other chemokines and cytokines may also be stored within the bronchial epithelium, and that they may be released under a similar mechanism.

To determine which chemokines and cytokines may share a common release mechanism, we first asked which mediators are present in preformed stores within the bronchial epithelium. To do this, we analyzed cell lysates via the multiplex Luminex assay, which measures numerous analytes simultaneously. We measured additional targets by ELISA. Table 1 includes a list of the targets we measured. We found a number of chemokines and cytokines that were stored in bronchial epithelial cells.
under homeostatic conditions (Fig 2a). Some targets that were pre-formed in the cell lysate at the highest levels include gro-α, IL-8, IL-6, and IL-1α. CCL20 stores were orders of magnitude lower than these higher concentration proteins. It was not the lowest measured chemokine stored, however. Additionally, a background level of CCL20 is released by these cells, similar to what has been observed previously, along with background release of other chemokines such as IL-8 and FGF-2. Low-level, constitutive IL-8 release from endothelial cells has been reported by other groups.\textsuperscript{38}

We next set out to determine which chemokines and cytokines that are present in the lysate are also released after HDM exposure. We treated 16HBE14o- cells with HDM for 2 hours and collected the supernatants to measure chemokine and cytokine release via multiplex assay (Fig 2b). We found that many, but not all, chemokines and cytokines that were pre-formed were released by HDM. Also, release was not directly proportional to initial levels of preformed stores. For example, preformed stores of CCL20 were much lower than those of FGF-2, but after HDM treatment we observed more CCL20 had been released into the supernatant than FGF-2. We saw this same phenomenon with IL-8 and IL-6. While slightly more IL-8 was measured in the cell lysate than IL-6, HDM-induced release of IL-6 was almost twice that of IL-8. These data suggest that significant amounts of pre-formed stores are not released in response to HDM exposure.
To test whether chemokines other than CCL20 are stored in similar intracellular compartments as CCL20, we used density-dependent fractionation in a Percoll gradient to separate the subcellular components of cell lysates. Fractions removed from the top of the gradient were tested for the presence of CCL20, gro-α, IL-17f, and TSLP via ELISA (Fig 3). We found that each of these chemokines and cytokines were stored within bronchial epithelial cells. Gro-α and CCL20 localized to the same cell fractions, while IL-17f and TSLP localized to unique fractions, and therefore presumably different subcellular locations. This experiment is insufficient to determine whether gro-α and CCL20 are colocalized, but only measures whether they reside in vesicles of similar specific gravity. Also, measurement of lysosomal markers is required to further determine which of these cell fractions represent lysosomal vesicles. However, lysosomes are known to have multiple specific gravities and hence to separate to multiple fractions during density-dependent centrifugation.39

To further determine the subcellular localization of chemokines and cytokines stored in bronchial epithelial cells, we visualized cells grown on slides using confocal microscopy. Cells were stained with antibodies against GM-CSF, gro-α, IL-8, and CCL20 (Fig 4). We saw robust staining of GM-CSF, gro-α, IL-1α, and IL-8; but very little staining of CCL20. This may reflect the absolute abundance of these proteins within the cells (indeed, this matches our observations in whole-cell lysates from figure 2A) or the antibodies used to stain. While subcellular localization appeared in punctate
bodies in GM-CSF, gro-a, and IL-8; staining of IL-1α appeared diffuse, with no apparent vesicular localization. Additionally, IL-1α and IL-8 staining localized primarily within the nucleus, with some staining outside the nucleus as well. This may explain why high levels of stored IL-8 in cell lysates did not translate to correspondingly high levels of IL-8 release after treatment with HDM (Fig 2). In contrast, GM-CSF appeared to have some nuclear staining, and gro-α showed no nuclear staining.

**Establishing a protocol for single organelle fluorescence analysis**

To further distinguish the movement and release of individual chemokine and cytokine-containing vesicles, we sought to analyze single organelles via flow cytometry. Previous groups have reported performing single organelle fluorescence analysis (SOFA) by transfecting cells with one or more fluorescent probes.40 We sought to modify this technique using antibody staining. First, we used beads of specific sizes to set flow gates within which we expect to find lysosomes and other vesicles. As lysosomes are reported to be found within a 0.2-1.2 µM range, we used beads of 0.2-1.25 µM size (Fig 5A) to set voltages for forward and side scatter photo multiplier tubes (PMTs). At this size, we were forced to reduce the exclusion threshold on the cytometer; therefore, we wanted to determine whether our measurements are affected by debris that were previously screened out under the previous threshold. We tested unfiltered sucrose (Fig 5B), 0.22 µM filtered sucrose (Fig 5C), and sucrose that was spun down by ultracentrifugation for 2 hours at 40,000 x g (Fig 5D) for a fixed period and
measured background events over a defined time period. We found that most debris
was removed via filtration, and centrifugation removed an even greater amount of
debri. Each of these measurements were taken over a five minute interval – about five
times longer than individual samples were subsequently run for – and therefore
represent what would be a much smaller representation of the background observed in
any specific sample. Additionally, none of these background events displayed auto-
fluorescence in any of the channels we measured. As such, this population represents a
minimal number of aberrant events that can only serve to dilute the target vesicles of
interest. We expect that once lamp-1+ vesicles are sorted out of the population this
effect will disappear.

We next sought to determine whether we could remove larger components, such
as nuclei and mitochondria, from cell lysates by centrifugation at 5,000 x g for 10
minutes, as has been reported by others. Using the same size exclusion reported
earlier (Fig 6A,B), we compared size and complexity (forward scatter and side scatter)
of: 1) vesicles that were directly lysed (Fig 6C), 2) the supernatant of lysates spun down
at 5,000 x g for 10 minutes (Fig 6D), 3) and the pellets of this centrifugation step (Fig 6E).
Direct measurement of the lysate revealed both large and small populations of
organelles. We found that the larger cellular components were successfully separated
by centrifugation, as evidenced by the concentration of large vesicles in the nuclear
pellet, and their relative absence in the post-nuclear supernatant (PNS). In subsequent
experiments, we used the PNS to measure lysosome localization. By doing so, we ensured cells that were not lysed during the sonication step were removed from the analysis.

We next sought to determine whether we could measure lysosomes via SOFA by staining with antibodies against lamp-1. When we directly stained the cells with lamp-1 and measured intact cells via flow cytometry, we found that all cells stain positively for the lysosomal marker, as expected (Fig 7A). Staining for SOFA was performed on intact cells. After staining, cells were lysed via sonication. To determine what amount of sonication is necessary to liberate subcellular organelles, we performed a series of increasing rounds of sonication on cells to determine the optimum number of cycles that would lyse the cells without compromising the antibodies themselves (Fig 7B). We found that we could successfully stain subcellular organelles with antibodies and visualize them via flow cytometry. We also observed an increased concentration of lamp-1 positive vesicles after removal of the nuclear pellet. Finally, we observed that increasing the number of sonication cycles did not improve the yield of lysosomes, but instead reduced the number of recovered lysosomes, presumably due to the sonication disrupting the lysosomes themselves over extended cycles.

Having established a protocol for antibody staining and measurement of subcellular organelles, we sought to determine whether we could replicate these results
with antibodies against various chemokines and cytokines. To do this, we stained cells with antibodies against gro-α (Fig 8A), lamp-1 (Fig 8B), CCL20 (Fig 8C), and TNF-α (Fig 8D). We found that we could detect positive antibody staining for all antibodies tested. The strongest staining was exhibited by lamp-1 and TNF-α, with significant staining of gro-α manifesting at higher concentrations. While we did observe a small amount of CCL20 staining, this small increase was insufficient, even at higher concentrations, to allow us to quantify CCL20 in vesicles using multiple antibody stains. The isotype controls displayed for each target correspond to the medium antibody concentration used. Higher isotype concentrations did not increase background staining within the concentrations we measured.

**Many chemokines and cytokines colocalize in vivo**

We next sought to determine whether chemokines and cytokines that are stored in the bronchial epithelium are stored in the same vesicles with CCL20. Because CCL20 staining in human cells was too dim to measure colocalization, we stained mouse lungs with antibodies against multiple chemokines/cytokines to determine whether they colocalize. We found that in mouse lungs CCL20 colocalized with lamp-1 (Fig 9A-C), similar to our observations in human cells. Additionally, we observed significant, though not complete, colocalization of CCL20 with IL-6 (Fig 9D-F) and gro-α (Fig 9G-I), suggesting that many, but not all, intracellular lysosomes containing CCL20 also contain IL-6 and gro-α.
Degranulation of bronchial epithelial cells is induced by a variety of allergens

Having observed that multiple chemokines and cytokines localize to the same secretory lysosomes, we hypothesized that this mechanism for chemokine/cytokine release may be common to allergens other than HDM. To test whether other allergens are able to induce secretory lysosome release, we performed a degranulation assay in the presence of a panel of allergens, including cockroach frass, LPS, ragweed pollen, and maple pollen. We found that the number of cells that displayed significant lamp-1 uptake increased with each of the allergens we tested (Fig 10A). In addition, MFI for each of the allergen treatments was increased in comparison to PBS control (Fig 10B). These data indicate that increased degranulation is occurring in these cells. We also found that different allergens produced different levels of degranulation; in particular, cockroach frass induced much more degranulation than other allergens we tested.

When we treated the cells with the chloride inhibitor DIDS, we saw a dramatic decrease in degranulation for all treatments. DIDS treatment further reduced cockroach-specific degranulation in addition to baseline decreases (Fig 10C). Additional tests are needed to determine whether DIDS, bafilomycin A, and other signaling pathway inhibitors also reduce this measure of degranulation, and whether this same mechanism is induced by each of the specific allergens we tested.
**Degranulation of specific vesicles is differentially induced by specific allergens**

We next sought to determine whether stimulation with various allergens was sufficient to induce release of specific chemokine and cytokine-containing secretory lysosomes using the SOFA technique outlined previously. After treating human bronchial epithelial cells with various allergens, we stained them with to identify lysosomes and specific chemokines. Our earlier observations showed sufficient subcellular staining to distinguish vesicles containing gro-α, TNF-α, and Lamp-1; but not CCL20. Therefore, we selected gro-α and TNF-α as a candidate chemokine/cytokine pair to determine whether they are stored in the same or separate vesicles, with lamp-1 used to identify lysosomes. We then lysed the cells and performed SOFA on the PNS.

We observed that levels of gro-α and TNF-α were generally enriched in lamp-1 containing vesicles when compared with lamp-1 deficient vesicles. This lends support to our hypothesis that these chemokines and cytokines are stored in secretory lysosomes (Fig 11). Additionally, we observed increased numbers of gro-α positive, TNF-α negative vesicles when we treated cells with frass, LPS, or maple; suggesting treatment with these allergens induces accumulation of gro-α in these lysosomes (Fig 11A). Additionally, gro-α and TNF-α double-positive vesicles accumulated in the presence of LPS, suggesting stimulation with LPS induces accumulation of both gro-α and TNF-α in lysosomes (Fig 11B). No accumulation of TNF-α was observed in lysosomes that did not contain gro-α (Fig 11D). Finally, we observed depletion of
vesicles that contain neither gro-α nor TNF-α when treated with any allergen, suggesting accumulation of these mediators into lysosomes is driven by allergen exposure (Fig 11C). Although this experiment has been repeated, the magnitude of the error bars in A and B suggest a larger n should be collected to further verify these results. Additionally, collection of an increased number of events could also help reduce the uncertainty in the data. There are, however, practical limits to the number of events that can be obtained when collecting a large number of samples.

Some co-loading of lysosomes is indicated by the increase in the number of Lamp-1+ vesicles containing both gro-a and TNF-a after treatment with LPS. However in each case we observed an increase in the number of vesicles containing chemokine and cytokine in response to allergen. This may indicate the target proteins are being loaded into vesicles after allergen stimulation. Since there is no change in the Lamp-1- population, this increase is likely to be derived from a non-vesicular source, such as the cytosol.

**Release of many mediators is induced by a broad spectrum of allergens**

We treated human bronchial epithelial cells with a panel of allergens, including ambient particulate matter collected from urban Baltimore (AUB); lipopolysaccharide (LPS); which is found on many bacterial cell walls; HDM; cockroach frass; fel d 1, the major cat allergen; ragweed pollen; birch pollen; alder pollen; and maple pollen (Fig 12, summarized in table 2). We found a number of interesting patterns governing release
of mediators. For example, HDM induced the greatest release of any allergen we treated cells with for any chemokine or cytokine. Additionally, stimulation with the other insect allergen, cockroach frass, often mirrored HDM stimulation, suggesting they share common pattern recognition receptor stimulation. Also, LPS induced a number of mediators, such as IL-1α, IL-6, IP-10 and TNF-α, suggesting that allergens containing significant amounts of LPS, such as HDM and frass, should induce release of these mediators. Interestingly, the pollen allergens induced less chemokine and cytokine release overall, and in the case of Alder even decreased MIP-1β release. This suggests a bias of early epithelial cells against reactivity to pollen allergens.
Discussion

Although when we blocked CCL20 alone we saw a small trend of reduced AHR and BAL cellularity, this did not appear to be effective in substantially reducing the airway response to HDM. One explanation for this muted response could be that the anti-CCL20 neutralizing antibody was delivered concurrently with HDM, and the CCL20 was released to the baso-lateral side of the epithelium before the neutralizing antibodies could travel across the epithelium. This co-delivery method was necessary to ensure the mice would not receive multiple i.t. treatments within a short period of time, with insufficient time to recover from the previous anesthetization. Pretreatment of the airways with CCL20 would allow time for penetration of anti-CCL20 prior to HDM-induced CCL20 release, given that we expect baso-lateral release of CCL20. Another option would be to directly measure the effect of blocking CCL20 by measuring recruitment of iDCs into the airways after treating with HDM, with and without CCL20-blocking antibodies. Although this method would more directly test the effect of blocking CCL20, measuring downstream endpoints, such as AHR and airway inflammation, is a better measure for whether blocking CCL20 alone is sufficient to diminish the development of asthma overall.

A number of therapies based on inhibition of individual chemokines or cytokines have been pursued recently.\textsuperscript{41,42} Although promising, many of these therapies have proved inadequate for reducing asthma symptoms.\textsuperscript{43} It is likely that targeting multiple
chemokine and cytokine pathways simultaneously will be necessary to dramatically impact AHR for some asthmatics. The prospect that multiple mediators might be released under similar cellular mechanisms led us to investigate whether multiple chemokines and cytokines are stored and released from bronchial epithelial cells, and whether chemokines and cytokines other than CCL20 are also stored and released from secretory lysosomes in response to allergen exposure.

Using a combination of subcellular flow cytometry, and confocal microscopy, we showed that multiple chemokines and cytokines may reside in distinct vesicles within bronchial epithelial cells. In lung sections visualized by confocal microscopy, a number of vesicles appear to be stained for both CCL20 and gro-a, or CCL20 and IL-6. This staining is not exclusive, suggesting multiple types of these vesicles may to reside within a single cell. This is similar to secretory granules in other cell types, such as in neutrophils that have azurophilic, specific, and gelatinase granules; with individual subsets of granules containing distinct patterns of protein and enzyme storage. We also showed that different allergens could induce release of different combinations of chemokines and cytokines. Our data also suggest that this difference may be due to release of different combinations of vesicles; with some containing gro-α, but not TNF-α induced by HDM; and some containing both gro-α and TNF-α induced by LPS. Thus, release from these vesicles was triggered by allergen exposure, and individual allergens triggered release of specific subsets of lysosomes. As mentioned previously, gro-α and
TNF-α were both found at high concentrations within the cell lysates, and these proteins were also both released after HDM treatment. Future studies will determine whether this differential sorting is true across other chemokines and cytokines.

Similar measurements of proteins that were found in lower abundance in the cell lysate, such as CCL20, were difficult due to the sensitivity constraints of the assay. Further improvements to the SOFA assay to improve sensitivity will be required to make comparisons between CCL20 and other chemokines and cytokines. Our attempts to improve sensitivity included increasing CCL20 antibody concentration, decreasing the number of sonication cycles to reduce dissociative stress, and employing a primary-secondary antibody system to enhance staining. A future approach may include creating a stable-transfected cell line expressing CCL20.

Despite the sensitivity limitations of SOFA, we demonstrated that staining and measurement of subcellular organelles, such as lysosomes, could be accomplished by flow cytometry. This is significant, because previous efforts at measurement of subcellular organelles by flow cytometry required either transfection of the cells with fluorescently labeled tags for the protein of interest, or relied on staining with less-specific dyes, such as MitoTracker. Here, we demonstrate a method for accomplishing subcellular flow by antibody staining, similar to traditional flow cytometry. This approach offers several advantages to using transfected plasmids.
Antibody staining allows for greater flexibility and a broader array of targets, as plasmids need not be prepared for each target and transfected into the cell line. Also, cells from existing animal models can be interrogated directly.

Additionally, as we showed here, staining with multiple antibodies allows us to identify colocalization within a subcellular organelle. This colocalization is superior to superposition observed in confocal microscopy. Superposition only suggests colocalization and must be validated to ensure the staining does not represent protein in the cytosol. In flow analysis, the vesicles are separated from their surrounding cytosol, eliminating this confounding factor. Indeed, SOFA could be useful as a general tool to validate colocalization observed by microscopy.

Another benefit of this method is that it is quantitative. While methods such as immuno-TEM are sufficient to show colocalization of two proteins within the same organelle, we demonstrate that SOFA has the added benefit of measuring populations of organelles, and changes in these populations after stimulation.

We also noted some significant limitations to this approach. First, we found that measurement of subcellular organelles requires increased sensitivity, and our efforts at SOFA were unsuccessful on older, less sensitive machines. This is similar to what was reported by researchers attempting SOFA with fluorescently tagged proteins. Second, we noted that increased sensitivity (and a decreased threshold for events on the
instrument) required increased purity of the sucrose buffer in which the cell lysates were suspended. Most fine debris could be removed by filtering the sucrose buffer through a 0.22 um filter. We found that additional debris could be eliminated by centrifuging the buffer at 40,000 x g for 2+ hours. This could be useful for identifying organelles of low abundance. Since lysosomes are plentiful within the cell, we only required filtration for debris removal.

The increased sensitivity requirement for SOFA is due to the smaller size of the target. When measuring an intact cell, the signal returns the sum fluorescence across a larger volume than when measuring subcellular features. Therefore, SOFA necessitates fluorescent probes with greater sensitivity, or a target molecule that is in high abundance. In microscopy, increased sensitivity can be easily accomplished by increasing the dwell time of the laser for each pixel, but for flow cytometry this is impossible, as the lysosome passes through the flow cell rapidly and does not allow for increased dwell time. Thus, we found a practical limit for SOFA that impeded our attempts to measure less abundant molecules, such as CCL20. As such, we were unable to determine whether vesicles containing CCL20 also contain other mediators. Another approach to this problem is addressed through the Amnis ImageStream instrument, which takes a continuous exposure of the event as it passes through the flow cell. This has the practical effect of increasing exposure time, similar to that seen on a confocal microscope. Unfortunately, one of the main limiting factors of the ImageStream is the
number of events that can be acquired, which is orders of magnitude lower than traditional flow cytometry. Given that we were already running somewhat high numbers of events for each sample, this practical limitation reduces the ImageStream’s effectiveness due to a smaller sample size, even though it may allow detection of otherwise low-abundance proteins such as CCL20.

A major strength of using a flow cytometry-based technique is that it was not necessary to separate the cell lysate by the time-consuming process of density-dependent centrifugation, because we had a specific marker to identify our organelle of interest. In this case we used lamp-1 to identify lysosomes, although this method could be applied to any subcellular organelle. A drawback of lysing the cell is that fragments of the cell membrane are also likely to form micelles and appear as a small number of events during analysis. These micelles are likely to be small, but may still affect our analysis. Our data suggest that after allergen exposure there is an increase in Lamp-1 that localizes to the cell surface. Specific cell surface stains could help remove this population in future experiments.

As previously discussed, multiple chemokines and cytokines are released into the BAL of asthmatics after exposure to allergen. Our finding that many allergens could induce the release of multiple chemokines and cytokines from bronchial epithelial cells supports this previous observation, and suggests that a primary source for many
mediators of inflammation is the bronchial epithelium. However, we also observed that while some mediators are stored within the same cell compartment, not all the mediators released upon allergen stimulation can be traced back to a single vesicle, or even vesicular storage at all in the case of IL-1α. Thus, the release of mediators governed by many different release mechanisms suggests that multiple signaling pathways contribute to the diverse set of mediators released after allergen challenge.

For IL-8, we observed significant levels of nuclear staining in addition to cytoplasmic staining. At present, it is unclear what role IL-8 plays in the nucleus. Additionally, other groups have reported IL-8 storage on the cell surface. We did not observe significant staining of surface IL-8 or CCL20. In contrast, surface staining of CCL20 by flow cytometry reveals some CCL20 localizes to the cell surface, although at significantly lower levels than intracellular staining. It is likely that surface concentrations of both CCL20 and IL-8 are significantly lower and more diffuse than intracellular vesicular concentrations, such that they are not visible by immunofluorescence microscopy.

As a potential treatment option, there are several advantages to targeting the mechanism for chemokine and cytokine release outlined above, as opposed to targeting a single signaling molecule. We found that targeting CCL20 alone had limited biological activity, possibly due to the many other inflammatory mediators induced by
HDM. Our studies suggest an alternate strategy of targeting the mechanism for release of multiple mediators. Broadly speaking, this would reduce the overall signal produced by the bronchial epithelium in response to HDM. Blocking the release pathway initiated by specific allergens, instead of the chemokine itself, may allow us to target activation in response to HDM (or other allergens) specifically, as opposed to more general immune components. This would allow attenuation of allergen-specific immunity, without the unwanted effect of compromising beneficial immune responses to unwelcome pathogens. For example, long-term treatment with glucocorticoids leads to osteoporosis, and increases susceptibility to infections as a result of its immunosuppressive effects.46,47 Finally, our finding that this pathway of secretory lysosome release is common to multiple allergens suggests targeting their release may be potent against multiple types of allergens.
References:


### Table 1 – Chemokines and cytokines measured in bronchial epithelial cell lysates.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Lysate Stores</th>
<th>HDM release</th>
<th>Mediator</th>
<th>Lysate Stores</th>
<th>HDM release</th>
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<td>MIP-1a</td>
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<td>MIP-1b</td>
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<td>IL-7</td>
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(Number of “+” equal to log stores or half-log released chemokine.)
Table 2 – Chemokine/cytokine release by various allergens

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<th>LPS</th>
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<tr>
<td>GM-CSF</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Gro-a</td>
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<tr>
<td>IL-1a</td>
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<tr>
<td>IL-6</td>
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<tr>
<td>Rantes</td>
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<td>TNF-α</td>
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Increased release compared to PBS treatment:

- + 25% increase
- ++ 50% increase
- +++ 75% increase
- ++++ 100% increase
- - 25% decrease
Figure 1 – CCL20 neutralization has little effect on airway responsiveness, and airway inflammation after HDM sensitization and challenge. (A) Experimental outline; mice were treated with HDM or PBS control intra trachea on days 0 and 21. Mice were sacrificed 72 hours after final treatment. Neutralizing antibody to CCL20 was given at either sensitization (B, D) or challenge (C, E). Data are represented as mean +/- SEM. *p<0.05 compared to control PBS treatment.
challenge (C, E). Airway hyper-responsiveness measured by APTI 72 hours after final HDM exposure are given in (B) and (C), and BAL cellularity for the same time point is given in (D) and (E). Data are represented as mean +/- SEM. *p<0.05 compared to control PBS treatment. n=8.
Figure 2 – *Many chemokines and cytokines that are stored in bronchial epithelial cells are released in response to HDM.* (A) Untreated cell lysates were measured by multiplex protein quantification assay for the presence of a panel chemokines and cytokines. (B) Supernatants of cells treated with HDM or PBS control for 2 hours were measured by multiplex assay for the presence of a panel of chemokines and cytokines. Proteins measured are listed in table 1; proteins below the limit of detection are not depicted. Data are represented as mean +/- SEM.
*p<0.05, **p<0.01, ***p<0.001 compared to control HDM treatment.  n=3.
Figure 3 –Chemokines and cytokines are stored in multiple cellular compartments. HBE cells were separated on a Percoll gradient. After centrifugation, fractions were removed from the top of the gradient and measured for various chemokines and cytokines.
Figure 4 – Chemokines and cytokines in lung epithelial cells localize to distinct cellular compartments. Unstimulated HBE cells stained with different antibodies, and imaged via confocal microscopy. Antibody staining appears green, and Nuclei are stained with DAPI and appear blue.
Figure 5 – Sucrose purification is necessary to remove debris prior to single organelle flow analysis. (A) Size beads were used to set appropriate gates for forward and side scatter on a log scale. Sucrose debris was then assessed by measuring events for 60 seconds at a constant flow rate. Debris is represented for direct, unfiltered sucrose (B), sucrose that was filtered through 0.22 μM pores (C), and sucrose centrifuged at 40,000 x g for 2 hours (D).
**Figure 6** – *Isolated post-nuclear supernatant is distinct from nuclear pellet.* Size exclusion gates were set for forward and side scatter using bead sizes of 0.2 µM (A) and 1.25 µM (B). Density plots for forward and side scatter are depicted for HBE cells that were lysed (C) and then centrifuged at 5,000 x g for 10 minutes prior to separation of the post-nuclear supernatant (D) and the nuclear pellet (E).
**Figure 7** – *Vesicles containing Lamp-1 are primarily located in the post-nuclear supernatant.*

After staining whole cells with Lamp-1, cells were lysed via sonication for 5, 10, or 15 cycles, and then centrifuged at 5,000 x g for 10 minutes prior to separation of the post-nuclear supernatant and the nuclear pellet. Whole cells (A) or vesicles (B) were analyzed by flow cytometry for the presence of Lamp-1.
Figure 8 – Vesicles containing chemokines and cytokines can be measured by SOFA. Unstimulated HBE cells stained with varying antibody concentrations for different chemokines and cytokines were lysed and PNS was recovered after centrifugation. Histogram plots for Gro-α (A), Lamp-1 (B), CCL20 (C), and TNF-α (D) are shown.
Figure 9 – Different vesicles in lung epithelial cells contain different subsets of chemokines and cytokines. Unstimulated mouse lung sections stained with different antibody combinations. (A-C) Lungs stained with Lamp-1 (A) and CCL20 (B) with a merged pseudo-color image (C). (D-F) Lungs stained with CCL20 (D) and IL-6 (E) with a merged pseudo-color image (F). (G-I) Lungs stained with CCL20 (G) and IL-33 (H) with a merged pseudo-color image (I). Nuclei are stained with DAPI and appear blue (C, F, I).
Figure 10 – Various allergens induce rapid degranulation of HBE cells. Degranulation measured by lamp-1 uptake after 30 minutes of culture with fluorescent lamp-1 and a panel of allergens. Data represented as percentage of positive cells (A), or mean fluorescence intensity of all cells (B). (C) Cells treated with chloride transporter inhibitor DIDS or vehicle control for 1 hour were treated with HDM, frass, or PBS control for 30 min. in the presence of fluorescently-labeled lamp-1 antibodies. PBS = phosphate buffered saline control, HDM = house dust mite, Fra = cockroach frass, LPS = lipopolysaccharide, Rag = ragweed pollen, Mpl = maple pollen. Data are represented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control HDM treatment.
Figure 11 – Allergens induce degranulation of specific vesicles. HBE cells treated with different allergens were stained with Gro-α and TNF-α, and then lysed and PNS was recovered after centrifugation. Mean fluorescence intensity of Gro-α (A) and TNF-α (B) for all vesicles. (C) Percent of Gro-α+, TNF-α vesicles. (D) Percent of Gro-α+, TNF-α+ vesicles. (E) Percent of Gro-α+, TNF-α+ vesicles. PBS = PBS control, Frs = cockroach frass, LPS = lipopolysaccharide, Rag = ragweed pollen, Mpl = maple pollen. n=3.
**Figure 12** – Different allergens induce release of distinct sets of chemokines and cytokines.

Chemokine and cytokine release from HBE cells treated with a panel of allergens for two hours. Supernatants were measured by multiplex assay.
Chapter 5. Summary, Discussion, and Conclusions

Summary

The aim of this dissertation is to determine the mechanisms by which bronchial epithelial cells respond to HDM and release inflammatory chemokines and cytokines. We have demonstrated that:

1. CCL20 is stored in secretory lysosomes under homeostatic conditions.
   a. Multiple other mediators are stored in bronchial epithelial cells under homeostatic conditions. Some, such as Gro-α and IL-33, are stored in secretory lysosomes, and may even be stored in the same secretory lysosomes as CCL20.
   b. Other mediators, such as IL-1α and TSLP, are stored in the cytoplasm and are released by a different mechanism than secretory lysosome release.

2. HDM induces rapid secretory lysosome release, including the release of CCL20.
   b. Multiple other allergens induce rapid secretory lysosome release from bronchial epithelial cells.

3. Regulation of rapid CCL20 release through secretory lysosomes is a complex process driven by Raf1 signaling and the generation of reactive oxygen species.
Discussion

**CCL20 is well-suited to the lysosomal environment**

Recruitment of immature dendritic cells (iDCs) into the bronchial mucosa has been well documented in animals and humans in response to allergen.\(^1\)\(^2\) This iDC recruitment is a rapid process. In patients challenged with HDM, an increase in the number of DCs in lung biopsies was observed within 4-5 hours of allergen exposure.\(^3\) Central to early DC recruitment, CCL20 is the only known ligand for CCR6, a receptor preferentially expressed on immature DCs.\(^4\) In humans, both CCL20 release and increased CCR6\(^+\) DCs in the lungs of asthmatics have been documented.\(^5\) Until now, little has been known about the mechanisms by which CCL20 is released from the bronchial epithelium in response to allergen. We have shown that CCL20 is rapidly released from the bronchial epithelium within minutes of allergen exposure, and that an important pathway leading to release includes storage of CCL20 in secretory lysosomes within bronchial epithelial cells. This storage and release mechanism has important implications for the function of CCL20, as well as potential insights into the storage and release of other chemokines and cytokines from the bronchial epithelium. Indeed, our data suggest that nearly half of the CCL20 release by bronchial epithelial cells within the first 24 hours that can be attributed to HDM is released within the first two hours of exposure.
Although lysosomes are often studied for their degradative effect on many proteins, some proteins are actively stored in lysosomes. These proteins are often better suited to the caustic environment of the lysosome. Some proteases, such as cathepsins, are resistant to degradation by other lysosomal proteins, allowing them to remain within the lysosomal environment. Other proteins evade lysosomal degradation by binding to the heparin-sulfate proteoglycans (HSPGs) that make up what is known as an electron-dense core in lysosomes (so-named because in electron micrographs these areas within the lysosome appear dark). Proteins residing in this HSPG-rich environment within the electron-dense core are not accessible to degradative lysosomal enzymes, and therefore they escape intact. Many chemokines and cytokines may potentially utilize this strategy to evade lysosomal degradation. Binding motifs to heparin-sulfate have been identified in IL-8, CCL20, and many other chemokines and cytokines. Several chemokines have been shown to bind to specific HSPGs in vivo, such as IL-8 binding to syndecan-1. This binding interaction is targeted at the heparin-sulfate side chains, not the protein stock, for both IL-8 and many other chemokines. Syndecans have generally been shown to localize to the cell surface, however syndecan-1 has also been identified within the lysosomes of breast carcinoma cells – presenting the possibility that this mechanism of intracellular syndecan storage may be present in other cell types as well. A more thorough investigation into the cellular localization of syndecans may reveal a trafficking pathway that leads through the lysosomal
compartment. Taken together, these studies suggest a mechanism by which chemokines and cytokines may evade degradation by binding to HSPGs located within lysosomes. In the absence of intracellular syndecan-1, this binding may still play a role in chemokine release. As chemokines are released from bronchial epithelial cells, they are exposed to syndecans on the cell surface, which may sequester them after release until these HSPGs are cleaved themselves.

Previous studies involving CCL20 indicate that it is not only tolerant of the lysosomal conditions, but that it displays greater functionality under conditions normally found only within lysosomes, such as increased acidity and high chloride concentrations. For example, under neutral pH CCL20 exists in a dimeric form, but acidic conditions cause this dimer to dissociate into its component monomers. These monomers display increased antimicrobial activity – potentially because CCL20 antimicrobial activity is dependent on insertion of its C-terminal domain into the target microbial cell membrane. This C-terminal domain is hidden in dimer form, thereby reducing antimicrobial activity. Dissociation of the CCL20 dimer reveals the two C-terminal domains. In this way, CCL20 antimicrobial activity is similar to that of β-defensin.\textsuperscript{4, 15} Dissociation of the dimer also increases the number of CCL20 molecules available for chemotaxis, which is thought to be mediated by the N-terminal domain of the protein.\textsuperscript{16} Although we would not expect CCL20 to induce chemotaxis or attack invading microbes from within the lysosome, the protein itself is clearly well-suited to
an environment in which many other proteins denature (due to the low pH) or are otherwise inactivated (due to exposure to proteases). In addition to low pH, lysosomes also contain elevated levels of chloride ions. Elevated chloride concentrations have also been shown to enhance CCL20 antimicrobial activity.\textsuperscript{17} Some of the same properties that make CCL20 resistant to lysosomal degradation also make it well-suited to the conditions found in asthmatic airways. For example, asthmatic airways display dramatically increased acidity, in the pH range of 5.2.\textsuperscript{18} Under these conditions, CCL20 would exist as a monomer and exhibit increased chemotactic activity.\textsuperscript{15} As discussed in previous chapters, CCL20 release is induced by a number of mechanisms that are not necessarily linked to airway pH and as such we expect it will induce chemotaxis independently of decreased airway pH. However, as asthma develops this may lead to a positive feedback mechanism in which, as a T\textsubscript{H}2 response develops CCL20 chemotaxis is enhanced, leading to increased recruitment of CCR6-expressing cells such as iDCs, T\textsubscript{H}17 cells, and B-cells.

Thus, although most proteins would be expected to denature and be readily degraded within lysosomes, CCL20 is highly suited to persistent storage in the acidic lysosomal environment, protected from degradation by binding to HSPGs in the electron dense core. Given the structural similarities between CCL20 and many other chemokines – such as IL-8, MIG, and IP-10 – we expect many of these may also be well-suited to residence within the lysosomal environment.\textsuperscript{4} Most of the chemokines and
cytokines we saw released from bronchial epithelial cells after HDM exposure display heparin-binding motifs (including IL-1α, IL-1β, IL-6, IL-8, IP-10, FGF-2, and Gro-α; but not TNF-α). Some of these proteins were not found in lysosomes in our hands, such as IL-1α, but would still be expected to bind extracellular sulfated heparin upon release from the cell, contributing to their role in chemotaxis. Others may reside either within lysosomal stores or in other intracellular vesicles. Future experiments are needed to determine whether multiple types of intracellular vesicles are used to export different chemokines and cytokines, or if secretory lysosomes are the sole intracellular storage vesicle for chemokines and cytokines.

As a chemokine, CCL20 functions both as an antimicrobial peptide and as chemotactic factor for iDCs and T<sub>H17</sub> T-cells. These are functionally separate events, as antimicrobial activity relies on the C-terminal domain and chemotaxis relies on the N-terminal domain. Thus, our finding that CCL20 is rapidly released via degranulation suggests a mechanism that allows the cell to respond to danger by simultaneously attacking a potential intruding microbe and initiating an adaptive immune response. These dual activities have also been demonstrated for a number of other chemokines in addition to CCL20; many chemokines display strong antimicrobial activity against gram negative bacteria (with an LD<sub>50</sub> in the range of 1 µg/mL), but weaker activity against gram positive bacteria (with an LD<sub>50</sub> in the range of 5 µg/mL). In comparison, DC chemotaxis has been shown to require only 100-300 ng/mL.
Additional storage and release mechanisms for CCL20 include extracellular stores on syndecan-1

Although much of our work is focused on intracellular mechanisms of chemokine release we also observed evidence of extracellular CCL20 stores. As mentioned earlier, stores of chemokines, such as IL-8, have been shown to be bound to syndecans on the cell surface. It is likely that some of the signaling pathways described above inhibit either or both intracellular and extracellular mechanisms of release. For example, we saw complete inhibition of CCL20 release in the presence of chloride transporter inhibitors or in chloride deficient solution. However, when we specifically targeted lysosome acidification by inhibiting V-ATPase or by knocking down CLC-7, which participates in lysosome acidification, we only saw about a 50% reduction in CCL20 release. This suggests that the presence of chloride affects CCL20 release through interactions other than through the lysosomal release pathway, such as through a separate chloride transporter.

Although the specific identity of this chloride transporter remains unknown, chloride and other ions are known to be involved in a number of cell processes that are important for chemokine release. In addition to lysosome formation, release of lysosomes by fusion with the external plasma membrane is also ion-mediated. In neuronal cells, synaptic vesicles have been shown to tether to the plasma membrane in preparation for release. Upon cell depolarization, calcium released into the cytosol
induces membrane fusion complexes, called the SNARE complex, to change conformation and fuse the vesicle with the plasma membrane. This change in membrane potential that initiates vesicle fusion is driven by ion changes in the cell. In different cell types, these changes have been shown to be associated with a variety of ions. An example of this with anions is external ATP-mediated vesicle release from medullary adrenal cells, which has been shown to be a chloride dependent process. A cation-specific example of this is synaptic vesicle release in frog neurons, which is induced by high external concentrations of potassium – similar to our observation that increased external potassium dramatically increased CCL20 release. In bronchial epithelial cells, chloride efflux, or potassium influx, could potentially drive release of docked vesicles by changing the electrical potential within the cell. The specific identity of these transporters remains to be determined; however some interesting candidates have been associated with synaptic vesicle release in neurons. CLC family members, such as CLC-2, have been associated with tethered synaptic vesicles in particular, which may suggest a direct role in vesicle release. Additionally, potassium transporters, such as calcium-gated potassium channels (gKca), have been shown to be in close proximity to the presynaptic membrane where synaptic vesicles are tethered, suggesting they may also play a role in vesicular release. Whichever transporters participate in vesicular release in bronchial epithelial cells, they are likely to do so by inducing rapid changes in voltage or ion concentration to trigger release.
In addition to intracellular changes in ion concentration, extracellular changes could also affect release of extracellular chemokine and cytokine stores. As has been noted previously, many chemokines and cytokines are stored on HSPGs via electrostatic interactions. We would therefore expect that changes in ion concentration at the basement membrane would interfere with these electrostatic interactions and cause these proteins to dissociate from the HSPGs to which they are bound. Indeed, we saw increased baseline release of CCL20 in the presence of various elevated cations, suggesting that changing the extracellular ion balance may be sufficient to passively dissociate CCL20 from the cell surface. Given that these cations did not induce an additional increase in CCL20 release after HDM exposure, this suggests the change in cations predominately affected release of extracellular stores. This mechanism requires further study. For example, we would expect that mouse lungs deficient in baseline syndecan release, for example in syndecan-1 knockout mice, resident lung DCs would be reduced in comparison with mice expressing syndecan-1. However, separating intracellular and extracellular impacts of syndecan-1 knockout mice may be difficult to do, as we discuss below.

As we have seen, a number of mechanisms involving ion transport may mediate release of chemokines and cytokines by bronchial epithelial cells. This is not surprising, given the role of the airway epithelium in maintaining the ionic composition of the sol in the airway lumen. Invasion of this environment by bacteria or other microbes would
dramatically alter airway ionic composition and pH, which would serve as a danger signal to the epithelium to release chemo-attractants to help fight off invaders.

In addition to direct release from HSPGs, our data suggest that release of extracellular chemokines and cytokines is potentiated by cleavage of syndecan-1. We observed that tracheal epithelial cells from syndecan-1 knockout mice showed about a 50% reduction in release of both CCL20 and IL-8 (data not shown), suggesting that the extracellular CCL20 pool previously discussed localizes to syndecan-1. Another possible explanation for this reduction is that if stores of CCL20 within the lysosome are bound to syndecan-1, these would no longer be protected from degradation in the lysosomes of knockout mice. Future research will need to determine whether the reduction of CCL20 release in syndecan-1 knockout mice is due the absence of an extracellular storage location, or to the degradation of intracellular stores. For example, if CCL20 is degraded in the lysosomes of syndecan-1 knockout mice, we would expect this to be reflected in an absence of CCL20 intracellular staining in immunofluorescence images of mouse lungs from knockout mice.

Syndecan shedding has been shown to be dependent on the PKC and MAP kinase signaling pathways, and potentially JNK signaling as well.\textsuperscript{32} Thus many, but not all, of the signaling pathways we found to be involved in CCL20 release from secretory granules have also been associated with syndecan release. From these studies, we
hypothesize that although CCL20 may be stored in multiple locations throughout the cell, release from these locations may not be mediated by mutually exclusive pathways but is rather mediated by similar mechanisms. Future studies will lead to a better understanding of the common pathways that lead to chemokine release from multiple cellular sources at once, as well as those pathways that lead to mobilization of only specific cellular sources. An enhanced understanding of this complex intracellular signaling network will help in further understanding why different allergens induce release of different subsets of chemokines and cytokines from the bronchial epithelium.
Chemokine and cytokine combinations found in secretory lysosomes work together to promote a Th2 response

In addition to antimicrobial activity; CCL20, IP-10, and other chemokines have also been shown to directly kill parasites such as Leishmania mexicana. It is not surprising, then, that we found other antimicrobial chemokines, such as gro-α, are controlled by a similar release mechanism as that of CCL20. However, while CCL20 recruits iDCs and Th17 T-cells; gro-α, IP-10, and IL-8 recruit neutrophils. These divergent activities allow rapidly released chemokines to actively respond to danger signals through direct killing, while also contributing in diverse ways to the downstream inflammatory response. Neutrophils, Th17 cells, and dendritic cells have all been documented in asthmatic lungs – and more particularly in the lungs of severe asthmatics. Our findings suggest a common mechanism initiating the release of the chemotactic agents responsible for recruiting these cell types, as well as cytokines that will act on the recruited cells to induce an asthmatic Th2/Th17 phenotype. In addition to those we specifically identified, we expect a number of other preformed chemokines may also be subject to this release mechanism, given their common chemotactic functions and observed rapid release. Some examples of chemokines we expect may are rapidly released include CCL20, gro-α, IL-8, and IP-10. Bronchial epithelial cells are not the sole source of chemokines in the lung, and mechanisms of storage and release in other cell types may by similar to that of epithelial cells.
Indeed, IL-8 stored in Weibel-Palade bodies in endothelial cells was shown to be rapidly released, which is strikingly similar to the mechanism we have described for chemokine release from bronchial epithelial cells.\textsuperscript{39} This may suggest that secretory lysosome release of vesicles containing chemokines and cytokines is common to additional cell types as well. If these vesicles resemble the vesicles we observed in bronchial epithelial cells, they may also contain a mixture of different chemokines and cytokines that are co-released.

As outlined previously, a mechanism of secretory granule release, similar to the one described in this study, has also been described for cells of the hematopoietic lineage as well as for endothelial cells. Interestingly, our findings are the first to describe granule release from cells that are not derived from the mesoderm, as epithelial cells are derived from the endoderm. This suggests that this mechanism may be found in other endoderm-derived cell types in other tissues. Particularly, we hypothesize that release of chemokines from secretory granules may also occur in nasal, gut, and skin epithelial cells, as well as other types of epithelial cells. Future studies would need to determine which, if any, other cell types retain persistent vesicular stores of chemokines and cytokines in this manner, as well as what agonists trigger their release.
SOFA is a powerful new tool for analyzing subcellular organelles

One major experimental difficulty we encountered was in positively identifying that intracellular chemokine stores were co-localized to the same vesicle. We showed that one promising avenue for identifying the contents of individual granules is through single organelle fluorescence analysis (SOFA). We demonstrated that multi-color flow cytometry, could be performed on subcellular components using SOFA. This technique could be useful in further understanding the nature and components of chemokine and cytokine-containing granules. Specifically, by taking advantage of flow-based sorting techniques, such as fluorescence-activated organelle sorting (FAOS), organelles containing different subsets of chemokines and cytokines could be isolated and analyzed. These sorted populations could then be separated by 2D gel electrophoresis in preparation for peptide mass fingerprinting via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which would allow specific identification of individual proteins residing in different types of granules. A major technical difficulty with this approach is that the sample size required to obtain enough secretory granules to form a sortable population could become prohibitively large.

Some proteins we expect to find in these secretory vesicles include proteins involved in secretory granule release, such as synaptotagmin and synaptobrevin – two proteins that make up part of the SNARE complex, and facilitate vesicle fusion with the
plasma membrane.\textsuperscript{40–42} Identifying the specific SNARE proteins localized to these secretory granules would be beneficial in developing new therapies to block their release. Unlike secretory granules found in some hematopoietic cells, we would not expect epithelial cells to contain effector molecules that drive cell death, such as perforin and granzyme.\textsuperscript{43–46} These effector molecules mediate cell death in natural killer cells and cytotoxic T-lymphocytes;\textsuperscript{47,48} however perforin-mediated cell death is not a function that has been observed in non-migratory airway epithelial cells.\textsuperscript{49}

\textit{CCL20 is sufficient, but not necessary to promote AHR}

The importance of CCL20/CCR6 in allergic sensitization was demonstrated in a cockroach model of allergic asthma in which CCR6 knockout mice displayed protection against development of asthma.\textsuperscript{50,51} Contrary to these findings, in which cockroach frass was the major allergen studied; we observed that in a HDM-exposure model of asthma, blocking CCL20 was insufficient to eliminate AHR. This discrepancy may be due to the presence of a number of signals, other than CCL20, released in response to HDM exposure that have also been demonstrated as important in the development of AHR. AHR that is driven by cockroach allergen may be primarily dependent on signaling through the CCL20/CCR6 axis, whereas AHR initiated by HDM may induce more robust response, including the release of a more broad array of chemokines and cytokines. Our data suggest that a potential reason for this discrepancy may be due in part to the different initial signals provided by HDM versus cockroach frass. In
addition to CCL20, HDM induced release of FGF-2, Gro-α, TNF-α, IL-6, IL-8, and IP-10, among others. In contrast, cockroach frass did not induce release of IL-6 and IP-10, and caused release of significantly lower levels of FGF-2, gro-α, TNF-α, and IL-8. In a cockroach model, blockage of CCL20 signaling may result in reduction of downstream signaling and eventual development of AHR because it is a large component of the bronchial epithelial cells’ initial response to the allergen. In contrast, HDM induces release of a much broader array of chemokines and cytokines from the airway epithelium. All these signals function to promote inflammation through recruitment of a number of cell types, such as DCs and TH17 cells for CCL20, T-cells for IP-10, and neutrophils for IL-8 and gro-α. IL-6 has been shown to inhibit the development of regulatory T cells in favor of Th17 cells,\textsuperscript{52} which augment a Th2 response in asthmatics to promote more severe AHR.\textsuperscript{53} Thus, our data suggest that HDM induces the release of multiple signals that combine to promote the development of a Th2 response. As such, loss of one signal, namely CCL20, appears to be insufficient to block the overall development of AHR.

Both cockroach frass and HDM are common, complex allergens. However, a number of factors contribute to the more robust signal provided by HDM in comparison to cockroach frass and many other allergens. HDM is a complex mixture of several different types of agonists including proteases, chitin, and LPS.\textsuperscript{54–56} Thus, multiple signals work together to promote a Th2 response. It is a potent allergen, with
atopy to HDM more common in asthmatics than any other allergen. This makes targeting pathways for HDM-induced AHR a valuable strategy for developing novel asthma treatments, since many of the mechanisms that are necessary for chemokine and cytokine release in response to HDM are also likely to be activated in response to other, less complex allergens. Cockroach frass is also a complex allergen, although it is not as potent and not as prevalent in atopic individuals. Frass also contains chitin, active proteases, and LPS, which may explain why it induced release of more chemokines and cytokines from the bronchial epithelium than other allergens we tested, such as tree and grass pollens. The mechanism we describe, in which chemokines and cytokines are stored in and released from intracellular vesicles, is a novel mechanism in bronchial epithelial cells, and may help to explain how upstream signaling pathways lead to the release of different subsets of chemokines and cytokines and therefore different outcomes from exposure to a variety allergens.

Different allergens induce release of unique subsets of chemokines and cytokines to promote AHR

The bronchial epithelium has recently been highlighted as an important source for a number of different chemokines and cytokines that are critical in the induction of atopic asthma. Much recent focus has turned to IL-33, as well as TSLP, as airway epithelial cell-derived mediators that play an important role in the development of type-2 immune responses. IL-33 expression in the bronchial epithelium correlated with
asthma severity in an OVA model of AHR, and it has been shown to induce production of the Th2 cytokines IL-4, IL-5, and IL-13 as well as airway inflammation. TSLP expression has also been shown to be increased in human asthmatic airways, and to induce a type-2 immune response. When we consider our finding that CCL20 and IL-33 may share a common release mechanism, this suggests a model in which airway epithelial cells co-release CCL20 and IL-33 from lysosomal stores upon stimulation with insect allergens, such as HDM or cockroach frass. Under these circumstances, CCL20 would work to promote recruitment of cells containing CCR6, such as iDCs, innate lymphoid cells, and Th17 cells, while IL-33 would promote the development of a type-2 response through DCs and ultimately the downstream development of a Th2 response.

In addition to our finding that IL-33 appears in punctate vesicles within bronchial epithelial cells, we postulate other potential release mechanisms for preformed IL-33. One example of a cytokine belonging to the IL-1 superfamily with multiple demonstrated release mechanisms is IL-1β. This cytokine has long been known to be stored within the cytoplasm, but recent evidence has indicated that it may also be stored in the secretory lysosomes of monocytes. IL-33 may be stored in a similar fashion, with both cytoplasmic and lysosomal storage locations. Although the process of protein release from within vesicles has long been established as simple fusion of the vesicle and plasma membranes, it is unclear how IL-1 family members...
stored in the cytosol are released from the cell, given the necessity of crossing a lipid bilayer in order to exit the membrane-bound area. Two potential mechanisms for cytoplasmic release of IL-1β that have been proposed include cell death and release of micro-vesicles that are the result of membrane blebbing. As a closely related cytokine to IL-1β, IL-33 may also be stored inside the cell by similar mechanisms. Indeed, staining overlays of both CCL20 and IL-33 in our confocal images suggest that these two proteins share many common secretory vesicles, but additional non-punctate staining of IL-33 is prevalent – leaving the possibility that additional cellular storage locations for IL-33 exist.

We also found cellular stores of IL-1α that were rapidly released by HDM. However, these stores did not appear to localize to secretory lysosomes or to discreet intracellular pools, indicating a potential cytosolic storage for this cytokine as well. A unique mechanism of pre-release processing for the IL-1 family member IL-1β has been reported previously, in which activation of the inflammasome by a number of factors, such as uric acid or the presence of external ATP, leads to cleavage of pro-IL-1β. This cleavage converts pro-IL-1β to IL-1β in preparation for release of the active cytokine. Although this mechanism shows how IL-1β is processed into its active form, little is known about how IL-1β is subsequently exported from the cell. We observed a diffuse distribution of IL-1α, suggesting a cytosolic distribution similar to IL-1β, and although the inflammasome may not be involved in IL-1α processing, this closely
related protein may share a similar release mechanism with IL-1β. A separate release mechanism for IL-1α that has previously been reported is unlikely to be involved in HDM-induced IL-1α release. In this mechanism, IL-1α acts as an alarmin; it translocates to the nucleus in the event of cell damage, and is passively released in the event of cell death. Our data suggest IL-1α was released from healthy bronchial epithelial cells, suggesting that cell death is not the mechanism of release for this cytokine. Additionally, our ELISA measurements are unlikely to detect membrane-bound cytokine, suggesting membrane blebbing is also not the mechanism governing release of IL-1α. Other possible mechanisms for release of proteins from the cytoplasm include active transport across the cell membrane or loading into intracellular transport vesicles. Additional experiments are needed to more fully understand the mechanisms governing allergen-induced release of these IL-1 family members.

Common storage and release patterns, especially for chemokines and cytokines stored in a common vesicle or released through a common pathway, suggest the signaling molecules are involved in a coordinated function. In bronchial epithelial cells treated with HDM, we saw nearly complete ablation of intracellular stores of CCL20. Stores of IL-33 that reside in the same vesicles as CCL20 would also be expected to be released after HDM exposure. Co-release would allow CCL20 to induce recruitment of iDCs and Th17 T-cells, while IL-33 induces DCs to promote a downstream Th2 response. Similar co-storage and release after HDM exposure was observed with gro-
α, which induces recruitment of neutrophils, as previously stated, also contributing to the development of an asthma phenotype.

In contrast to IL-33 and gro-α, we did not detect any preformed stores of TSLP; although we did see TSLP release after two hours of ragweed exposure (data not shown), similar to what other groups have observed. Of the panel of cytokines and chemokines we looked at, only CCL20 and TSLP were released in response to ragweed pollen. Therefore, ragweed exposure suggests an alternate route to the development of a Th2 response, in which CCL20 works to recruit iDCs and TH17 cells, and TSLP acts on these recruited iDCs to promote a Th2 phenotype.

We have reviewed two major pathways for release of preformed cytokine from bronchial epithelial cells that appear to play important roles in the development of a Th2 response in asthma, although only specific pathways are activated by any individual allergen. Thus, different classes of allergens may initiate a Th2 response in unique ways. In addition to these two pathways leading to protein release, two other release mechanisms are suggested by our findings: de novo synthesis, and cell surface storage. A better understanding of each of these mechanisms, and the signaling pathways that lead to their release, may help in the development of future targeted treatments for asthma.
Given that individual allergens appear to induce the release of specific subsets of chemokines and cytokines, the specific pathways triggered by an individual allergen could be blocked in asthmatics based on atopy to specific allergens, thereby providing targeted inhibition and reducing overall immune suppression. For example, in our hands ragweed pollen predominantly induced release of TSLP and CCL20, but not other mediators, from the bronchial epithelium. In patients who display atopy to ragweed, blockade of TSLP – directly through anti-TSLP neutralizing antibodies or indirectly through blocking the signaling pathway leading to TSLP release – may be an effective treatment to reduce airway inflammation. As CCL20 induced recruitment of iDCs to the airway epithelium, TSLP would be blocked from promoting a Th2 response in these recruited iDCs, preventing the development of AHR. This same treatment would likely prove ineffective in patients who are atopic to HDM, as HDM does not induce immediate release of TSLP from bronchial epithelial cells but does induce release of broad range of different chemokines and cytokines that lead to the development of a Th2 response.

*Targeting cytokine release mechanisms may be a more effective therapy than targeting individual cytokines for some types of atopic asthma*

Our research has focused on better understanding the mechanisms of allergen-induced chemokine and cytokine release, with the ultimate goal that this may lead to better therapies for asthmatics. Currently, a number of cytokine-based therapies are
undergoing clinical trials for the treatment of asthma. These therapies rely on anti-cytokine antibodies to neutralize the effects of the target cytokine. Some targets that are being pursued include IL-4, IL-5, and IL-13 (key mediators of the type-2 response in asthma) as well as TNF-α. Although anti-cytokine therapy showed great promise in animal models of disease, trials in humans have proven less effective at alleviating AHR and airway inflammation, with some authors concluding that blockade of multiple mediators may provide more robust results. Additionally, new targets for therapy are being considered, including anti-IL-33 and anti-TSLP, among others.

Our work suggests a number of potential therapeutic approaches for suppression of chemokine and cytokine release by targeting release pathways as opposed to directly targeting individual cytokines. A few examples include targeting epithelial secretory lysosomes directly, targeting pattern recognition receptors, or targeting the downstream signaling pathways they initiate. We will consider each of these targets and their potential issues in using targeting them for therapy. MAPK signaling pathway activation in a number of different cell types has been implicated in allergic airway inflammation. ERK1/2 and p38 phosphorylation have been shown to contribute to IL-8 release, and activation of Raf1 has been shown to induce gro-a in epithelial cells. Ras-Raf1-Syk signaling mediates secretory lysosome function. Additionally, ICAM-1 expression in the bronchial epithelium was shown to be dependent on signaling through both ERK and JNK. General inhibition of MAPK signaling led to a decrease
in airway inflammation and AHR.\textsuperscript{98,99} Thus, signaling through the MAPK pathway; including Raf1, ERK, and JNK; is an attractive target to inhibit the initial airway epithelial response to allergens, as well as the treatment of asthma in general.

There are a number of potential drawbacks with this broader approach. Directly targeting lysosomal release could potentially lead to impaired neurological function if used as a long-term therapy, similar to individuals suffering from lysosomal storage diseases such as Tay-Sachs or Salla disease.\textsuperscript{100,101} Long-term inhibition of lysosomal release causes a build-up of waste products within the cell, as proteins targeted for degradation are loaded into endosomal bodies, but are never degraded. These endosomal bodies swell the size of the cell, and interfere with normal cell function. Neurons are particularly susceptible to inhibition of lysosome function.\textsuperscript{102} Another promising treatment target is the Raf1 signaling pathway. Inhibitors to Raf-1 have been developed for treatment in a number of different cancers, including hepatocellular carcinoma.\textsuperscript{103} The treatment has been well-tolerated, with skin rashes of the hand and foot being the most common adverse effect documented to date.\textsuperscript{104}

A larger problem with targeting the release of a broad range of chemokines and cytokines is that these proteins play an important protective role against invading pathogens. For example, in response to respiratory syncytial virus (RSV) a number of chemokines and cytokines are released by the bronchial epithelium, including RANTES,
IL-8, and IL-6 – all of which are released in response to HDM in our hands.\textsuperscript{105} This suggests that blocking mechanisms of HDM-induced chemokine and cytokine release would likely target RSV-induced immune responses, thereby enhancing susceptibility of these patients to infection. However, some evidence suggests that blocking CCL20 release during RSV infection may diminish adverse responses to RSV by blocking conventional, but not plasmacytoid DC recruitment.\textsuperscript{106} Thus, it is not immediately clear that blocking chemokine and cytokine release would dramatically exacerbate RSV infection.

One way to minimize these adverse effects would be to focus the more broad lysosomal-release-targeted therapy on severe asthmatics. Severe asthmatics display increased neutrophil inflammation in the lungs, as well as increased Th17 T-cells.\textsuperscript{38,107} Our data suggest that inhibiting the upstream mechanisms that control release of intracellular CCL20 would also reduce release of IL-8 and gro-\(\alpha\), which have been shown to induce neutrophil recruitment, and IP-10, which has been shown to contribute to recruitment of T-cells and dendritic cells.\textsuperscript{108–110} A more ideal treatment paradigm would be to identify patients who are susceptible to developing asthma, and expose them to allergen while blocking lysosomal chemokine release. This would have the added benefit, as a short-term therapy, of avoiding many of the long-term adverse effects mentioned above. Unfortunately, this approach would have little benefit for
individuals who already have asthma and have developed a T_{H2} response to an allergen.

One of the benefits of targeting common release pathways instead of individual chemokines and cytokines is that it would reduce the release of multiple mediators simultaneously. This has the practical effect of targeting multiple downstream pathways that lead to AHR, such as neutrophil recruitment, DC recruitment, and T-cell recruitment, among others. Our own data suggest that blocking CCL20 alone had a limited effect on HDM-induced AHR and inflammation. Other groups showed reduced inflammation in CCR6 knockout mice stimulated with either cockroach frass\textsuperscript{111} or particulate matter.\textsuperscript{51} In our hands, HDM induced the release of a broader variety of chemokines and cytokines than any other allergen, including cockroach frass. Thus, for some atopic individuals targeting a single chemokine/receptor pair, like CCL20/CCR6, may be sufficient to reduce asthma symptoms. But for atopic asthmatics sensitive to HDM, the effect of singly targeting CCL20/CCR6 may be blunted by the release of a number of other inflammatory mediators. For these individuals, targeting a release mechanism for chemokines and cytokines should be considered, as it would inhibit the release of a broader range of inflammatory mediators. Since a majority of atopic asthmatics respond to HDM, we would not expect studies that target individual chemokines and cytokines released from the bronchial epithelium to have sufficient
power to detect the potentially potent effect these therapies might have on minority populations of asthmatics who are not atopic to HDM.

Currently, many trials involving anti-cytokine therapies are focused on stratification of patients based on asthma severity.\textsuperscript{112,113} Our data suggest a different method for stratification: allergen sensitivity. Although we would not expect to observe a difference in treatment efficacy for downstream cytokines such as IL-4 and IL-5, we would expect that stratification of patients based on allergen sensitivity for anti-chemokine/cytokine therapy targets such as IL-33, TSLP, and CCL20 would reveal groups of patients susceptible to treatment. These groups would potentially include individuals who are atopic to cockroach (CCL20), and ragweed (TSLP). An additional benefit to this approach is that separation based on atopy can be determined fairly easily and inexpensively.

Conclusions

The work we have presented in this dissertation contributes to our understanding of chemokine and cytokine storage and release mechanisms in bronchial epithelial cells. Release of a number of important chemokines and cytokines – including CCL20, Gro-\(\alpha\), IL-8, IL-33, and TNF-\(\alpha\) – previously thought to be dependent on \textit{de novo} synthesis, have been shown to exist within pre-formed stores in bronchial epithelial cells in both intracellular and extracellular pools. These stores may then be selected for
release, depending on a number of stimuli.

Intracellular stores of some chemokines, such as CCL20, are found in secretory lysosomes; and multiple mediators may localize to the same secretory granules. A number of diverse signaling pathways lead to secretory granule loading and release, including Raf1 and JNK.

Of the vesicles that are stored within the bronchial epithelium, the specific vesicles that are released depend on the particular allergen the cell is exposed to. A diverse array of chemokine- and cytokine-specific responses are mounted to each allergen the bronchial epithelium is exposed to. Of all the allergens we tested, HDM was the most potent at inducing release of preformed stores of chemokines and cytokines. Although intracellular storage and release is not the sole means by which these mediators are delivered to the extracellular environment, our findings illustrate that they play an important role in inflammation. These studies expand our understanding of how chemokines and cytokines are released from the bronchial epithelium and provide a number of potential targets for treatment of allergic airway diseases.
References


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