THE ROLE OF HYALURONAN SIZE IN MURINE ASTHMA

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June 2010

Submitted in partial fulfillment of requirements for the degree
DOCTOR OF PHILOSOPHY IN CLINICAL/BIOANALYTICAL CHEMISTRY
at the
CLEVELAND STATE UNIVERSITY
May 2016
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DEDICATION

This work is dedicated to my incredible family.

To my utterly remarkable parents, David and Melanie – you gave me the freedom to learn and the tools to pursue my dreams, and for that, I will be forever grateful.

Proverbs 1:8-9

To my siblings, Kristin, Joshua, Lydia, and James – I am unbelievably blessed to be your big sister. Thank you for visiting me whenever I was homesick, and for encouraging me whenever I began to doubt myself. You four are my role models and my best friends. I look forward to share in all of your future accomplishments.
ACKNOWLEDGEMENTS

There are many individuals who are responsible for my achievements as a graduate student while at the Cleveland Clinic Lerner Research Institute and at Cleveland State University, and I will do my best to give everyone the recognition and acknowledgment that they deserve.

First and foremost, I want to express my heartfelt appreciation to my research advisor, Dr. Mark Aronica, who consistently supported me throughout my graduate career. I cannot thank you enough for graciously allowing me to join your lab in 2012, and for introducing me to asthma and matrix biology research. Your encouragement, kindness, and patience kept me going whenever I struggled, and for that I am thankful.

To my committee members, Dr. Aimin Zhou, Dr. Yan Xu, Dr. Vincent Hascall, and Dr. David Anderson, I am truly indebted to each of you for being a part of my doctoral committee, for your astute feedback regarding my thesis project, and for your dedicated encouragement. To my graduate program advisor, Dr. Michael Kalafatis, I am indebted to you for your help in addressing my numerous concerns. I want also to thank the Department of Chemistry at CSU, including Dr. David Ball, Ms. Richelle Emery and Mrs. Michelle Jones for their tireless efforts on behalf of students and faculty alike.

To Dr. Georgiana Cheng, my fellow lab member, you have my utmost respect and gratitude for your assistance, direction, and patience throughout my time at the Cleveland Clinic Lerner Research Institute. I want to thank for always being available to answer my questions and for consistently encouraging me to reach my goals. And to Lisa Ruple and Dr. Alana Majors, also a members of the Aronica Lab, many thanks to you for your
efforts on my behalf. Many others at Lerner have also been directly responsible for mentoring me, and teaching me a wide array of new techniques. Those I want to particularly thank are: Dr. Amina Abbadi, Dr. Kewal Asosingh, Dr. Carol de la Motte, Dr. Sean Kessler, Yeojung Kim, and the late, and greatly missed, Dr. Mark Lauer. To Amina, a special thanks for her unceasing support and expertise.

And finally, to Dr. Chanda Mullen, who I want to thank from the bottom of my heart for reading all my papers, listening to all my presentations, and for being a truly amazing friend. Remember all those times we were mistaken for sisters? I think they had it right all along. We are soul sisters, and I have no doubt that we will be so for life.
THE ROLE OF HYALURONAN SIZE IN MURINE ASTHMA

JENNIFER LEIGH RODGERS

ABSTRACT

Hyaluronan (HA), a component of extracellular matrix (ECM), helps maintain tissue integrity and regulates water homeostasis. HA is a high molecular weight (HMW) polymer of repeated D-glucuronic acid and N-acetyl-D-glucosamine disaccharides exceeding $10^6$ Daltons in mass. ECM turnover results in accumulation of HA fragments of lower molecular weight (LMW). Asthma is a chronic lung disease, characterized by inflammation, airway hyperresponsiveness (AHR), airway remodeling, and ECM turnover. HA is elevated in asthmatics as has been found correlate with disease severity, but its role in asthma is not well understood. In this study, we utilized two models of murine asthma to provide evidence that treatment with HA impacted AHR \textit{in vivo}, mediated by interleukin-13 (IL-13), a Th2 cytokine. IL-13 is strongly linked to development of the asthmatic phenotype, including AHR. In our study, we treated mice intratracheally with either LMW or HMW HA in either an ovalbumin (OVA) or house dust mite (HDM) model of antigen-induced murine asthma. While both HA sizes impacted AHR in our OVA model, only HMW HA significantly impacted AHR in our HDM model. The observed increase in AHR in both models correlated to increased mRNA expression of IL-13 within the lung. In an \textit{in vitro} model, airway epithelial cells (AECs) treated with either LMW or HMW HA demonstrated significantly elevated levels of certain Th2 cytokines, and SAA3. We propose that our observed results \textit{in vivo} are linked to HA signaling in AECs.


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<tr>
<td>AEC</td>
<td>airway epithelial cells</td>
</tr>
<tr>
<td>ALI</td>
<td>air-liquid interface</td>
</tr>
<tr>
<td>Alum</td>
<td>aluminum hydroxide</td>
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<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
</tr>
<tr>
<td>ASMCs</td>
<td>airway smooth muscle cells</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD44</td>
<td>cluster of differentiation 44</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
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<tr>
<td>DAMP</td>
<td>danger associated molecular pattern</td>
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<tr>
<td>DM</td>
<td>dust mite</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DS</td>
<td>dermatan sulfate</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>acetylglucosamine</td>
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</table>
GlcUA  glucuronic acid
GM-CSF  granulocyte macrophage colony-stimulating factor
HA  hyaluronan
HA-HC  hyaluronan cross-linked with heavy chain
HAS  hyaluronan synthase
HC  heavy chain
HDM  house dust mite
HMW HA  high molecular weight hyaluronan
HS  heparan sulfate
HYAL  hyaluronidase
HYBID  hyaluronan binding protein involved hyaluronan depolymerization
HβD2  human beta-defensin 2
IαI  inter-alpha-trypsin inhibitor
IBD  inflammatory bowel disease
IL  interleukin
IN  intranasal
IP  intraperitoneal
IP-10  IFN-gamma-inducible protein 10 (CXCL10)
IT  intratracheal
IV  intravenous
KIAA1199  hyaluronan binding protein involved in hyaluronan depolymerization
KC  CXCL1
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>KS</td>
<td>keratan sulfate</td>
</tr>
<tr>
<td>LMW HA</td>
<td>low molecular weight hyaluronan</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LYVE-1</td>
<td>lymphatic vessel endothelial hyaluronan receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-associated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<tr>
<td>MIP-2</td>
<td>macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
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<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>Poly:I:C</td>
<td>polyinosinic acid:polycytidylic acid</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation, normal T expressed ad secreted (CCL5)</td>
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<tr>
<td>RHAMM</td>
<td>receptor for hyaluronan-mediated motility</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
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<tr>
<td>TGF-β1</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TJ</td>
<td>tight junctions</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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<td>TSG6</td>
<td>tumor necrosis factor-stimulated gene-6</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
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<td>UDP</td>
<td>uridine diphosphate</td>
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CHAPTER I

BACKGROUND

1.1 EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is comprised of an intermingling network of water, proteins and polysaccharides, which perform essential structural roles in maintaining tissue integrity (1). The ECM includes the connective tissue ECMs, which underlie the epithelium, the basement membrane that separates them, and epithelial surfaces. Vertebral ECM may be organized to form different types of matrix, based upon its biochemical composition. Aqueous connective tissues are found in the eye, while more fibrous connective tissues are found within muscles (1). The ECM was traditionally considered to have primarily a mechanical role in tissues. However, recent studies of the ECM have now begun to focus on its roles in cell migration, proliferation, survival, and signaling (1, 2, 3).

The protein components of the ECM are predominantly fibrous, including collagen, elastin, fibronectin, and laminins (2). Collagen is one of the most abundant proteins in multicellular animals, constituting as much as 30% of total protein mass, and has a main role in providing structural support to the matrix (1, 3). Elastin is a highly
elastic fibrous protein that associates with collagen fibrils, thereby allowing tissues to maintain their shape after stretching (1, 4). Fibronectin is a glycoprotein that has roles in cell adhesion and migration, giving it a crucial role in organizing the interstitial ECM (1). Laminins provide structural support for the basement membrane, and also play an important role in cell adhesion, migration, differentiation, and proliferation (5).

The second major macromolecular components of the ECM include proteoglycans and glycosaminoglycans (GAGs) (1, 6). Proteoglycans are glycosylated proteins that contain one or two of the members of the sulfated GAG family, chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), and keratan sulfate (KS). The other GAG hyaluronan, (HA) does not contain a core protein (6). Proteoglycans, including aggrecan and versican, are almost entirely comprised of carbohydrates (GAGs) by weight, with as much as 90% abundance, distinguishing them from glycoproteins (6). Aggrecan is the most abundant proteoglycan found in cartilage, consisting of a core protein with large numbers of attached CS and KS chains to form bottlebrush structures that provide necessary load-bearing properties in articular cartilage (6, 7). Aggrecan also binds to HA, which allows for mediation of interactions between chondrocytes and matrix (7). Versican is abundant proteoglycan that contains either CS or DS, and it is also able to bind to HA (8). Versican has an important role in cell adhesion, migration, and proliferation (8).

1.2 HYALURONAN

The discovery of hyaluronan (HA) dates back to the 1930s when Karl Meyer purified HA directly from the vitreous humor from bovine eyes (9). HA was so named by
Meyer because of its “hyaloid”, or transparent, appearance in water, and also because of its likely hexuronic acid composition (9). HA was later characterized as a polymer of repeating \( D \)-glucuronic acid (GlcA) and \( N \)-acetyl-\( D \)-glucosamine (GlcNAc) disaccharide units linked by a glucosidic bond (10, 11, 12). It was also noted that HA acts as an anion in physiologic conditions; preferring to associate with cations, such as sodium (10). The salt form of HA is named 'sodium hyaluronate’, while ‘hyaluronan’ is used to reference all forms of the molecule (13).

**Figure 1. Fundamental disaccharide unit of hyaluronan.** Polymer includes repeated \( D \)-glucuronic acid (GlcA) and \( N \)-acetyl-\( D \)-glucosamine (GlcNAc) disaccharide units linked by glucosidic bonds (10, 11, 12).

HA is found ubiquitously throughout most vertebrate tissues, and is detectable in massive quantities in synovial fluid (1400-3600 µg/g) and vitreous humor (140-338 µg/g) (14). Blood serum HA concentration typically ranges from 0.01 to 0.1 µg/g (14). HA is
typically found in ‘normal’ tissues in high molecular weight (HMW) form up to 10 MDa in size (15).

HA is a member of the glycosaminoglycan (GAG) family, all of which consist of unbranched, polysaccharides of repeated disaccharide units, including an amino sugar, either N-acetylglucosamine or N-acetylgalactosamine, and a hexuronic acid, either glucuronic or iduronic acid (CS, DS, HS), or galactose (KS) (1). Structural characterization by X-ray diffraction and NMR demonstrates that HA forms stiff, helical formations in the solid state (17). However, utilizing tapping mode atomic force microscopy (AFM), HA was found to form a more extended, relaxed condensed coil in physiologic conditions (17).

Unlike other members of the GAG family, HA is normally not covalently associated with a core protein, and it does not undergo modification by sulfation, nitration, or phosphorylation (16). The negative charge imparted by the sugar’s carboxyl group allows HA to attract cations, such as Na+, and also water, giving it a central role in the maintenance of tissue homeostasis (16). The hydrodynamic properties of HA are governed by its concentration and molecular weight. HA also readily interacts with ECM components, including proteoglycans and link proteins, allowing it to act as a supportive framework within the extracellular and pericellular spaces (17). In addition to its purely mechanical role in tissue, HA has also been suggested to impact disease as a signal molecule. To date, HA has been linked to a wide array of chronic inflammatory disorders and cancers, including those of the lung, heart, joints kidneys, and gut (18, 19, 20, 21, 22).
1.2.1 HYALURONAN SYNTHESIS AND CATABOLISM

HA is synthesized by three mammalian hyaluronan synthases (HAS1-3) located on the inner surface of the plasma membrane, primarily by fibroblasts and other mesenchymal cells (16, 23). During synthesis, HA is extruded out into the extracellular space, so that the immense molecule does not overwhelm the intracellular space (23). Uridine diphosphate glucuronic acid (UDP-GlcUA) and uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) are alternately added to the growing chain by two glycosyltransferase activities located in HAS (23). HAS1, 2, and 3 are expressed in a variety of tissues and are localized on chromosome 19 (19q13.3-q13.4), chromosome 8 (8q24.12), and chromosome 16 (16q22.1), respectively (24). HAS1 or 3 knockout mice are viable, whereas the HAS2 knockout causes lethal cardiac defects in embryonic mice (25).

Enzymatic degradation of hyaluronan is carried out by the action of hyaluronidase, β-D-glucuronidase, and β-N-acetyl-hexosaminidase (27). β-D-glucuronidase and β-N-acetyl-hexosaminidase degrade HA oligosaccharides, and other GAGs, by hydrolyzing the terminal sugar at the non-reducing end (27). Given the high amounts of HA in tissues, HA turnover is a relatively well-controlled, quick process. Two hyaluronidases (Hyals), Hyal1 and 2 are primarily responsible for hydrolysis of HA into smaller fragments for HA turnover (28, 29). Other hyaluronidase proteins expressed in humans include Hyal3 and 4, and PH20 (28). Genes which expression Hyal 1, 2, and 3 are located on chromosome 3q21.3 (28).

Hyals are predominantly active at low pH, with the exception of hyaluronidase PH20, which is expressed in the testes, and plays a role in fertilization, and it is active at
neutral pH (25). Hyal2, located on the cell surface, catalyzes initial cleavage of HA in collaboration with CD44 (26). Hyal1, located within the lysosome, will then catalyze cleavage of HA into oligosaccharides that are then further cleaved into monomers by activity of β-D-glucuronidase and β-N-acetyl-hexosaminidase (26). Hyals have been shown to improve uptake of certain drugs at subcutaneous injection sites, by increased fluidity allowing for improved diffusion of injected treatment (29).

Another important protein involved in HA degradation is HYBID (hyaluronan binding protein involved in hyaluronan depolymerization), also called KIAA1199 (30). KIAA199 has been shown to have an important role in fragmentation of HA by synovial fibroblasts, which is typically associated with osteoarthritis (OA) and rheumatoid arthritis (RA) (31). KIAA1199 is also associated with HA degradation in human dermal fibroblasts (31). A murine homologue of the KIAA1199 gene has also been linked to HA degradation (32).

1.2.2 HYALURONAN BINDING PROTEINS

Proteins that bind HA, also called hyaladherins, contain a common domain termed a LINK module (33). The LINK module is designated as a conserved region of ~100 amino acid residues located in a variety of proteoglycans, including aggrecan and versican, as well as in cluster of differentiation 44 (CD44), lymphatic endothelium-specific hyaluronan receptor 1 (LYVE-1), and tumor necrosis factor-stimulated gene 6 (TSG-6) (33). CD44 is a widely expressed receptor of hyaluronan, and can interact with other ligands, including osteopontin, collagens, matrix metalloproteinases (MMPs), and is involved in a range of biological processes, including inflammation and cancer (34, 35,
LYVE-1 is expressed by lymph vessel endothelial cells, and is responsible for sequestering HA in lymphatic vessels leading to HA catabolism (33). TSG-6 is upregulated in response to inflammation and is involved in heavy chain (HC) transfer from inter-alpha-inhibitor (IαI) to HA, allowing for HA-HC cross-linking (38). IαI, is a serum proteoglycan consisting of two heavy chains (HC) and one light chain, bikunin, covalently linked to a CS chain (38). In the presence of TSG-6, HCs are transferred to HA, forming a meshwork of HA-HC cross-linkages (38).

Additional hyaladherins, which do not contain a LINK module, include receptor for hyaluronan-mediated motility (RHAMM). RHAMM exists in two isoforms in humans on the cell surface, and in the cytoplasm (39). Activation of RHAMM by HA has been linked to proliferation and migration of malignant and non-malignant cells (39). RHAMM has also been shown to initiate inflammation, independent of CD44 (39).

HA has also been demonstrated to bind with receptors that recognize pathogen-associated molecular patterns (PAMPs), which have been shown to result in activation of innate immunity via toll-like receptors (TLRs) in connection with a variety of disease processes (40, 41, 42, 43, 44). Endogenous initiators of inflammation that trigger these receptors, such as HA, are termed damage-associated molecular patterns (DAMPs) (45).

1.2.3 HYALURONAN IN DISEASE

Abnormal ECM turnover results from tissue injury and is associated with accumulation of HA fragments. While HA was originally considered to serve a primarily biomechanical role in tissues, acting as inert scaffolding, it has since been linked to a variety of human diseases (18, 19, 20, 21, 22). Increased levels of HA have been demonstrated in patients with diseases that involve tissue injury and inflammation,
including in diseases of the liver, lung, vasculature, kidney, and bowel (22). HA accumulation is also present in certain types of cancers, including in breast and pancreatic cancers (46, 47, 48). HA accumulation has also been shown in experimental animal models of disease, including in mouse models of lung injury and colitis (22, 43, 49, 50, 51). The appearance of markedly increased amounts of HA in human and animal disease has led to further studies of HA’s likely role in the progression of certain disorders, as well as its potential impact as a signal molecule in disease processes.

1.2.3.1 LOW MOLECULAR WEIGHT VS HIGH MOLECULAR WEIGHT HA

The biologic activity of HA in tissues is closely related to HA size, specifically regarding LMW HA (10-500 kDa) vs. HMW HA (>1000 kDa) (52). LMW HA has been shown to be primarily pro-inflammatory, whereas HMW HA is predominantly anti-inflammatory. Accumulation of LMW HA produced by increased synthesis and degradation processes, including fragmentation by reactive oxygen species (ROS), correlates to progression of a wide array of disease processes, including inflammation and cancer (22). LMW HA has been shown to induce CD44-dependent signal pathways that promote cell proliferation and migration, as well as angiogenesis, inflammation and chemosensitivity (53, 54). Additionally, tumor cell adhesion and migration in fibrosarcoma have been demonstrated to be principally facilitated by interaction between LMW HA and RHAMM (61).

While HMW HA is highly abundant in malignant cells, amplified synthesis of HMW HA in the naked mole rat is associated with this species increased longevity and cancer resistance (56, 62). HMW HA is also critical for normal embryo development, and has also been determined to be crucial for wound healing (57, 58). HMW HA
demonstrates an anti-inflammatory impact in a model of T-cell-mediated liver injury by inhibition of pro-inflammatory cytokines (59). A similar anti-inflammatory impact was observed in a sepsis-induced model of lung injury, where HMW HA was shown to significantly improve overall inflammation (60).

The impact of HA size in the pathogenesis of disease has been linked to a number of facilitators, including HA synthases, hyaluronidases, and receptors. Several of these processes and associated molecules are discussed below, as they relate to either LMW or HMW HA.

**1.2.3.2 HYALURONAN SYNTHASE (HAS)**

Up-regulation of hyaluronan synthases (HAS1, 2, and 3) have been demonstrated to contribute to pathological accumulation of HA. Each HAS is responsible for synthesis of unique size ranges of HA; HAS1 and HAS2 primarily produce long chain HA of high molecular weight (HMW), while HAS3 produces shorter chains (62). High levels of HMW HA found in breast tumors were found to correlate with increased expression of HAS2 (63, 64). HAS3 was demonstrated to be associated with proliferation and progression of pancreatic cancer (48). Furthermore, protein activities of all three HASs (HAS1-3) were increased in endometrioid and endometrial carcinomas (65).

In human asthma, HAS2 mRNA was found to be upregulated in lung fibroblasts (66). In synovia taken from osteoarthritis and rheumatoid arthritis (OA and RA) patients, HAS1 and 2 mRNA expressions were significantly decreased (31, 32). In a murine model of colitis, HAS3 knockout mice were shown to have decreased inflammation (67). Conversely, in a murine model of knee cartilage injury, HAS1 knockout mice were shown to have worsened inflammation as well as intra-articular fibrosis (68). These
studies demonstrate that the role of HAS may be disease specific, and thus, the role of HA may also be disease specific.

### 1.2.3.3 HYALURONIDASE (HYAL)

HA degradation by Hyals has also been confirmed to contribute to pathological formation of HA in tissues. Hyal1 and 2 activities are detectable in nearly all somatic cells, and are primarily responsible for degradation of HA (22, 26). In addition to their role in HA clearance, Hyals have also been shown to produce HA fragments that are capable of inducing a biologic response. Platelet-derived Hyal2 has been shown to produce HA fragments that are capable of initiating pro-inflammatory cytokines (26). Increased Hyal1 expression has been linked to prostate and bladder cancers, and has been further indicated as a prognostic tool in these types of tumors (69, 70). Additionally, certain aggressive breast cancer cells lines have been shown to express high levels of Hyal2 (71).

### 1.2.3.4 TOLL-LIKE RECEPTORS (TLRS)

The structure of HA mimics features of pathogen-associated molecular patterns (PAMPs) and has been shown to interact with toll-like receptors (TLRs), indicating a role for HA in innate immunity (41, 43). TLRs are a family of pattern recognition receptors (PRR) that are located in sentinel cells, such as macrophages and dendritic cells, and are also found in epithelial cells (41, 43). HA has been linked with TLR-signaling in these cell types (41,43).

LMW HA is capable of initiating activation of dendritic cells (DCs) and macrophages by interaction with TLR4 (72). TLR2 and TLR4 have been further
characterized as potential receptors for HA in inflammation in certain animal models of disease. In an ozone model of induced lung injury, TLR4, MyD88 and TIRAP were found to be required for development of airway hyperresponsiveness (AHR) in ozone challenged mice (42). It was further demonstrated that TLR4-/-, MyD88-/- and TIRAP-/- knockout mice exhibited abated airway constriction as a result of ozone exposure, compared to wild-type mice (42). Furthermore, direct challenge with LMW HA fragments was found to increase airway constriction in wild-type mice in the absence of ozone exposure. This effect was not observed in TLR4-/-, MyD88-/- and TIRAP-/- knockout mice in response to HA challenge.

Certain inflammatory markers were elevated as a result of HA challenge, including IL-1β, IL-2, MCP-1 and TNFα. However, production of inflammatory factors by HA challenge was arrested in TLR4-/-, MyD88-/- and TIRAP-/- knockout mice. These results indicate that HA fragments may act as an endogenous ligand in the activation TLR4-MyD88-TIRAP signaling pathways to induce AHR and cytokine/chemokine production in ozone-induced lung injury. TLR2, in addition to TLR4, may also play an integral role in HA signaling (41, 43).

1.3 ASTHMA

Asthma is a chronic lung disease often associated with shortness of breath, difficulty breathing, and characteristic “wheezing.” The World Health Organization (WHO) estimates that nearly 150 million people currently suffer from the disease (73). The yearly mortality rate for the disease is estimated to be around 180,000 (73).
Treatment typically includes inhaled steroids in order to control underlying disease symptoms, but there is currently no cure.

Asthma is driven by chronic inflammation and is characterized by airway remodeling, airway hyperresponsiveness (AHR), mucus plugging, epithelial shedding, and abnormal ECM deposition (74). Airway remodeling refers to thickening of the airways, resulting in narrowing of the airway lumen (77). Airway hyperresponsiveness (AHR) characterizes sensitivity of asthmatic patients to bronchospasm (77). Lung injury associated with asthma is also characterized by abnormal extracellular matrix (ECM) turnover (42).

Clinical diagnosis of asthma may include measurements of forced expiratory volume in one second (FEV1), also called spirometry; or by measurement of total respiratory resistance, Rrs, as measured by the forced oscillation technique (FOT) (78, 79). Both FEV1 and FOT testing are useful to assess airway obstruction in asthmatics. Asthmatic patients are generally found to have diminished FEV1 and increased Rrs, or lung constriction. FEV1, however, is not easily performed on young children because it requires a high level of interaction, including comprehension of the patient to verbal commands given by the test performer. FOT, by contrast, requires little to no cooperation of the patient, as is therefore often more useful in diagnosis of younger patients (78).

Further diagnostic assessment may be acquired by utilizing airway-constricting agents during testing including by methacholine (Mch) (82). Inhaled Mch or histamine leads to bronchoconstriction, which is typically augmented by pre-existing chronic lung conditions, such as asthma. Nitric oxide (NO) tests may also be used, which measure
amounts of NO in breath, since asthmatics tend to have higher NO concentrations in their
breath due to chronic lung inflammation (89). Diagnostic assessment of asthma in mice is
discussed in Chapter 2.

1.3.1 CHRONIC INFLAMMATION

Chronic inflammation associated with asthma is first initiated by a stimulus, such
as dust mites, pollen, or mold spores, all of which are common allergens. Other
environmental stimuli that induce inflammation include air pollutants and other irritants,
both indoor and outdoor, such as ozone, air fresheners, cosmetics, and many others.
Irritants or allergens, which are introduced into the airways by inhalation, are recognized
by antigen-presenting cells (APCs), such as dendritic cells (DCs). DCs, in conjunction
with the airway epithelium, are believed to be primarily responsible for initiation of
inflammation in asthma by presenting digested allergen peptides to Th0 lymphocytes in
the lymph nodes, which results in induction of Th2 type T-lymphocytes in
the lymph nodes, which results in induction of Th2 type T-lymphocytes, a common
phenotype in allergic asthma (75, 77, 76). Th2 cells induce specific chemokines and
cytokines, which induce production of other inflammatory cell types, such as
macrophages, eosinophils, neutrophils, mast cells, and B-lymphocytes (80). Activation of
B-lymphocytes by Th2 cells is mediated by IL-4 and IL-13, and results in production of
IgE. CD4+ T-cells are the most abundant Th2 lymphocyte population in asthmatics; CD8+
T-cells are another abundant cell type (81). Antigen-specific IgE will then bind to mast
cells, causing them to become primed to release certain inflammatory factors, including
histamines and leukotrienes, upon subsequent exposure to the antigen (82). The release of
histamine and leukotrienes will result in bronchospasm and mucus hyper-secretion (82).
Asthmatic inflammation has also been shown to be associated with activation of Th17 cells. The presence of Th17 cells, and their associated cytokines, has been shown to be elevated in asthmatic patients (81).

Lavage from asthmatics patients reveals that many inflammatory cells types persist in the airways, including in the airways of patients with milder forms of the disease. The continuous presence of these, and their associated cytokines, is suggested to play a role in the chronic changes associated with asthma, including smooth muscle hypertrophy, increased collagen deposition, and increased numbers of goblet cells in the airway epithelium (82).

1.3.2 AIRWAY EPITHELIUM

The airway epithelium functions as the first line of defense for the airway, and thus plays an important role in asthmatic inflammation. The airway epithelium acts as a physical barrier that serves as a boundary between the internal mucosa and the external environment. Ciliated airway epithelial cells (AECs) represent the majority of cells within the airway epithelium (84). Particles such as allergens and other pathogens are moved out of the airways by mucociliary clearance, a process in which debris is caught by secreted mucus (secretory AECs) and moved out of the airways by ciliated AECs (85). Other AEC types that comprise the airways include: columnar, undifferentiated, and basal (85). Cultured murine AECs in air-liquid interface (ALI) have been shown to synthesize HA on their ciliated surface, in the absence of inflammatory stimuli (116). Synthesized HA is modified by HC, donated from IαI, which then remains on the surface of these cells to form rafts that are adhesive to leukocytes (116). Another crucial role of
AECs is in the maintenance of tight junctions (TJ), the purpose of which is to regulate flux of water, ions, macromolecules and inflammatory cells (86). Once disrupted, increased permeability of the airway epithelium is associated with many disorders of the lung, including asthma, chronic bronchitis, and cystic fibrosis (86).

Airway epithelium plays a role in the remodeling of asthmatic airways, characterized by hyperplasia and metaplasia of epithelial goblet cells, smooth muscle cell hyperplasia, angiogenesis, and increased collagen deposition by initiation of innate immunity (87). Damage to the epithelium results in the production of growth factors in order to facilitate cell migration. Growth factors produced primarily by the airway epithelium include: PDGF, TGF-β, FGF, EGF, and IGF (82). Fibroblasts, which underlie the compromised barrier, will produce matrix in order to temporarily bridge the gap of damaged cells (87). Collagen and other ECM components are also increased, as well as differentiation of fibroblasts to myofibroblasts (87). Once the barrier has been rehabilitated, new AECs will undergo differentiation, which will also lead to goblet cell differentiation in order to return secretory processes to normal, including mucociliary clearance (87). During the final resolution of damage, myofibroblasts will undergo apoptosis, and matrix will be degraded (87). Cultures from asthmatic AECs shown an overall deregulation in the repair process described above, demonstrating the break down in repair processes as a result of chronic activation (87).

AECs have also been shown to play an early role in asthma by production of certain inflammatory mediators, such as IL-25, TSLP, IL-13, and nitric oxide (NO) (88, 89, 90). IL-25 has been shown to promote Th-2 immunity in asthma (91). Thymic
stromal lymphopoietin (TSLP) has similarly been shown to play an important role in the initiation asthmatic inflammation. IL-13 has been linked to mucus hypersecretion, eosinophilia, and development of AHR, and has been shown to have a direct impact on AECs to induce AHR and mucus hypersecretion (92). NO has been shown to be elevated in human asthmatics, and is attributed to induction of nitric oxide synthases, including iNOS in AECs (93). NO has been shown to play a role in relaxing airway smooth muscle cells (ASMCs) (94), and may also impact lung remodeling (95, 96). NO also acts as a neurotransmitter for noradrenergic noncholinergic nerves, and plays an important role in response to vascular injury (97).

1.3.2.1 INTERLEUKIN-13

Interleukin-13 (IL-13) has been linked to common phenotypic features of human asthma, including airway hyperresponsiveness (AHR), inflammation, and airway remodeling (98). In murine asthma, IL-13 has been further linked to mucin secretion, fibrosis, as well as being strongly linked to AHR (98). Importantly, signaling by IL-13 has also been demonstrated as having a role in the development of corticosteroid-resistant asthmatic inflammation (99).

IL-13 shares structural similarities to IL-4 of roughly 25% homology (100). Primarily Th2-polarized CD4+ cells secrete IL-13, but other cells types, including Th1-type cells and non T-cell type inflammatory cells, have been indicated as an alternative source (101). Eosinophils, a non T-cell inflammatory cell, have been demonstrated to be a significant source of IL-13 (102, 103). Other sources of IL-13 include, human airway smooth muscle cells (hASMCs) (104). Regulation of IL-13 production involves a wide
array of mediators and cytokines (100). These factors include IL-9, IL-25, histamine, adenosine, and endothelin-1. The action of IL-13 is mediated by three receptors, IL-4a, IL-13Ra1, and IL-13Ra2 (100). IL-13 recruits an IL-4a chain into its primary receptor complex (IL-13Ra1) to activate STAT6 and induce an asthma-related allergic phenotype, including AHR and inflammation (100).

1.3.2.2 SERUM AMYLOID A

Serum amyloid A (SAA) is an acute-phase protein generated within the liver, which, after circulation, associates with high-density lipoprotein (HDL) particles (105). SAA levels are known to increase dramatically, up to 1000-fold, as a result of acute inflammation (105). Increased SAA mRNA gene expression has been linked to several inflammatory disorders, including those associated with the kidney and lung (106). SAA has been linked with chronic inflammatory diseases, including rheumatoid arthritis and atherosclerosis, and is considered a potential target for the development of new therapies in the treatment of these disorders (105).

SAA is also elevated in asthmatics, and has been suggested as a biomarker for the disease (107). Human pulmonary production of SAA in response to infection is regulated by epithelial expression of SAA1 and 2 (107). SAA has been shown to worsen airway hyperresponsiveness in ovalbumin (OVA) and house dust mite (HDM) sensitized and challenged mice in response to methacholine (Mch) challenge (107). Pulmonary production of SAA in mice is regulated by epithelial expression of SAA3 (107). SAA1 and 2 in mice are expressed in the liver only; there is no true equivalent of SAA3 in humans (107).
SAA and hyaluronan have been suggested to play an important role in facilitating monocyte adhesion (108), and thus inflammation. A complex formed by adipocyte-derived SAA3 and hyaluronan was found to induce monocyte chemotaxis, an effect that was hindered by siRNA silencing of SAA3 (108). SAA is also demonstrated to induce leukocyte chemotaxis through Formyl Protein Receptor 2 (FPR2) (109).

SAA engages inflammasome in human neutrophils, resulting in the production of active IL-1β, a pro-inflammatory cytokine (113). The conversion of pro-IL-1β to its active form occurs when NOD-like receptor Pyrin domain containing 3 (NLRP3) is induced to oligomerize with adaptor protein (ASC), thereby forming the inflammasome complex and allowing activation of caspase-1 (113). NLRP3 recognizes pathogen and damaged associated molecular patterns, PAMPs and DAMPS, respectively (113). SAA itself has been shown to activate pattern recognition receptors, including TLR2, to induce cytokine production (102).

### 1.3.3 AIRWAY SMOOTH MUSCLE

Airway smooth muscle (ASM) is responsible for contractibility of the airway lumen in response to non-specific stimuli in order to increase resistance of airflow (107). Symptoms of asthma arise primarily from restricted airflow (108). ASM contractibility is coordinated by a series of extracellular agonists that act on airway smooth muscle cell (ASMC) receptors. Once these receptors are activated within the plasma membrane of ASMCs, a cascade of intracellular events result in ASMC contraction, and thus airway constriction. Contraction is ultimately initiated by an increase in the amount of cytosolic 
Ca2+ released primarily from intracellular sarcoplasmic reticulum (SR) (108). Mediators
that result in contraction in ASMCs primarily originate from neurotransmitters, airway epithelium, and inflammatory cells (107).

Another important facet of airway narrowing arises from abnormalities of ASM structure and morphology (108). An increased in overall ASM mass has been observed from patients with fatal asthma, compared to patients who died from other non lung-related causes (108, 109). Increased ASM mass is a crucial factor in the development of airway remodeling in asthmatics (108). ASMC proliferation is initiated by certain growth factors, including epidermal growth factor (EGF) and platelet derived growth factor (PDGF), by activation of receptor tyrosine kinase, and other kinase receptors (110). Certain inflammatory factors have been shown to promote AMSC proliferation in vitro, including IL-1β and TNFα (110). IL-1β and TNFα increase the expression of histamine, bradykinin, leukotrienes, and other mediators that stimulate airway constriction (110).

Other abnormalities include hypercontractility of ASM in asthmatic patients (108). ASM contractibility, as assessed by gel contraction assay, demonstrated that asthmatic ASM contracts to a significantly higher degree than non-asthmatic ASM in response to an agonist, such as histamine or bradykinin (108). One observed abnormality in ASM contraction, which plays a likely role in ASM hypercontractility, is increased expression of myosin light-chain kinase (MLCK). MLCK mRNA is increased in asthmatics (108). Additionally, expression of MLCK is also correlated with disease severity, with higher levels observed in patients with a more severe disease form. Mast cell (MC) infiltration is also known to result in increased expression of α-actin and ASM contraction (108). MCs release histamine and IL-13, both of which result in contraction
in ASMCs (108). Another potential mechanism for ASM hypercontractility is in Ca^{2+} dysregulation, or homeostasis (108). Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) expression was decreased overall in a population of asthmatics, compared to non-asthmatic controls (108, 111). When SERCA2 was subsequently inhibited in vitro in non-asthmatic ASMCs, cells assumed an asthmatic phenotype with increased cytosolic Ca^{2+}.

1.3.4 HYALURONAN IN ASTHMA

HA was first discovered in the pulmonary secretions of asthmatic patients more than three decades ago in 1978 (112). Furthermore, HA was reported as the only GAG present in lavage samples taken from asthmatic patients, as confirmed by digestion with a HA-specific hyaluronidase (112). More recent studies have demonstrated that deposition of HA and proteoglycans is increased within the submucosa of asthmatic airways, when compared to non-asthmatic airways, strongly contributing to airway remodeling (66). In addition, HA found in the lavage of asthmatic patients is demonstrated to correlate with disease severity, with higher HA levels found in patients with a more severe form of the disease (38, 66, 113, 114).

The mechanism of HA accumulation in asthma is coordinated by depolymerization of existing HA, as well as synthesis of new HA. All three hyaluronidases (HYAL 1-3) have been shown to be upregulated in airway epithelium as a result of treatment with certain asthma-related cytokines, including IL-1β and TNFα, contributing to ECM turnover resulting in increased HA fragments (114). Additionally, fibroblasts synthesize HA in response to certain inflammatory cytokines, including IL-1β,
TNFα, and TGFβ (66). Reactive oxygen species (ROS) generated by the inflammatory response are also known to induce fragmentation of HA on the airway surface (114). Hyaluronan synthase 2 (HAS2), the predominant type expressed in human lung fibroblasts, is considered to be a primary source of pathologic HA seen in asthma (66).

HA is suggested to play an early role in asthmatic inflammation, and has been demonstrated to initiate collagen deposition and fibrosis in a mouse model of allergen-induced asthma (49, 59, 115). HA in BAL of asthmatic mice is significantly increased compared to non-asthmatic mice. Lung sections from asthmatic mice demonstrate increased HA deposition within the lung as a result of treatment with antigen, as early as 12 hours after the first antigen exposure (49). In addition, HA deposition has been found to co-localize with inflammatory cell infiltration within the lung (50, 115).

Airway smooth muscle cells (ASMCs) cultured from tracheal tissue have been shown to produce HA as a result of treatment with compounds that are known to cause endoplasmic reticulum (ER) stress. ASMCs isolated from murine tracheas and treated with either polyinosinic-polycytidylic acid (Poly(I,C)) or tunicamycin, to simulate viral infection, were found to synthesize long HA cables that were also leukocyte-adhesive (38). In pathological conditions, HA is degraded by inflammatory cells, leading to accumulation of HA fragments, that may lead to stimulation of subsequent inflammatory processes. In addition to ASMCs, airway epithelial cells (AECs) are also known to synthesize leukocyte-adhesive HA in response to inflammatory stimuli (116).

HA is covalently modified by heavy chains (HC), from IαI, in response to increased levels of TSG-6, as previously discussed. The presence of modified HA, HA-
HC, is observed in the BAL of human asthmatic patients, and is also present in the basement membrane and submucosa of the lower asthmatic airways (38). The addition of HC within the framework of HA increases leukocyte adhesion, suggesting that modification of HA plays an important role in organizing inflammatory cell migration (38). In addition, asthmatic TSG-6 knockout mice were found to have a less severe form of the disease than did wild-type asthmatic control mice (38).

While an association between HA and asthma has been firmly established, its precise role in the disease is still not well understood. HA may have an early role in providing the preliminary matrix needed for collagen synthesis and fibrosis observed in asthmatic airways (69, 117, 118). Evidence for an early role of HA in inflammation is further demonstrated by its early appearance in the lungs (within 12 hours) of antigen-exposed mice in murine asthma (49). Co-localization of HA with inflammatory cells within the lungs of asthmatic mice supports an additional role for HA in localizing inflammatory cells, thereby maintaining their presence within the lung (49, 50).

The impact of HA size in asthma has yet to be studied, but may prove to be crucial for clarifying the role of HA in asthma. While HMW HA has been shown to favor tissue homeostasis and is anti-inflammatory in certain models of disease, including in a murine model of lung injury (41, 43), its impact in asthma has not been well characterized. Additionally, while LMW HA is considered to be primarily pro-inflammatory, it was found to induce an anti-inflammatory impact in a murine model of colitis (51). It is therefore necessary to further characterize the impact of LMW HA, as well as HMW HA, in asthma.
1.4 OVERVIEW OF PROJECT HYPOTHESES AND AIMS

The goal of this project is to elucidate the role of HA size in asthma. In Hypothesis 1, we proposed that treating asthmatic mice exogenously with either size would amplify the respective impacts of LMW and HMW HA, thus allowing for a better understanding of the role of HA size in asthma. In order to test this hypothesis we proposed two aims: Aim 1: Treatment with exogenous LMW or HMW HA in Ovalbumin (OVA) antigen-induced asthma, and Aim 2: Treatment with exogenous LMW or HMW HA in House dust mite (HDM) antigen-induced asthma. Our in vivo studies in Aims and 2 included studies of inflammation and airway hyperresponsiveness (AHR) in mice, in order to determine the impacts of either exogenous HA size on these two crucial characteristics of asthma. Based on these studies, we determined a role for LMW and HMW HA in the development of AHR, which is related to induction of the Th2 cytokine, IL-13.

Results from Aim 1 and 2 indicate that exogenous HA remained primarily in the airways of treated mice. In Hypothesis 2, we proposed that AECs are the primary cell type encountered by exogenous HA, thus by treating these cells with exogenous HA in vitro we propose a mechanism for the observed impact of exogenous HA in vivo. To test our second hypothesis, we proposed an additional aim for this study: Aim 3: Treatment of exogenous LMW or HMW HA in a culture model of murine airway epithelial cells (AECs). In our in vitro experiments, we utilized an air-liquid interface (ALI) cultured model utilizing AECs isolated from murine tracheas.
1.5 REFERENCES


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CHAPTER II
ANIMAL PROTOCOLS

2.1 ANIMAL MODELS OF ALLERGIC ASTHMA

Many aspects of the cellular and biochemical processes, which underlie the common characteristics of human asthma, are still poorly understood (1). Animal models can be utilized in an attempt to investigate these processes, since it is not typically feasible to perform the necessary experiments on humans (1). A variety of animal asthma models have been implemented, including those that involve the use of mice, rats, dogs, monkeys, and horses (2). Murine asthma models, however, have several advantages over other models, including availability to inbred strains, easy breeding, and relatively short gestation (2). Importantly, in murine models of asthma, the humoral immune response is driven by IgE as the principal allergic antibody (2). Disadvantages of these models mostly surround the obvious anatomical differences between mouse and human respiratory systems, including limited airway musculature, branching, and vasculature in mice (2).

Models that are currently used in order to replicate human asthma include ovalbumin (OVA) and house dust mite (HDM) models (1). These disease models have
been adapted to both acute and chronic disease protocols (1, 3, 4, 5). OVA and HDM murine models successfully replicate features of asthma, including AHR and inflammation, by utilizing antigen sensitization and challenge techniques, since mice do not typically develop asthma without sufficient provocation (1). Additionally, studies involving these models typically use BALB/c strains, since these mice give a robust immunological response (6). However, other strains, including C57BL/6, have been successfully adapted to asthma models as well (5).

2.1.1 OVALBUMIN-INDUCED MODEL OF ASTHMA

Ovalbumin (OVA) models generate a T helper cell 2 (Th2) immune response in mice that produces similar features to the human asthma, including, production of Th2 cytokines (IL-4, IL-5, and IL-13), and subsequent development of airway inflammation and AHR (5). OVA is a protein isolated from chicken egg (1). It is important to note that OVA is rarely associated with development of human asthma. In this model, animal subjects, such as mice, are first sensitized with OVA and adjuvant in order to prime the adaptive immune system resulting in a Th2 phenotype when mice are later challenged with OVA, see Table 1 (1, 7). Aluminum hydroxide (AlOH₃) is typically employed as an adjuvant with OVA (1). OVA/alum sensitization is successful when performed via intraperitoneal injection (IP), and not by means of inhalation (1). The period of time between sensitization and challenge in this model ranges from 14 to 21 days (1).

During antigen challenge, OVA solution is administered directly into the airways, either by aerosol by nebulizer or by intratracheal (IT) or intranasal (IN) instillation (1). The number of days in the challenge period varies by protocol, and is generally determined by preference and experimental goals.
IP priming, or immunization, results in systemic inflammation (7). OVA-Alum recruits both monocytes and DCs into the peritoneum, where they internalize antigen, proliferate, mature and migrate into the lymph nodes where they interact with T and B cells to initiate a Th2 response and adaptive immunity (7). OVA/Alum will also activate resident DCs already present in the peritoneum.

OVA preparations may be contaminated by endotoxin, including LPS, which presents a disadvantage to this model (2). One study utilizing LPS-free OVA demonstrated that mice sensitized and challenged with endotoxin-free OVA become tolerant to challenge and therefore do not develop AHR and allergic inflammation (8). Purified protein extracts of either HDM or cockroach are utilized in antigen-challenge models in order to avoid possible contaminated agents.
Table 1. Overview of OVA antigen-induced murine model of asthma. The ovalbumin (OVA) model of asthma is successful for multiple strains including: BALB/c, C57BL/6, and A/J. OVA sensitization requires the addition of adjuvant into the peritoneum. OVA challenges include introduction of OVA into the airways via aerosol. The OVA model induces common features of asthma in mice, including AHR, airway remodeling, and inflammation.

2.1.2 HOUSE DUST MITE-INDUCED MODEL OF ASTHMA

Models that incorporate house dust mite (HDM) are considered clinically relevant because HDM is commonly implicated in human asthma, and are a significant cause of indoor allergies (1, 9). As in the OVA model, HDM models also induce a Th2 phenotype that is associated with common features of human asthma by induction of Th2 cytokines, such as Il-4, Il-5, and Il-13.

Sensitization with HDM produces a Th2-type response in mice without the use of a peritoneal adjuvant, allowing for a mucosal origin of inflammation in this model that more closely mirrors human exposure (9). HDM, like OVA, will interact with APCs to activate naïve T cells in the lymph nodes in order to generate a Th2-driven immune
response, including production of IgE (10). In addition, HDM directly activates AECs in the lining of the mucosa, resulting in release of early Th2 cytokines from AECs, including IL-25, IL-33, and TSLP in an innate immune response (11 12, 13). These early response cytokines result in activation of DCs (TSLP), innate helper cells, fibroblasts, eosinophils, naïve CD4+ cells, mast cells, and macrophages, which ultimately leads to development of Th2 immunity (14). HDM has been shown to directly activate AECs through TLRs, protease activation receptor 2 (PAR2), and C-type lectin receptor (CTR) (10).

Features of mice sensitized and challenged with HDM, include airway inflammation and AHR, see Table 2 (3, 9). In this model, mice are sensitized with HDM via intranasal instillation (IN). It is important to note that some HDM protocols do not distinguish between sensitization and challenge exposures because they include a continuous antigen challenge (9). Antigen concentration may range from 5-100 mg HDM extracts for both sensitizations and challenges, depending on the protocol (9). HDM sensitizations and challenges protocols vary in length, but usually last for up to 10-12 days in an acute challenge model, followed by 2 days of ‘rest’ before tissue harvest and BAL collection (1, 9).
Table 2. Overview of HDM antigen-induced murine model of asthma. The house dust mite (HDM) model of asthma is effective in multiple strains including: BALB/c, C57BL/6, and A/J. HDM sensitization does not require adjuvant, and is performed intranasally, as is HDM challenge. The HDM model induces common features of asthma in mice, including AHR, airway remodeling, and inflammation.

2.2 RESPIRATORY MECHANICS IN MURINE ASTHMA

Measurements of airway hyperresponsiveness (AHR), an important feature of asthma, may be generated in animal models of disease by challenging mice with an aerosolized bronchoconstrictor and then measuring subsequent changes within the airways and lungs by the Forced Oscillation Technique (FOT). The flexiVent (SCIREQ Inc, Montreal, Qc, Canada) utilizes FOT to measure airway mechanics and lung function in a variety of animal subjects, including in mice, rats and rabbits (15). Studies that incorporate FOT are used both in pre-clinical testing of new therapeutics, as well as in providing crucial information regarding the impact of certain factors on the respiratory system (16, 17, 18, 19). A common application of flexiVent is in assessment of AHR by
inhaled methacholine challenge in a variety of animal disease models, including chronic obstructive pulmonary disease (COPD), lung fibrosis, acute lung injury, and asthma (15).

Animal studies involving assessment of AHR in asthma begin by delivering an aerosolized bronchoconstrictor into the airways followed by subsequent collection of automated measurements. A paralytic is typically injected prior to attachment of animal to the nebulizer, in order to ensure artificial breathing. The nebulizer allows direct attachment of the animal subject to mechanical ventilation, as well as providing delivery of inhaled bronchoconstrictor, or even other inhaled compounds.

A computer-controlled piston pump (flexiVent) delivers a precise volume of air into to the subject’s airways. Measurements of respiratory mechanics are subsequently generated by analysis of small changes in pressure and volume that are acquired as a result of precise oscillatory waveforms, also called perturbations, applied at the subject’s airway opening (15, 20). Software included with flexiVent (flexiWare) is capable of reporting reproducible dose-response respiratory mechanic parameters. Respiratory parameters include Crs, Ers, G, and H (defined below) (15). A single frequency FOT is used to generate Rrs, Crs, and Ers. Broadband frequency FOT generates Rn, G, and H.

Rrs, measures quantitatively the overall level of constriction in the lungs. Airway compliance, Crs, is a measurement of the ease of lung expansion. Tissue elastance, Ers, represents the rigidity of the lungs. In single frequency FOT measurements of asthmatic subjects, both Rrs and Ers are typically elevated compared to non-asthmatic subjects, whereas Crs will be decreased compared to non-asthmatic subjects. Newtonian resistance, Rn, is a measurement of resistance in the conducting airways, and is also called central airway resistance. Tissue damping, G, measures tissue viscoelasticity and
resistance within the small airways (15, 20). Tissue elastance, $H$, measures conservation of energy in the alveoli. Parameters measured during broadband frequency oscillations, including $R_n$, $G$, and $H$, are all typically elevated in asthmatic subjects, compared to non-asthmatic subjects (15, 20).

### 2.3 REFERENCES


CHAPTER III
EXPERIMENTAL METHODS

3.1 ANIMALS AND ANIMAL CARE

The Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic Foundation approved all animal procedures and protocols. Animals were maintained at the Cleveland Clinic Facility and maintained under a pathogen-free environment using microisolator cages in compliance with appropriate animal care regulations. Mice were given 24 hr access to food and water and kept at appropriate temperature and humidity with 12 hr light/dark cycle. Immunocompetent, non-irradiated, BALB/c wild-type females, aged 6-8 weeks, were obtained from the Jackson Laboratory (Bar Harbor, ME). Each new mouse order was kept in quarantine at the Cleveland Clinic Animal Facility for 72 hrs directly following their arrival from Jackson before being released for experimental use.
3.2 *IN VIVO PROTOCOLS*

3.2.1 OVALBUMIN ANTIGEN-INDUCED MODEL OF ASTHMA

Table 3 outlines the methods of OVA sensitization and challenge in mice. Mice aged 8 weeks were sensitized with 100 mg/mL OVA (Sigma-Aldrich, St. Louis, MO) in 200 mg/mL aluminum hydroxide Al(OH)$_3$ via intraperitoneal injection (IP) at day 1. Following sensitization, beginning at day 15, mice were nebulized with 40 mL 1% OVA/PBS for 45 min. Nebulized OVA challenges were repeated for 6 consecutive days (days 15-20) at 24 hr intervals between each challenge. Naive mice did not undergo IP sensitization and were given no subsequent OVA exposure. 24 hours following the final OVA challenge, mice underwent methacholine challenge with *flexiVent* (Scireq, Montreal, Quebec), and tissues were harvested, including collection of blood, BAL, and lungs, on day 21. Intratracheal (IT) instillation in mice is described in Section 3.2.3.

<table>
<thead>
<tr>
<th>BALB/C wildtype mice</th>
<th>n</th>
<th>OVA Sensitization</th>
<th>OVA Challenge</th>
<th>IT</th>
<th>Methacholine challenge and tissue harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Day 21</td>
</tr>
<tr>
<td>Vehicle (OVA/OVA)</td>
<td>13</td>
<td>Day 1</td>
<td>Day 15-20</td>
<td>Day 15-20</td>
<td>Day 21</td>
</tr>
<tr>
<td>LMW HA (OVA/OVA)</td>
<td>11</td>
<td>Day 1</td>
<td>Day 15-20</td>
<td>Day 15-20</td>
<td>Day 21</td>
</tr>
<tr>
<td>HMW HA (OVA/OVA)</td>
<td>7</td>
<td>Day 1</td>
<td>Day 15-20</td>
<td>Day 15-20</td>
<td>Day 21</td>
</tr>
</tbody>
</table>

Table 3. OVA antigen-induced murine experimental model. Animal subject groups include naïve, vehicle (OVA/OVA), LMW HA (OVA/OVA), and HMW HA (OVA/OVA). Experimental protocols adapted from (1, 2).
3.2.2 HOUSE DUST MITE ANTIGEN-INDUCED MODEL OF ASTHMA

House dust mite (HDM) *dermatophagoides farinae* was purchased from Greer (*D. farinae*, XPB81D3A2.5). Table 4 demonstrates protocol for HDM sensitization and challenge. Mice were sensitized at 8 weeks intranasally (IN) at day 1 with 100 mg HDM in 50 ml PBS while anesthetized with isoflurane. At day 8, mice were challenged with 10 mg HDM in 50 ml PBS via IN for five consecutive days. Mice were then allowed to rest for 72 hours following final HDM challenge, before beginning Mch challenge. At day 15, mice underwent methacholine challenge with *flexiVent* (Scireq, Montreal, Quebec) and tissues were harvested, including collection of blood, BAL, and lungs. Intratracheal (IT) instillation in mice is described in Section 3.2.3.

<table>
<thead>
<tr>
<th>BALB/c wildtype</th>
<th>n</th>
<th>DM Sensitization</th>
<th>DM Challenge</th>
<th>IT</th>
<th>Methacholine challenge and tissue harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Day 15</td>
</tr>
<tr>
<td>Vehicle (DMDM)</td>
<td>6</td>
<td>Day 1</td>
<td>Day 8-12</td>
<td>Day 8-12</td>
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<td>Day 8-12</td>
<td>Day 8-12</td>
<td>Day 15</td>
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<tr>
<td>HMW HA (DM/DM)</td>
<td>5</td>
<td>Day 1</td>
<td>Day 8-12</td>
<td>Day 8-12</td>
<td>Day 15</td>
</tr>
</tbody>
</table>

**Table 4.** HDM antigen-induced murine experimental model. Animal subject groups include naïve, vehicle (DM/DM), LMW HA (DM/DM), and HMW HA (DM/DM). Experimental protocols adapted from (1, 2).
3.2.3 TREATMENT WITH LMW AND HMW HA IN VIVO VIA INTRATRACHEAL INSTILLATION

For the duration of nebulized OVA challenge, mice were pre-treated with 100 mg LMW or HMW HA in 50 ml saline via intratracheal instillation (IT) from Days 15-20. Vehicle control mice were treated with 50 ml saline only. ITs were performed 6 hours prior to nebulized OVA challenge. Research grade LMW (21-40 kDa) and HMW (1-2 MDa) sodium hyaluronate were purchased from Lifecore Biomedical and stored at -20°C. LMW and HMW HA solutions were prepared and aliquoted 24 hrs prior to start of IT and temporarily stored at -20°C, as suggested by the manufacturer.

3.2.4 RESPIRATORY MECHANIC MEASUREMENTS

AHR and lung mechanic parameters were collected by flexiVent (Scireq, Montreal, Quebec). On Day 21, 24 hrs following the final IT and OVA challenges, mice were sedated with 80-90 mg/kg pentobarbital via IP and intubated with a 19-gauge cannula following tracheotomy incision attached by silk suture ligation. Mice were attached to artificial ventilation (150 breaths/min) coordinated by Flexiware 7.2.2 (FlexiVent; Scireq, Montreal, Quebec) software in which a piston forces air into the mouse in order to acquire AHR parameters by the forced oscillation technique at single and broadband frequencies. A tidal volume of 10 ml/kg was used for all Mch dose-response experiments, with a positive end-expiratory pressure (PEEP) of 3 cm H₂O.

To ensure continuous inhalation and exhalation patterns during Mch challenge, a muscle paralytic, pancuronium bromide at 0.8 mg/kg (Sigma-Aldrich, St. Louis, MO), was used in order to prevent mice from breathing on their own. Once mice were not
observed to breath on their own, between 2-5 min following paralytic treatment, the lungs were allowed to expand twice to 30 cm H₂O amplitude pressure (total lung capacity, TLC). Mch dose-response was obtained for LMW HA treated, HMW HA treated, and vehicle treated asthmatic mice. Non-asthmatic naïve mice also underwent Mch dose-response. Aerosol Mch challenge was conducted from 0 (baseline) up to 50 mg/mL per mouse.

AHR parameters were calculated by flexiWare 7.2.2 from (Scireq, Montreal, Quebec) with the data acquired during each perturbation (volume waveform), and include measurements of the single compartment model (single frequency) parameters, including airway resistance (Rrs), lung compliance (Crs), and elastance (Ers); as well as from constant phase model (broadband) parameters, including Newtonian resistance (Rn), tissue damping (G), and tissue elastance (H). Coefficient of determination (COD), a quality control of model fit, was measured by software during both single and broadband frequency perturbation events. Events that produced COD of less than 0.90 indicated a poor fit and were therefore excluded.

3.2.5 LUNG HISTOLOGY

The right superior and middle lobes were collected for RNA isolation, and the right inferior lobe was collected for protein analysis. The inferior left lobe was used for HA sizing and quantification (FACE). The superior left lobe was collected for histology by fixation in 10% formalin and embedding in paraffin for preparation of sections. Sections were then cut at 5 µm and stained with hematoxylin and eosin (H&E) to stain nuclei of inflammatory cells. Stained sections showing HA deposition utilized
hyaluronan-binding protein (HABP) staining of paraffin embedded sections. Cleveland Clinic Imaging Core Services provided stained sections.

3.2.6 BRONCHOALVEOLAR LAVAGE AND BLOOD

Completion of Mch challenge by FlexiVent was followed immediately by tissue harvest, including collection of lungs, blood, and bronchoalveolar lavage (BAL) for each mouse. BAL was collected by inserting 700 ml 1x DPBS into the mouse airways by 1 ml syringe, through attached 19-gauge cannula, and then drawing liquid back into the syringe by gentle suction. Between 400-600 ml BAL was typically recovered from each mouse. From raw BAL, 10 ml was used to count total nucleated cells by hemocytometer and fluorescent microscopy, utilizing ethidium bromide and acridine orange to stain cell nuclei. Remaining BAL was spun down at 750 g for 5 min. The supernatant was saved and stored at -20°C for HA sizing, and for quantification analysis by FACE or ELISA-like assays. The pellet was re-suspended in 1xDPBS for cytospin (2 x 10^5 cells/slide) using Shandon EX funnel Cytofunnel (Thermo Scientific, Kalamazoo, MI). Slides were then stained with HEMA 3 (Fisher Diagnostics, Middletown, VA) and used for cell differential count by light microscopy. Counted cells included eosinophils, neutrophils, macrophages, and lymphocytes. Total inflammatory cell numbers in BAL for each mouse were calculated by multiplying percentage of each inflammatory cell type with the total nucleated cell count in BAL. Blood was collected by syringe with EDTA (50 ml) via cardiac puncture using a 25-gauge needle. Blood was spun down at 6500 rpm for 5 min in order to collect plasma. Plasma and blood were both stored at -20°C.
3.2.7 HA QUANTIFICATION OF BAL BY ELISA-LIKE ASSAY

HA from BAL supernatant was quantified by an ELISA-like assay utilizing biotinylated HA binding protein (HABP). A 96-well plate was incubated overnight at room temperature (RT) with 200 ml of 25 mg/ml HA in 20 mM sodium carbonate (Na₂CO₃) coating buffer. After overnight coating step, wells were washed with 1x PBS/0.05% Tween-20. 200 ml of BSA was added to wells and allowed to incubate at RT for 1 hr in order to block nonspecific binding. After a second washing step, 200 ml BAL sample or HA standard (10 ng/ml – 10 ug/ml) was added to wells in duplicate and allowed to incubate at RT for 90 min. Washing was repeated with 1x PBS/0.05% Tween-20, followed by addition of 200 ml horseradish peroxidase (HRP) for 30 min. Wells were then washed up to five times in order to remove any free HRP. 200 ml of o-phenylenediamine substrate was added for 5-10 min until strong color development. The reaction was quenched with HCl and the plate was read at 492 nm by spectrophotometer. HA in BAL was calculated from HA standard curve.

3.2.8 HA SIZING IN BAL

Methods for HA sizing have been adapted from Lauer et. al and are replicated exactly in this study, and are described in the following paragraphs (1). Pooled BALs for LMW HA and HMW HA treated groups were digested, 2-3 per group, with 1 mg/ml proteinase K (Invitrogen, Carlsbad, CA) in 100 mM ammonium acetate, adjusted to pH 7.0 with 0.01% sodium dodecyl sulfate, and incubated overnight at 60°C. After overnight incubation, 100% ethanol was added to each sample for HA recovery by ethanol precipitation. Samples were spun at 13200 rpm for 10 min. Precipitates were then
collected, suspended and washed with 1 mL cold 75% ethanol, and spun again at 13200 rpm for 10 min. Supernatants were removed and pellets air dried for 20 min. 100 mL 1 mM ammonium acetate was added to resuspend each pellet by brief vortex. Samples were allowed to rest at room temperature for 20 min.

HA was recovered from BAL or lung tissue by ethanol precipitation, described above, and then further digested with 3 ml 2U/ml DNase (Ambion, Austin, TX) and 3 ml 1.28 mg/ml RNase (Roche, Indianapolis, IN). 10 ml (half of total sample volume equaling 20 ml) of each purified HA sample was treated with Streptomyces hyaluronidase (1 ml at TRU/ml) for 4 h at 37°C. Hyaluronidase enzyme was afterward deactivated by heating samples at 90-100 °C for 5 min. Both untreated and enzyme treated samples were lyophilized and then redissolved in 10 ml of tris-acetate-EDTA (TAE; 4 mM tris, 50 mM EDTA, pH 8.26). Enzyme treated and untreated samples were loaded on a 1% agarose gel (SeaKem HGT, Cambrex, Walkersville, MD) for electrophoresis using TAE for 60-90 min at 100 V (0.5 cm thick, 11x8 cm gel) at 4°C.

HA molecular weight standards (Select-HA™ LoLadder, HiLadder, and Mega-HA™ Ladder; Hyalose, Oklahoma City, OK) were used for gel band size comparison. Gels were stained with freshly made Stains-All at 200 ml at 6.25 mg/mL in 30% ethanol (E-9379, Sigma-Aldrich) overnight in complete dark. Gels were washed with DI water and exposed to light for 20-30 min. The gels were then imaged with a standard color digital camera on a light box.
3.3 IN VITRO PROTOCOLS

3.3.1 TRACHEA ISOLATION AND PREPARATION

BALB/c wild-type females aged 5-6 weeks were sacrificed utilizing pentobarbital via IP injection. Mice were dissected once heartbeat was undetectable, at around 15-20 minutes following injection. Tracheas were removed with surgical scissors, placed into Ham’s F12 with 1x pen/strep, and maintained at 4°C. A total of 10 tracheas were collected for each cell experiment.

Microdissection of tracheas was completed under a light microscope following macrodissection, in order to remove any adhering connective tissue, using only no. 15 scalpel to cut tissue away from each trachea and a needle probe to hold tissue in place. Once each trachea was cleaned, a single linear incision was made along the length of each trachea in order to expose the inner portion. Tracheas were then placed into fresh Ham’s F12 and then transferred to pronase for overnight digestion at 4°C for 18 hrs.

3.3.2 EPITHELIAL CELL ISOLATION AND AIR-LIQUID INTERFACE CULTURE

The protocol for culture of epithelial cells by air-liquid interface (ALI) was adapted from the lab of Dr. Vince Hascall in the Department of Biomedical Engineering at the Cleveland Clinic Lerner Research Institute (3). Primary airway epithelial cells (AECs) were isolated from murine tracheas by limited proteolytic digestion in pronase. Purification of AECs was followed by culture on a native basement membrane pre-deposited by Madine-Darby Canine Kidney (MDCK) epithelial cells on a transwell 12-well insert. For 10 total mice, cells were transferred to 10 inserts in two separate 12-well
plates. AECs are allowed to reach confluence in serum-free media and then cells were lifted from apical media, thereby generating an “air-liquid interface.” 10-14 days after lifting, AECs in ALI conditions will differentiate into mature, pseudostratified, and ciliated airway epithelium.

3.3.3 HA TREATMENT IN AIRWAY EPITHELIAL CELLS

Research grade LMW (21-40 kDa) and HMW (1-2 MDa) sodium hyaluronate was purchased from Lifecore Biomedical and was stored at -20°C. LMW and HMW HA solutions were prepared at 5 mg/ml in MQ water and stored at -80°C. AECs were treated with either 350 ug/ml LMW or HMW HA diluted in appropriate cell culture media. Cells were treated at confluency and differentiation.

For treatment of AECs, media was changed 24 hrs prior to treatment of cells. Directly before treatment, media was removed from both apical and basal chambers of experiment wells. Cells were treated apically and basally with 500 ul LMW HA, HMW HA or vehicle. Cell supernatants were saved for ELISA assay, and cell layers were collected in Trizol for mRNA isolation, or in cell lysis buffer for western blotting.

3.3.4 SAA ELISA IN AIRWAY EPITHELIAL CELL BASAL SUPERNATANTS

SAA concentration was determined with Mouse SAA ELISA kit (Tridelta Development Ltd., Greystones, Wicklow, Ireland). Supernatants were measured in duplicate, and without additional dilution, from two separate AEC ALI experiments according to the kit protocol. Included cell conditions for this assay were vehicle, LMW HA, and HMW HA treated AEC supernatants. Standards were provided in the kit and diluted serially as directed to generate a standard curve. Supernatants and standards were incubated at 37°C with horseradish peroxidase (HRP) labeled anti-SAA antibody.
3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added after washing in order to generate a blue product that is directly proportionate to the amount of SAA present in the original sample. Absorbance was read at 450 nm.

3.3.5 TH2 CYTOKINE AND CHEMOKINE MEASUREMENT BY MULTIPLEX MAGNETIC BEAD ASSAY

MILLIPLEX® MAP mouse cytokine/chemokine 25-plex magnetic bead panel kit was purchased from EMD Millipore™. Basal cell supernatants from three separate assays were undiluted and run in duplicate on one purchased kit. The kit included two cytokine/chemokine controls that were used as positive controls for our assay. Also included were lyophilized standards, which were serially diluted according to the supplied instructions. Basal cell supernatants were stored at -70°C prior to use, and vortexed at RT before addition. Pre-mixed beads were allowed to incubate with standards, controls, and samples overnight at 4°C. Detection antibodies were added to each well for 1 hr at RT, followed by addition of Streptavidin-Phycoerythrin for 30 min at RT. Bead fluorescence was determined by Luminex® (Bio-Plex®100). Luminex® software provided concentrations of each cytokine/chemokine for every sample, as determined by standard curves. Cytokine/chemokine results are reported as concentrations in pg/mL. Data is included for G-CSF, GM-CSF, IP-10, KC, MIP-2, and RANTES only.

3.4 REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME PCR

RNA was extracted by the Trizol method (Invitrogen, Carlsbad, CA), and subsequently cleaned utilizing RNAeasy Cleanup Kit (Qiagen). Nanodrop was used to
measure the quality of total RNA, and only samples with A260/A280 from 1.8-2.2 were used for qRT-PCR analysis. Total RNA for mouse lung samples in the same group, either LMW HA, HMW HA, vehicle or naïve, were pooled for qRT-PCR. For cells, RNA was measured per well using Trizol method without RNAeasy Cleanup Kit.

Primers were designed from mRNA sequences in GenBank and Primer Express (Applied Biosystems, ABI, Foster City, CA). NCBI BLAST was used to check all primers for appropriate specificity. Primers used were between 60-100 bps and were checked by dissociation curves and melting temperatures. Mouse glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control for all cDNA samples, and was used to normalize samples for relative transcript abundance. Mean fold changes were calculated by average of triplicate gene measurements. The 2-ΔΔCT method was used to calculate relative fold difference.

Primer sequences are included in Table 5 for IL-13, and SAA3. Superscript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) was used for RT-PCR in order to generate cDNA. SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) was used for qPCR using a Bio-Rad CFX96 Touch Real-time PCR detection system (Bio-Rad, Hercules, CA).
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Orientation</th>
<th>Primer sequences 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-13</td>
<td>Forward</td>
<td>CAGCATGGTATGGAGTTGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGGGTCTCTGAGATGATG</td>
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<tr>
<td>SAA3</td>
<td>Forward</td>
<td>TGCCATCATTCTTTCATCTTTGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCGTGAACCTCTGAACAGCCT</td>
</tr>
</tbody>
</table>

**Table 5. Sequences of primers used for qRT-PCR.** Included are the forward and reverse primers for mouse IL-13 and SAA3. Primers were all acquired from Applied Biosystems (ABI, Foster City, CA). Sequences for SAA3 forward and reverse were provided by the lab of Dr. Xiaoxia Li, at the Cleveland Clinic Lerner Research Institute, in the Department of Immunology.
3.5 REFERENCES


CHAPTER IV

EXOGENOUS LMW HA AGGRAVATES AHR IN AN OVALBUMIN MODEL OF ASTHMA BY INDUCTION OF IL-13

4.1 INTRODUCTION

HA was first found in the pulmonary secretions of asthmatic patients more than three decades ago (1). Excess HA accumulation has since been shown to correlate with asthma severity, with higher HA levels associated with a more severe form of the disease (2, 3). In a mouse model of allergic asthma, HA was found to accumulate within the lungs and bronchoalveolar lavage (BAL) of asthmatic mice, when compared to non-asthmatic mice (4, 5). Furthermore, histology from asthmatic mice reveals co-localization of HA with both eosinophils and lymphocytes (4, 5). These data support a likely integral role for HA in allergic asthma, but further study is necessary in order to determine its precise role.

We proposed to elucidate the impact of HA treatment in an ovalbumin (OVA) model of murine asthma. Given the importance of HA size in the determination of its potential role in disease processes, we further proposed to treat asthmatic mice with a
commercially available exogenous, endotoxin-free source of LMW and HMW HA (Lifecore Biomedical, Inc). The OVA model generates a robust Th2 immune response that induces AHR and inflammation, characteristic of allergic asthma (6-8). Utilizing this model in BALB/c wild-type mice, we were able to determine the impact of either LMW or HMW HA on each feature of the asthmatic phenotype.

4.2 RESULTS

4.2.1 LMW HA IMPACTS RESPIRATORY MECHANICS IN ASTHMATIC MICE

Figure 2 represents airway mechanic results from our study of treatment of OVA-induced asthmatic mice with vehicle, HMW HA, or LMW HA via IT. It is noted that naïve mice have considerably reduced AHR as determined by all six airway mechanic parameters (Fig. 2A-E) compared to all asthmatic groups; including in vehicle, LMW HA, and HMW HA treated mice (p** < 0.01 and p*** < 0.001). Lung constriction, Rrs, was not impacted by either LMW or HMW HA treatment in asthmatic mice (Fig. 2A). However, HMW HA treated asthmatic mice have significantly diminished lung constriction compared to LMW HA treated asthmatic mice at 25 mg/mL Mch (p* < 0.05). Airway compliance, Crs is not impacted overall by either LMW or HMW HA treatment in asthmatic mice (Fig. 2B); but, HMW HA treated mice have significantly increased Crs when compared to LMW HA treated mice at 25 and 50 mg/mL Mch (p* < 0.05). Ers, tissue elastance, was significantly increased in LMW HA treated asthmatic mice, compared to HMW HA treated asthmatic mice at 25 mg/mL Mch (p* < 0.05) (Fig. 2C). Tissue damping, G, was not significantly impacted by LMW HA and HMW HA treatment in asthmatic mice, compared to vehicle treated asthmatic mice (Fig. 2D).
However, HMW HA treated mice were shown to have significantly diminished G when compared to LMW HA treated mice (p** < 0.01).
Figure 2: AHR in murine model of OVA antigen-induced asthma. Airway mechanic parameters were generated in naïve mice, as well as in asthmatic mice, including vehicle, LMW HA, and HMW HA treated asthmatic mice. Airway parameters measured during methacholine challenge, include: A) lung constriction, Rrs, B) airway compliance, Crs, C) tissue elastance, Ers, and D) tissue damping; all plotted against methacholine doses from 0 (baseline) to 50 mg/mL. The number of mice per group are as follows; Naïve: 9 mice; Vehicle (OVA/OVA): 13 mice; LMW HA (OVA/OVA): 11 mice; HMW HA (OVA/OVA): 7 mice. Statistical analysis was analyzed by Student’s t test (p* < 0.05; p** < 0.01; p*** < 0.001). Error bars shown represent SEM.
4.2.2 LMW AND HMW HA ELEVATE TOTAL HA LEVELS IN BAL

HA was measured in the BAL in naïve, vehicle (OVA/OVA), LMW HA (OVA/OVA), and HMW HA OVA/OVA) groups, utilizing an ELISA-like assay with HABP. HA concentrations were elevated in all asthmatic groups compared to the naïve group (Fig. 3). Both exogenous LMW and HMW HA in BAL significantly increased total HA levels in asthmatic mice. LMW HA treated mice demonstrated up to 25-fold elevation in HA in their BAL compared to vehicle treated mice (p*** < 0.001). BAL from HMW HA treated mice demonstrated 8-fold elevation in HA concentration compared to vehicle treated mice (p** < 0.01). LMW HA treated mice have 3-fold higher HA concentration in their BAL than HMW HA treated mice (p** < 0.01).
Figure 3. HA concentration in OVA and naïve BAL analyzed by ELISA-like assay.

Total HA concentrations were measured from BAL in all experimental groups, including: naïve, vehicle (OVA/OVA), LMW HA (OVA/OVA), an HMW HA (OVA/OVA). Measurements were run in duplicate utilizing an ELISA-like assay for each BAL sample. Sample concentrations were extrapolated from standard curve. Statistical analysis was analyzed by Student’s t test (p* < 0.05; p** < 0.01; p*** < 0.001). Error bars shown represent SEM.
4.2.3  LMW AND HMW HA DO NOT IMPACT HA WITHIN THE LUNGS OF ASTHMATIC MICE

Lung sections from asthmatic and naïve mice were immunohistochemically stained with biotinylated HABP (brown) to show HA in the lung, and hematoxylin with Bluing Solution to show cell nuclei (blue) (Fig. 5). Asthmatic sections, including vehicle, LMW HA, and HMW HA treated groups, show overall increased deposition of HA around the airways and blood vessels compared to the naïve section. However, each asthmatic group demonstrates similar HA deposition, with HA co-localized with inflammatory cells in the lung.
Figure 4. Immunohistochemical HA staining of OVA and naïve lung sections with biotin-HABP. Lung sections included in this figure are: A) naïve, B) vehicle (OVA/OVA), C) LMW HA (OVA/OVA), and D) HMW HA (OVA/OVA). Deparaffinized sections were stained with biotinylated hyaluronan acid binding protein (5 mg/mL) and counterstained with hematoxylin and Bluing Solution. Prepared slides were imaged with light microscopy. Airways are indicated by Δ, and blood vessels by ➔.
4.2.4  LMW AND HMW HA DO NOT IMPACT INFLAMMATORY CELLS IN BAL

Figure 6 demonstrates the quantitative analysis of inflammatory cells acquired from mouse bronchoalveolar lavage (BAL). All asthmatic groups, including vehicle, LMW HA, and HMW HA treated groups, have significantly elevated inflammatory cell counts (total cells, eosinophils, neutrophils, macrophages, and lymphocytes) compared to naïve mice (p* < 0.05; p** < 0.01; p*** < 0.001). Based on data given in figure 6A, LMW HA and HMW HA did not impact total inflammatory cells from BAL in asthmatic mice, compared to vehicle treated asthmatic. Similarly, LMW HA or HMW HA did not impact cell differential counts of eosinophils, neutrophils, macrophages, and lymphocytes (Fig. 6B-E) in asthmatic mice, compared to vehicle treated asthmatic mice. The naïve BAL analyses showed minimal leukocytes, indicative of absence of inflammation.
C  Neutrophils

D  Macrophages
E

Lymphocytes

![Bar graph showing lymphocyte counts for different groups: Naive, Vehicle (OVA/OVA), LMW HA (OVA/OVA), and HMW HA (OVA/OVA). The graph indicates a significant difference between the groups with *** and * symbols.]
Figure 5: Inflammatory cells in BAL of OVA and naïve mice. Inflammatory cells were isolated from bronchoalveolar lavage (BAL) in naïve and asthmatic mice, directly following methacholine challenge. Analyses include total cell counts by hemocytometer and fluorescence microscopy, and subsequent cell differential counts by light microscopy. Cell data include: A) total inflammatory cells, B) eosinophils, C) neutrophils, D) macrophages, and E) lymphocytes, plotted against calculated cells per mL. The number of mice per group are as follows; Naïve: 9 mice; Vehicle (OVA/OVA): 13 mice; LMW HA (OVA/OVA): 11 mice; HMW HA (OVA/OVA): 7 mice. Statistical analysis was analyzed by Student’s t test (p* < 0.05; p** < 0.01; p*** < 0.001). Error bars shown represent SEM.
4.2.5  LMW AND HMW HA TREATMENT DO NOT IMPACT INFLAMMATORY CELL INFLUX IN THE LUNGS

Figure 7 compares H&E stained lung sections from naïve, vehicle treated asthmatic, LMW HA treated asthmatic, and HMW HA treated asthmatic mice. The naïve lung section has minimal inflammatory cell infiltration, as noted by increased appearance of stained cell nuclei. Sections from each asthmatic group, including vehicle, LMW HA, and HMW HA treated groups, demonstrate overall increased inflammatory cell infiltration around the airways and in the peripheral lung, compared to the naïve section. However, there is no distinct differences noted between vehicle, LMW HA, and HMW HA treated groups; which demonstrates that these groups have similar inflammatory cell influx within the lung.
Figure 6: Lung histology in OVA and naive mice. Lung sections were taken from larger left lobes of experimental mice following methacholine challenge. Lobes were stored temporarily in 10% formalin for 24 hrs prior to embedding in paraffin, followed by sectioning. Sections were stained with hematoxylin and eosin (H&E) and imaged under light microscope at 10X, including for: A) naïve, B) vehicle (OVA/OVA), C) LMW HA (OVA/OVA), and D) HMW HA (OVA/OVA). Blood vessels are indicated by ➔ and airways by Δ.
4.2.6 LMW AND HMW HA INDUCE GENE EXPRESSION OF INTERLEUKIN-13

RNA was isolated from the lungs of asthmatic mice. Relative expression of IL-13 mRNA was increased in the lungs of LMW and HMW HA treated asthmatic mice, compared to vehicle treated mice (p* < 0.05 and p** < 0.01, respectively) (Fig. 8). Other measured asthma-related lung Th2 cytokines and chemokines showed no impact of either LMW or HMW HA treatments on asthmatic mice. Other cytokines and chemokines were measured, but these did not show significant between groups.
**Figure 7: IL-13 lung gene expression in the lungs of OVA asthmatic mice.** RNA was isolated from fresh frozen lung samples taken from the right lobes. Lung samples were isolated from three mice per group, including from vehicle (OVA/OVA), LMW HA (OVA/OVA), and HMW HA (OVA/OVA) groups. Analysis was performed in triplicate. Statistical analysis was analyzed by Student’s t test (p* < 0.05; p** < 0.01; p*** < 0.001). Error bars shown represent SEM.
4.3 DISCUSSION

Our study of exogenous treatment of HA in an OVA model of murine asthma reveals a potential role for HA in the development of AHR. Based on our results, we determined that LMW HA treatment in asthmatic mice significantly elevates overall AHR, compared to treatment with HMW HA. Our study also shows that LMW HA and HMW HA induce IL-13, a pro-inflammatory Th2 cytokine. Our data correlate with several studies, both in vivo and in vitro, in which exogenous LMW HA was found to exert a pro-inflammatory impact (14, 17, 23, 24).

LMW HA treated asthmatic mice exhibited overall worsened AHR compared to HMW HA treated mice, as demonstrated by increases in Rrs, Ers, and G in LMW HA treated mice, as well as by decreased Crs. The impact of exogenous LMW HA on AHR is concurrent with an observed increase in gene expression of IL-13 within the lungs of LMW HA treated asthmatic mice, compared to vehicle treated asthmatic mice. IL-13 has been previously linked to development of AHR in asthma (25). While HMW HA treated mice demonstrated diminished AHR compared to LMW HA treated mice, these mice did not show significant reduction in AHR when compared to vehicle treated mice. While we observe a minimal impact of HMW HA on AHR, gene expression of IL-13 in the lungs of HMW HA treated asthmatic mice is significantly elevated compared to vehicle treated mice, similar to gene expression results observed in LMW HA treated mice.

Notably, there was no observed impact of either exogenous LMW or HMW HA in asthmatic mice on inflammatory cells, either regarding total cell counts or cell differential counts. Histology from asthmatic mice revealed similar inflammatory cell influx in the lungs of all asthmatic groups, including LMW HA, HMW HA, and vehicle treated
asthmatic mice. Similar observations were made for total inflammatory cell counts and differentials between asthmatic groups in the BAL. We conclude that neither HA size had an impact on recruitment or maintenance of inflammatory cell populations within the airways and lungs of asthmatic mice in our OVA experimental model.

Data from HA quantification in the BAL of exogenous LMW HA and HMW HA treated asthmatic mice demonstrate that HA is significantly elevated in both treatment groups, compared to vehicle treated asthmatic mice. Although equal amounts of both HA sizes were added into the airways, total HA recovery was diminished in HMW HA treated mice compared to LMW HA treated mice. However, HA staining in the lung demonstrates no similar HA levels between all asthmatic groups, including LMW HA, HMW HA, and vehicle treated mice. We conclude that exogenous HA stays primarily in the airway, and does not enter into the lung. In a preliminary study utilizing HA sizing of BAL in an agarose gel, we saw evidence that HMW is degraded in the airways to a certain degree (data not shown). Degradation of exogenous HMW HA may account for the similar impacts of exogenous HMW HA and LMW HA on induction of IL-13 in asthmatic mice. Accumulation of new LMW HA from exogenous HMW HA may contribute to IL-13 gene expression, with minimal subsequent impact on AHR.

Our results provide novel insights into the role of HA size in asthma. To summarize, we conclude that exogenous LMW HA aggravates AHR, induces IL-13 expression, but has little impact on inflammatory cells either in the lung or BAL. Exogenous LMW HA appears to remain primarily in the airways of treated mice, leading us to further conclude that its induction of IL-13 may be linked to interaction of HA with the airway epithelium. Mice in our OVA experimental model demonstrate increased sensitivity to HA fragments.
in the range of 20-40 kDa, as demonstrated by increased AHR in exogenous LMW HA treated mice compared to exogenous HMW HA treated mice.

Treatment of mice with exogenous HMW HA produced a minimal impact on AHR, but was shown to induce IL-13 gene expression. While exogenous HMW HA does not appear to enter the lung, we have determined that it may be degraded in the airways of asthmatic mice. We presume that the impact of exogenous HMW HA on IL-13 induction may be primarily due to HA turnover. However, we cannot rule out an impact of intact HMW HA in the airways to induce IL-13 and AHR.
4.4 REFERENCES


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CHAPTER V

EXOGENOUS HMW HA AGGRAVATES AHR IN AN HOUSE DUST MITE MODEL OF ASTHMA BY INDUCTION OF IL-13

5.1 INTRODUCTION

We propose to study the impact of HA size in a murine model of HDM antigen-induced asthma. HA is a major component of ECM and has been shown to be elevated in inflammatory disease, including asthma (1). While HA was discovered in the pulmonary secretions of asthmatic patients over three decades ago, its role in asthma is still not well understood (2). In a mouse model of allergic asthma, HA was found to accumulate within the lungs and bronchoalveolar lavage (BAL) of asthmatic mice, when compared to non-asthmatic mice (3, 4). Furthermore, histology from asthmatic mice reveals co-localization of HA with both eosinophils and lymphocytes (3, 4). Accumulation of HA in asthma has been linked with increased ECM turnover, as well as increased synthesis of new HA fragments (5).

In our previously reported OVA murine asthma experiments, we determined that LMW HA impacts AHR by induction of IL-13 gene expression, while HMW HA
induced IL-13 but exerted only a minimal impact on AHR. We further determined that exogenous HMW HA is degraded in the airways, producing a range of HA fragment sizes that may contribute to its impact on IL-13 gene expression. In our current study, we proposed to broaden our study of the impact of exogenous LMW and HMW HA in an additional model of asthma, utilizing HDM antigen exposure.

While an OVA murine model of asthma produces characteristics of Th2 immunity, including AHR and inflammation, an HDM antigen-induced model of allergic asthma in mice is more clinically relevant (6). HDM is a significant source of indoor allergies, and is frequently linked with human asthma (6, 7). Furthermore, in an HDM-induced asthma, mice are challenged with antigen directly into the nasal passages, which more closely mirrors human antigen exposure (6, 7).

5.2 RESULTS

5.2.1 HMW HA IMPACTS RESPIRATORY MECHANICS IN ASTHMATIC MICE

Figure 9 represents airway mechanic results from our study of treatment of dust mite antigen-induced asthmatic mice with vehicle, HMW HA, or LMW HA via IT. Naïve mice present considerably reduced AHR as demonstrated by all six airway mechanic parameters compared to all three asthmatic groups; including in vehicle, LMW HA, and HMW HA treated mice (p** < 0.01 and p*** < 0.001). Lung constriction, Rrs, was significantly impacted by HMW HA treatment in asthmatic mice at 25 and 50 mg/mL Mch, compared to vehicle treated asthmatic mice (p*** < 0.001) (Fig 9A). Additionally, our HMW HA treatment group demonstrated significantly elevated lung constriction
compared to our LMW HA treatment group, at Mch dose 25 mg/mL only (p* < 0.05). LMW HA was not found to impact overall lung constriction.

Airway compliance, Crs was not impacted overall by treatment of asthmatic mice with LMW HA (Fig 9B). However, HMW HA treatment in asthmatic mice is shown to significantly diminish Crs at 25 and 50 mg/mL Mch, compared to vehicle treated asthmatic mice (p* < 0.05). Tissue elastance, H, was impacted in asthmatic mice by treatment with HMW HA at Mch doses 25 and 50 mg/mL, when compared to vehicle treated mice at these doses (p*** < 0.001 and p* < 0.05, respectively) (Fig 9C). Treatment with LMW HA in asthmatic mice did not significantly impact H at all Mch doses.
Figure 8: AHR in murine model of HDM antigen-induced asthma. Airway mechanic parameters were generated in naïve mice, as well as in asthmatic mice, including vehicle, LMW HA, and HMW HA treated asthmatic mice. Airway parameters measured during methacholine challenge, include: A) lung constriction, Rrs, B) airway compliance, Crs, and C) tissue elastance, H, are plotted against methacholine doses from 0 (baseline) to 50 mg/mL. The number of mice per group are as follows; Naïve: 6 mice; Vehicle (DM/DM): 6 mice; LMW HA (DM/DM): 6 mice; HMW HA (DM/DM): 5 mice. Statistical analysis was analyzed by Student’s t test (p* < 0.05; p** < 0.01; p*** < 0.001). Error bars shown represent SEM.
5.2.2 LMW HA ELEVATED TOTAL HA CONCENTRATION IN BAL

HA was measured by an ELISA like assay and was measured in the BAL of naïve, vehicle (DM/DM), LMW HA (DM/DM), and HMW HA (DM/DM) treated mice. Naïve mice had similar concentration levels of total HA to vehicle (DM/DM) and HMW HA (DM/DM) treated asthmatic mice. Total HA concentration was significantly elevated in LMW HA treated asthmatic mice compared to naïve, vehicle, and HMW HA treated groups (Fig. 10) (p*** < 0.001).
Figure 9. HA concentration in HDM and naïve BAL analyzed by ELISA-like assay.

Total HA concentration was measured from BAL in all experimental groups, including: naïve, vehicle (DM/DM), LMW HA (DM/DM), an HMW HA (DM/DM). Measurements were run in duplicate utilizing ELISA-like assay for each BAL sample. Sample concentrations were extrapolated from a standard curve. Statistical analysis was analyzed by Student’s t test (p* < 0.05; p** < 0.01; p*** < 0.001). Error bars shown represent SEM.
5.2.3 LMW AND HMW HA DO NOT IMPACT ENDOGENOUS HA IN THE LUNGS

Lung sections from HDM-induced asthmatic, and naïve mice, were immunohistochemically stained with biotin-HABP (brown) to show HA in the lung, and hematoxylin and Bluing Solution to show cell nuclei (Fig. 11). Asthmatic sections, including vehicle, LMW HA, and HMW HA treated groups, show overall increased deposition of HA around the airways and blood vessels compared to naïve section. Similar HA deposition is observed between all asthmatic groups, with HA co-localized with inflammatory cells in the lung.
Figure 10. Immunohistochemical HA staining of HDM and naïve lung sections with biotin-HABP. Lung sections included in this figure are: A) naïve, B) vehicle (DM/DM), C) LMW HA (DM/DM), and D) HMW HA (DM/DM). Deparaffinized sections were stained with biotinylated hyaluronan acid binding protein (5 mg/mL) and counterstained with hematoxylin and Bluing Solution. Prepared slides were imaged with light microscopy. Blood vessels are indicated by ➔ and airways by ∆.
5.2.4 LMW HA TREATED ASTHMATIC MICE DEMONSTRATE DIMINISHED EOSINOPHILS AND NEUTROPHILS IN THE BAL

Total inflammatory cells in the BAL of HDM asthmatic groups, including vehicle and HMW HA treated mice, were demonstrated to be significantly increased compared to non-asthmatic mice (Fig. 12A) (p* < 0.05). In contrast, total inflammatory cells in LMW HA treated asthmatic mice were not significantly elevated compared to non-asthmatic mice (naïve). Eosinophils from BAL of HMW HA and vehicle treated mice were significantly elevated compared to non-asthmatic mice (Fig. 12 B) (p* < 0.05 and p** < 0.01, respectively), whereas eosinophils from BAL of LMW HA treated asthmatic mice were significantly diminished compared to vehicle treated asthmatic mice (p* < 0.05). Eosinophils from BAL of LMW HA treated asthmatic mice were also significantly diminished compared to HMW HA treated asthmatic mice (p* < 0.05).

Neutrophils from BAL of HMW HA and vehicle treated asthmatic mice were significantly elevated compared to non-asthmatic mice (Fig. 12C) (p** < 0.01 and p*** < 0.001, respectively), whereas neutrophils from BAL of LMW HA treated asthmatic mice demonstrated significantly decreased cells compared to both vehicle and HMW HA treated asthmatic mice (p* < 0.05). Cell differential count of macrophages demonstrated similar elevation of cells in all experimental groups, including for all asthmatic and non-asthmatic mice (Fig. 12D). Lymphocytes from BAL of vehicle and HMW HA treated asthmatic mice were significantly elevated compared to cells from BAL of non-asthmatic mice (Fig. 12E) (p* < 0.05), whereas lymphocytes from BAL of non-asthmatic and LMW HA treated asthmatic mice were similarly elevated.
E

Lymphocytes

![Bar chart showing lymphocyte counts for different groups.](image)
**Figure 11: Inflammatory cells in BAL in HDM and naïve mice.** Inflammatory cells were isolated from bronchoalveolar lavage (BAL) in naïve and asthmatic mice, directly following methacholine challenge. Analyses include total cell counts by hemocytometer and fluorescence microscopy, followed by subsequent cell differential counts by light microscopy. Cell data include A) total inflammatory cells, B) eosinophils, C) neutrophils, D) macrophages, and E) lymphocytes. Each cell type is plotted against calculated cells per mL. The number of mice per group are as follows; Naïve: 6 mice; Vehicle (DM/DM): 6 mice; LMW HA (DM/DM): 6 mice; HMW HA (DM/DM): 5 mice. Statistical analysis was analyzed by Student’s t test (p* < 0.05; p** < 0.01; p*** < 0.001). Error bars represent SEM.
5.2.5 LMW AND HMW HA DO NOT IMPACT INFLAMMATORY CELL INFLUX IN THE LUNGS

Figure 13 compares H&E stained lung sections from naïve, vehicle treated asthmatic, LMW HA treated asthmatic, and HMW HA treated asthmatic mice, in a dust mite experimental model of asthma. Naïve lung demonstrates minimal inflammatory cell influx, whereas vehicle (DM/DM) lung exhibits increased influx of stained cells around the vessels and airways. LMW HA (DM/DM) and HMW HA (DM/DM) lung sections also reveal increased inflammatory cell influx compared to naïve lung, but the appearance of cells in these sections is similar in magnitude to that of vehicle (DM/DM) lung. All asthmatic lung sections, including vehicle (DM/DM), LMW HA (DM/DM), and HMW HA (DM/DM), display airway thickening compared to naïve lung.
Figure 12: Lung histology in HDM and naïve mice. Lung sections were acquired from upper left lobes of experimental mice following methacholine challenge. Excised lobes were stored in 10% formalin for 24 hrs prior to embedding in paraffin for subsequent sectioning. Sections were stained with hematoxylin and eosin (H&E) and imaged under light microscope at 10X for A) naïve, B) vehicle (DM/DM), C) LMW HA (DM/DM), and D) HMW HA (DM/DM). Blood vessels are indicated by ➔ and airways by Δ.
5.2.6 HMW HA INDUCES GENE EXPRESSION OF INTERLEUKIN-13

RNA was isolated from the lungs of asthmatic groups, including vehicle, LMW HA, and HMW HA treated mice. Relative expression of IL-13 mRNA was significantly increased in HMW HA treated the lungs of asthmatic mice (p** < 0.01) only (Fig. 14). Other measured asthma-related Th2 cytokines and chemokines showed no impact of either LMW or HMW HA treatments on asthmatic mice (data not shown).
**Figure 13: IL-13 gene expression in the lungs of HDM asthmatic mice.** RNA was isolated from fresh frozen lung samples taken from the right lobes. Lung samples were isolated from three mice per group, including from vehicle (DM/DM), LMW HA (DM/DM), and HMW (DM/DM) groups. Analyses were performed in duplicate. Statistical analysis was analyzed by Student’s t test (p* < 0.05; p** < 0.01; p*** < 0.001). Error bars shown represent SEM.
5.3 DISCUSSION

By utilizing an HDM model of asthma we provide further evidence of the importance of HA size in the development of AHR. Our current study shows that exogenous HMW HA exerts a pro-inflammatory effect in HDM sensitized and challenged mice by exacerbating AHR in asthmatic mice. As in our previous study utilizing an OVA model of murine asthma, the impact of HMW HA on AHR can be linked to induction of IL-13 gene expression in our current study utilizing an HDM model of asthma.

In this study, exogenous HMW HA treatment in asthmatic mice in a HDM model increased overall AHR by increasing lung constriction (Rrs) and tissue elastance (H), as well as decreasing lung compliance (Crs). Conversely, exogenous LMW HA did not have an impact on Rrs, H, or Crs. We conclude that HMW HA had an impact on overall AHR. Furthermore, gene expression of IL-13 was demonstrated to be significantly induced in the lungs of HMW HA treated asthmatic mice, compared to vehicle treated asthmatic mice. Exogenous LMW HA, however, was not found to significantly impact gene expression of IL-13 in asthmatic mice. In our HDM model, unlike in our previous OVA study, AHR in asthmatic mice appears to be impacted by treatment with HMW HA.

HMW HA in our current study was not observed to significantly impact inflammatory cells in either the lungs or in the BAL. Lung histology demonstrated similar inflammatory cell influx between all asthmatic groups. Total cell and cell differential analysis demonstrated similar levels of total cells, eosinophils, neutrophils, macrophages, and lymphocytes in the BAL of HMW HA treated asthmatic mice. Similarly, LMW HA treatment did not impact inflammatory cell influx in the lungs of
asthmatic mice, as determined by histological analysis. However, LMW HA treated asthmatic mice demonstrated significantly reduced amounts of eosinophils and neutrophils compared to vehicle treated asthmatic mice, while LMW HA treated asthmatic mice did not have significantly elevated total cell levels compared to naïve mice.

Immunohistochemistry of lung sections demonstrated elevated HA in the lungs of asthmatic mice, compared to naïve mice. Additionally, there was no significant difference in HA staining observed between vehicle, LMW HA, and HMW HA mice. However HA measured in the BAL demonstrated that HA is not elevated in the BAL in either vehicle or HMW HA treated mice, compared to naïve. Significant elevation in HA concentration was observed for LMW HA treated mice only. We conclude that, while increased HA levels persist in the asthmatic lung; HA levels appear to return levels consistent with naïve mice in the BAL. Treatment with exogenous HMW HA does not impact total HA in the BAL, while exogenous LMW HA results in persistence of HA in the BAL.

We conclude from our data that HDM sensitized and challenged mice exhibit a worse asthmatic phenotype as a result of treatment with exogenous HMW HA, and to a lesser extent with treatment with exogenous LMW HA. From our previous study utilizing an OVA model of asthma, we suggested that exogenous HMW HA is turned over in the airways of asthmatic mice, resulting in generation of new LMW HA fragments from 100-500 kDa. Based upon the near-absence of HA in the BAL from HMW HA treated HDM-induced asthmatic mice, we suspect HA turnover may also play a role our current experimental model. We postulate that it is the generation of new HA fragments in exogenous HMW HA treated asthmatic mice that results in increased AHR and induction
of IL-13 gene expression, rather than the impact of HMW HA itself. Furthermore, given the diminished impact of exogenous LMW HA (20-40 kDa) in our current model, we propose that HDM mice are predominantly sensitive to the HA size range produced by HA turnover (100-500 kDa).

As in OVA study of HA size in murine asthma, we cannot rule out the possibility that intact HMW HA induces IL-13 and AHR in asthmatic mice. We propose that studies utilizing treatment of HMW HA in a cell culture model of airway epithelial cells will further elucidate the potential impact of HMW HA in the airways. Utilizing a cell model will allow us to study the impact of HA size in the airways in the absence of inflammation, and other factors that result in HA turnover.
5.4 REFERENCES


CHAPTER VI

IMPACT OF HA SIZE IN MURINE AIRWAY EPITHELIAL CELLS

6.1 INTRODUCTION

The results from our study utilizing LMW and HMW HA treatment in either OVA- or HDM-induced asthmatic model of asthma demonstrated an impact of HA in the airways. In our OVA model, LMW HA was found to exacerbate AHR in asthmatic mice, compared to HMW HA treated mice, it did not induce overall AHR compared to vehicle treated mice, with the exception of Rn induction. HMW HA similarly showed no overall impact on AHR compared to vehicle treatment induced AHR, however, it was also found to induce Rn compared to vehicle treated mice. Neither LMW nor HMW HA was found to impact inflammatory cells in the lungs or BAL, while both did induce IL-13 gene expression in the lungs, compared to vehicle mice. Given these results, we could not conclude an overall impact of either HA size on AHR induction in asthmatic mice, while the presence of LMW HA in the range of 20-40 kDa did appear to have some impact on AHR, compared to HMW HA treated mice. We suspected that the impact of HMW HA on Rn may have been due to generation of HA fragments by HA turnover in the airways. However, the induction of IL-13 gene expression in the lungs of both HA treated groups,
compared to vehicle treated mice, led us to continue our study in an HDM model of asthma, in order to determine the impact of HA size in a more clinically relevant model of murine asthma.

In our HDM model, we found that LMW HA did not exert a primary impact on AHR and gene expression of IL-13 in asthmatic mice. HMW HA treatment in HDM-induced asthmatic mice had a significant impact on AHR and induction of IL-13 gene expression in asthmatic mice, compared to vehicle mice. Interestingly, while HMW HA did not impact inflammatory cells in the lungs or BAL of asthmatic mice, certain cell types, including eosinophils and neutrophils, were found to be diminished in LMW HA treated mice compared to HMW HA treated mice. We suggest that LMW HA may have some impact on movement of cells from the lung parenchyma into the airways.

We concluded that generation of HA fragments from HA turnover in the airways may be responsible for our observed results, however, we could not determine that intact HMW HA had no impact on AHR and induction of IL-13 in the airways of asthmatic mice. Analysis of HA in BAL and in the lungs demonstrated that both exogenous HA treatments remain primarily in the airways of mice. Thus, we hypothesized that LMW and HMW HA impose a primary impact in the airways of mice, resulting in elevated AHR during Mch challenge. We chose to study airway epithelial cells (AECs) because these cells represent the first line of defense in the airways, thus making them likely to interact with exogenous HA which is known to remain predominantly in the airways of asthmatic mice. In order to test our hypothesis, we proposed to study the impact LMW and HMW HA on primary cell cultures derived from airway epithelial cells (AECs) isolated from strain and age-matched murine tracheas.
For this study, we focused on the impact of HA size on inflammatory Th2 cytokines/chemokines and SAA3 in AECs. We utilized an air-liquid interface (ALI) model of cell culture, which includes AECs grown on a basement membrane so that they are exposed apically to air, and basally to media. Our experimental methods included treating cells apically with LMW and HMW HA for 24 hrs, after which cell lysates and basal media were analyzed.

6.2 RESULTS

6.2.1 SAA3 mRNA EXPRESSION INCREASED IN LMW AND HMW HA TREATED AIRWAY EPITHELIAL CELLS

RNA was isolated from cell lysates using the Trizol method for two separate experiments. Figure 15 shows that gene expression of SAA3 was increased in both LMW and HMW HA treated AECs (24 hr), compared to vehicle treated cells (p* < 0.05). Gene expression of IL-13 was also measured in AECs, but mRNA for this cytokine was not detectable by qPCR.
Figure 14. SAA3 mRNA expression in AEC-ALI cell lysates. RNA was extracted by the Trizol method (Invitrogen, Carlsbad, CA). Relative transcript abundance of SAA3 was normalized to mouse glyceraldehyde phosphate dehydrogenase (GAPDH). Mean fold change was calculated by average of duplicate gene measurements. Gene mRNA expression of LMW HA and HMW HA treated cells (24 hr) is reported as relative expression compared to vehicle treated cells. Statistical analysis was analyzed by Student’s t test (p* < 0.05). Error bars shown represent SEM.
6.2.2 TOTAL SAA CONCENTRATION INCREASED IN LMW AND HMW HA TREATED AIRWAY EPITHELIAL CELL SUPERNATANTS

A commercial ELISA measured total SAA in AEC supernatants from three separate experiments. Basal cell supernatants were collected for each group, including for vehicle, LMW HA, and HMW HA treated cells. Cells were treated for 24 hrs only. Basal cell supernatants from LMW and HMW HA treated cells were found to have elevated levels of SAA, compared to vehicle treated cells (p* < 0.05). These results are shown in Figure 16.
Figure 15. SAA concentration from AEC-ALI supernatants. SAA concentration was determined with Mouse SAA ELISA kit (Tridelta Development Ltd., Greystones, Wicklow, Ireland). Supernatants were measured in duplicate, without additional dilution, from two separate AEC-ALI experiments. Final concentrations were combined per cell condition for both basal and apical media. Results include total SAA concentrations for vehicle, LMW HA, and HMW HA treated cell supernatants. SAA concentrations were measured from standard solutions provided in the kit. Statistical analysis was analyzed by Student’s t test (p* < 0.05). Error bars shown represent SEM.
6.2.3 TH2 CYTOKINES INCREASED IN LMW AND HMW HA TREATED AIRWAY EPITHELIAL CELL SUPERNATANTS

A multiplex magnetic bead assay was used to measure 25 different cytokines and chemokines in AEC basal cell media from three separate cell assays, after 24 hr treatment with either vehicle, LMW HA, or HMW HA. In Figure 17, LMW and HMW HA treated cells showed elevated levels of six total Th2 cytokines and chemokines, including, G-CSF, GM-CSF, IP-10, KC, MIP-2, and RANTES, compared to vehicle treated cells (p* < 0.05).
A

G-CSF

B

GM-CSF

C

IP-10
Figure 16. Th2 cytokines/chemokines in AEC-ALI basal supernatants in HA treated cultures. Th2 cytokine/chemokines were measured from basal cell supernatants utilizing MILLIPLEX® MAP mouse cytokine/chemokine 25-plex magnetic bead panel kit (EMD Millipore™). Supernatants from three assays were run in duplicate for vehicle, LMW HA, and HMW HA treated cells. Cytokines/chemokine concentrations are shown in pg/mL for A) G-CSF, B) GM-CSF, C) IP-10, D) KC, E) MIP-2, and F) RANTES. Statistical analysis was analyzed by Student’s t test (p* < 0.05). Error bars shown represent SEM.

6.3 DISCUSSION
The results from this study indicate that both LMW and HMW HA initiate an inflammatory response in the airway epithelium. The airway epithelium acts as the first line of defense against inhaled pathogens by initiating innate immunity. AECs recognize pathogens largely by toll-like receptors (TLRs) that results in the induction of a variety of inflammatory signal molecules, including cytokines and chemokines, which promote DC recruitment and activation, as well as CD4+ T cell polarization (13).

Both HA sizes were found to induce production of SAA3 and certain Th2 cytokines/chemokines, including KC, RANTES, G-CSF, GM-CSF, IP-10, and MIP-2. All of these inflammatory molecules are commonly elevated in asthmatics (13). KC (CXCL1) is a murine analog of IL-8 and has been linked to neutrophil chemoattractant activity (1). Certain Th2 cytokines, including IL-4 and IL-13, are shown to result in induction of KC in AECs (2). RANTES is a C-C chemokine that has been shown to act as a potent chemoattractant for inflammatory cells, including monocytes, T lymphocytes, basophils, and eosinophils (3). Up-regulation of RANTES has also been linked to ASM proliferation (4). C-Granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) have been shown to be upregulated in AECs in response to pro-inflammatory cytokines, including IL-1 and TNF-alpha (5, 6). Airway epithelial derived GM-CSF has been strongly linked to DC maturation (13). IFN-γ-inducible protein 10 (IP-10/CXCL10) is directly involved in the development of AHR and inflammation in murine asthma (7). IP-10 attracts Th1 lymphocytes and is found to be upregulated in asthmatics (7). Overexpression of IP-10 in mice is linked to increased AHR and eosinophilia (7). Up-regulation of macrophage inflammatory protein (MIP)-2,
also called CXCL2, in AECs has been linked to recruitment of neutrophils and lymphocytes (8).

HA fragments have been previously shown to induce IL-8 and IP-10 in AECs, utilizing air-liquid interface (ALI) (9). Our use of a 25-plex cytokine/chemokine allowed us to identify several additional inflammatory mediators that are induced by HA signaling in the airway epithelium that have not yet been published. One study published the impact of AEC production of cytokines/chemokines, including G-CSF, M-CSF, GM-CSF, KC, IL-6, MIP-1α, MIP-2, and IP-10, when cells were stimulated by an array of factors, including LPS, flagellin, TNF, and others (10). Our study presents a novel finding in which a similar profile of cytokines/chemokines may be induced by HA alone.

SAA3 was also found to be upregulated in AECs treated with either LMW or HMW HA. Increased SAA has been linked with inflammation, including in disorders of the lungs, kidneys, joints, and vasculature (11, 12). Lung production of SAA in humans has been observed in response to infection, and is regulated by epithelial expression of SAA1 and SAA2 (13). SAA has further been linked to worsened AHR in ovalbumin (OVA) sensitized and challenged mice in response to Mch challenge (13). SAA is also known to associate with HA to induce monocyte adhesion and inflammation, and has also been shown to induce certain cytokines (14). SAA3 expression has been linked to activation of airway epithelial NF-κB (9). Certain inflammatory mediators have been shown to induce SAA, such as IL-1, IL-6, and TNFα (15). SAA itself has been linked to induction of certain inflammatory mediators, including G-CSF (13).

Our current results demonstrate an additional mechanism in which HA exerts a pro-inflammatory impact by the airway epithelium, potentially mediated by SAA3. SAA
has been shown to be important in the establishment and maintenance of early inflammation (9). Since HA has also been linked with early inflammation in asthma, we suspect that HA signaling of SAA3 in the airway epithelium may play a key role in these processes. HA has not been previously linked with induction of SAA3 in the airway epithelium, and more study is needed in order to clarify this potential mechanism of HA signaling in asthma.

The impact of HA on AECs in this study appears to be independent of molecular size in our current model, since both LMW and HMW HA induce similar responses in cells. However, a comprehensive study of multiple sizes of HA in both the LMW and HMW ranges are needed in order to further clarify the potential role of HA size in the airway epithelium.

HA signaling has been previously linked to activation of toll-like receptors (TLRs) due to their structural similarities to pathogen associated molecular patterns (PAMPs). Additionally, certain models of disease have linked the impact of HA to TLRs, including in models acute lung injury (15). Th2 cytokine and chemokine production, as well as induction of SAA, have been linked to TLR signaling (13, 16). Thus, we present TLR-signaling as a possible mechanism for induction of Th2 cytokines and chemokines, and SAA3, as a result of treatment of AECs with LMW and HMW HA in our culture experiments. However, further study is needed in order to confirm this theory.
6.4 REFERENCES


7.1 THE IMPACT OF HA SIZE ON AHR IN MURINE ASTHMA IS MODEL-SPECIFIC

In our study of the role of HA size in AHR in murine asthma, we determined that the impact of HA size is specific to either OVA or HDM models of asthma. In our OVA model, asthmatic mice treated with LMW HA were shown to have elevated AHR, compared to HMW HA treated mice. One parameter, Rn, demonstrated that both LMW and HMW HA treated mice had increased AHR levels compared to vehicle treated mice. By contrast, in our HDM model, HMW HA treated asthmatic mice demonstrated overall increase AHR compared to vehicle treated mice, with LMW HA treated mice demonstrating a lessened increase in AHR compared to vehicle mice.

It has been previously reported that the impact of HA size may be disease-specific. LMW HA in a murine model of colitis was found to induce an anti-inflammatory response, while LMW HA in a murine model of acute lung injury was demonstrated to induce a pro-inflammatory response (1 – 3). We conclude from our data that the impact of HA size may also be dependent on disease models.
While the OVA and HDM asthma models initiate a similar generalized asthmatic phenotype, there are also unique differences. The HDM model bypasses peripheral sensitization with adjuvant, as in the OVA asthma model, and instead involves a more localized Th2 response within the lung mucosa (4). In addition, our lab has determined that OVA/Alum exposed mice have a greater influx of inflammatory cells in the BAL and the lungs, than do HDM exposed mice. It is possible that this latter difference impacts observed outcomes from both of our experimental models. Specifically, the impact of exogenous LMW and HMW HA treatment in murine asthma may be correlated to the degree of inflammation. In asthmatic mice with more severe inflammation, as in our OVA study, mice appear were more susceptible to exogenous LMW HA, while in our HDM model, which is characterized by diminished inflammation compared to the OVA model, mice are more susceptible to exogenous HMW HA.

The impact of HA in an OVA model may also be overshadowed by the severity of inflammation observed in this model. More specifically, HA signaling is not readily distinguished in OVA-induced asthmatic mice due to the severity of inflammation. The impact of HA signaling is more easily detected in HDM-induced asthmatic mice, because these mice have reduced inflammation.

7.2 HA SIZE IMPACTS AHR IN MURINE ASTHMA BY INDUCTION OF IL-13

Results from this study point to a mechanistic role the impact of HA size on AHR by induction of IL-13. While it has been shown in our study that the impact of HA size may be model-specific, in both OVA and HDM models of asthma, either LMW or HMW HA’s impact on AHR was linked to IL-13 gene expression in the lung. IL-13 is known to
have an important role in the development of AHR (5). Additionally, IL-13 has been shown in animal models of asthma to be potent regulator of bronchospasm and AHR, and has been found to independently induce AHR in naïve mice (6). However, the potential role of HA in the progression of AHR in asthma, via induction of IL-13, has not been previously studied.

Sources of IL-13 include Th2-polarized CD4+ cells, Th1-type cells and non T-cell type inflammatory cells, and ASMCs (7). Stimulation of AECs also results in subsequent production of IL-13 by innate immune cells, such as Th2 lymphocytes, while AECs themselves have been shown to secrete IL-13 as a result of mechanical injury (8, 9). We are particularly interested in the impact of HA size in the airway epithelium, since we conclude that both exogenous LMW and HMW stay primarily within the airways. We hypothesized that HA induces IL-13 as a result of its interaction with AECs. In a preliminary experiment, AECs treated with either LMW or HMW HA for 24 hrs, in an ALI culture model, did not demonstrate increased expression of IL-13, compared to vehicle treated cells. We conclude that HA does not directly induce AECs to express IL-13. However, indirect induction of IL-13 by HA’s interaction with AECs cannot yet be ruled out.

7.3 THE IMPACT OF HA ON AHR AND IL-13 IN VIVO ORIGINATES FROM ITS IMPACT ON THE AIRWAY EPITHELIUM

We have previously discussed that treatment of LMW and HMW HA in AECs results induction of SAA3 and certain Th2 cytokines (Section 6.3). We also suspect that our observed results may arise from HA’s interaction with TLRs in the airway epithelium. Stimulation of AECs has been previously shown to induce factors that result
in IL-13 production (8). In our *in vitro* model, stimulation of AECs with either LMW or HMW HA was sufficient to induce several Th2 cytokines, including G-CSF, GM-CSF, MIP-2, KC, RANTES, and IP-10. However, as previously discussed, HA treatment did not directly induce IL-13 by AECs. We hypothesize that this impact may result in downstream induction of IL-13, which would account for our observed results *in vivo* (Section 8.2).

While SAA is known to associate with HA to induce monocyte adhesion and inflammation, there is no obvious link between HA induction of IL-13 and up-regulation of SAA3 in AECs (11). However, SAA has been shown to worsen airway hyperresponsiveness in ovalbumin (OVA) and house dust mite (HDM) sensitized and challenged mice in response to methacholine (Mch) challenge (107). Thus, the up-regulation of SAA by either LMW or HMW HA may be partially responsible for subsequent induction of AHR in our in vivo models. Although, SAA3 was not found to be significantly elevated in HA treated mice in either OVA or HDM asthmatic mice (data not shown), we cannot determine if there was an early impact of this molecule on the development of AHR without a time-course study.

It is likely that the compounded impact of either LMW or HMW HA on inflammatory cell recruitment by stimulation of SAA3 and Th2 cytokines could reasonably result in a worsened asthmatic phenotype, as observed in our *in vivo* models. AECs have been shown to induce Th2 cytokines in mast cells by production of TSLP and IL-1 after stimulation with certain inflammatory factors (12). Stimulation of AEC, followed by subsequent activation of certain cell types, such as DC and T cell activation, is thought to play a significant role in asthmatic inflammation (12).
7.4 REFERENCES


CHAPTER VIII
FUTURE DIRECTIONS

8.1 TIME COURSE STUDY IN ANIMAL MODELS UTILIZING OVA AND
HOUSE DUST MITE MODELS OF ASTHMA

An acute model of disease was used in our study of either OVA- or HDM-induced asthma in mice. It was determined that HA size impacted AHR in both models, after a 6-day antigen challenge in our OVA model, and a 5-day challenge in our HDM model. HA size was also found to induce IL-13 gene expression, which is known to be closely involved in the development of AHR in asthma (1). It is not known, however, whether treatment with exogenous LMW HA or HMW HA impacts either AHR or IL-13 in the inflammatory response, or if it is subsequently maintained in a chronic model. A time-course can be performed in order to determined whether these characteristics at either early or late time points.

In addition, HA size did not greatly impact inflammatory cells in our OVA model, while exogenous LMW HA was found to diminish eosinophils and neutrophils in our HDM model compared to vehicle treated mice. A time-course study in both models may further clarify the impact of HA size on inflammatory cells. Early time points may help
determine whether or not HA size impacts recruitment of inflammatory cells. Our lab has previously shown that HA co-localizes with inflammatory cells during early inflammation in an OVA model of asthma, which leads us to suspect that either size may play an important role in the early response (2, 3).

8.2 POTENTIAL IMPACT OF ADDITIONAL HA SIZES IN VITRO AND IN VIVO

In both our culture and murine disease models, we utilized one molecular HA size each for exogenous LMW or HMW HA treatment. With the availability of finite molecular weights available for purchase (Lifecore Biomedical, Inc), additional sizes for either the low or high MW may be utilized in future in vivo and in vitro studies. The LMW range for HA is generally accepted to be in the range of 10-500 kDa, whereas the accepted range for HMW HA is usually above 1MDa (4, 5).

In an experiment utilizing an additional HA size in the HMW range, we determined that HMW HA of 1 MDa in size did not result in Th2 cytokine production, when added to AECs (Figure 18). Furthermore, in a preliminary study utilizing an HDM model of asthma, treatment of asthmatic mice with 1 MDa HA was found to have lower neutrophils and eosinophils, although this trend did not reach significance with one experiment (Figure 19). In the same HDM model experiment, 1 MDa HA did not impact AHR in asthmatic mice compared to vehicle treated mice (Figure 20). Further study of this particular size is needed in order to clarify its potential role in murine asthma.
Figure 17: Th2 cytokines/chemokines in AEC-ALI basal supernatants in HA treated cultures. Th2 cytokine/chemokines were measured from basal cell supernatants utilizing MILLIPLEX® MAP mouse cytokine/chemokine 25-plex magnetic bead panel kit (EMD Millipore™). In this figure, HA of 1 MDa (Lifecore Biomedical, Inc) was used to treat AECs. Also included is data from vehicle treated cells. Supernatants from three assays were run in duplicate for all treatment groups. Statistical analysis was analyzed by Student’s t test (p* < 0.05). Error bars shown represent SEM.
A  
**Eosinophils**

![Eosinophils graph]

B  
**Neutrophils**

![Neutrophils graph]
**Figure 18: Eosinophils and neutrophils in HDM asthmatic mice.** Inflammatory cells were isolated from bronchoalveolar lavage (BAL) in naïve and asthmatic mice, directly following methacholine challenge. BAL analyses were performed for one experiment. Cell data includes A) eosinophils and B) neutrophils. Each cell type is plotted against calculated cells per mL. The number of mice per group are as follows; vehicle (DM/DM): 2 mice; 1 MDa HA (DM/DM): 4 mice; 2 MDa HA (DM/DM): 2 mice. Statistical analysis was analyzed by Student’s t test (p* < 0.05). Error bars represent SEM.
Figure 19: Rrs in HDM asthmatic mice. Airway mechanic parameters were generated in naïve mice, 1 MDa HA and 2 MD HA treated asthmatic mice. Rrs, lung constriction is shown plotted against methacholine doses from 0 (baseline) to 100 mg/mL. The number of mice per group are as follows; Naïve: 2 mice; vehicle (DM/DM) 4 mice; 1 MDa HA (DM/DM): 4 mice; 2 MDa (DM/DM): 2 mice. Statistical analysis was analyzed by Student’s t test. No statistical significant is found between experimental groups.
8.3 HA TREATMENT WITH HYALURONIDASE IN VITRO

Results from our AEC-ALI assays demonstrated that LMW and HMW HA elevated certain Th2 cytokines and SAA3. It is important to determine if the response is due to HA, or if there may be some other contaminant. Historically, many published results in past years that details HA signaling have been since found to be primarily due to contaminating agents, primarily with lipopolysaccharide (LPS) (11). A proven method to test for contamination, either by LPS or some other agent, is to treat cells with HA and Streptomyces hyaluronidase, in order to degrade either LMW or HMW HA. Any resulting impact from treatment of cells with HA and hyaluronidase is presumed to be the result of interaction of cells with contaminant.

8.4 HA TREATMENT IN TLR2-/− AND TLR4-/− AECS

We have previously discussed that HA signaling in animal models has been linked with TLR2 and TLR4 (6,12, and 13). For this reason we hypothesized that treatment of AECs with exogenous LMW and HMW HA induced inflammatory mediators in our experiments by interaction with TLRs. In order to test this theory in future experiments, we propose to repeat our experiments on cultured AECs that have been isolated from either TLR2-/− or TLR4-/− mice. In this experiments, we hypothesize that if HA-TLR signaling is required for production of inflammatory factors in AECs, we will observe diminished production of these same inflammatory factors in our knockout cultures.
8.5 REFERENCES

1. Rael E, Lockey R. Interleukin-13 signaling and its role in asthma. WAO Journal. 2011;4:54-64


