The Immunological Response to Respiratory Syncytial Virus Infection has both Age-Related and Extracellular Matrix Provisions

Gerald George Kellar

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Reading Committee:

Steven F. Ziegler, Chair

William A. Altemeier

Ram Savan

Program Authorized to Offer Degree:

Department of Immunology

University of Washington

Abstract

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Gerald George Kellar

Chair of the Supervisory Committee:

Steven F. Ziegler, PhD

Department of Immunology

Early life respiratory syncytial virus (RSV) infection has been linked to the onset of asthma; despite this association knowledge as to the initial viral infection progression is limited while no safe or effective vaccine currently exists. Bronchioalveolar lavage, whole lung cellular isolation, and gene expression analysis were performed on 3-week (juvenile) and 8-week old (adult) RSV-infected C57BL/6 mice to investigate age-related differences in immunologic responses; juvenile mice displayed a sustained myeloid infiltrate (including monocytes and neutrophils) with increased RNA transcript production of *Ccl2*, *Ccl3*, and *Ccl4*, when compared to adult mice, at 72-hours post infection. Juvenile mice demonstrated *αSma* expression (which is indicative of myofibroblast activity), increased hyaluronan deposition in the lung parenchyma (which has been attributed to asthma progression), and a lack of CD64 upregulation on monocytes (which in conjunction with serum amyloid P is responsible for clearing residual hyaluronan (HA) and cellular debris). RSV infection of human airway epithelial cell/monocyte co-cultures (at air-liquid interface) were in concert with the CCL expression while suggesting matrix metalloproteinase-7 (*MMP7*) and *MMP9* as possible matrix modifiers.

Versican is an extracellular matrix component whose overexpression is often associated with exacerbations in asthma; however, RSV infection was shown to depress versican expression in human cell cultures. Therefore, we transferred our infection model into mice lacking versican in the epithelium (utilizing surfactant protein C-Cre (SPC-Cre) as the vehicle) to ascertain the impact; we found that our SPC-Cre versican floxed mice had a sustained myeloid recruitment at 72-hours, the CCL expression to support that recruitment, the expression of factors which would also impact HA metabolism (including hyaluronidase 1 (*Hyal1*) and *Hyal2*, hyaluronan synthase 1 (*Has1*) and *Has2*, and tumor necrosis factor stimulated gene-6), and an increase in the protein and hyaluronan content of the lavage fluid when compared to their RSV infected littermates and PBS controls.

These data suggest that RSV infection supports the sustained influx of myeloid cells, hyaluronan deposition in the lungs of juvenile mice during acute infection and could potentiate a modification in extracellular matrix components facilitating the conditions necessary for airway remodeling and hyperresponsiveness.

Dedication

To my dad, Allan J. Kellar, SR, the best father-figure a boy could have; your work ethic put me where I am today, and your simple humor will always be missed.

Acknowledgements

This adventure called life is full of personal trials, scrapes & bruises, and some peril; I am lucky enough to have been graced with some wise, resilient, and plainly colorful characters along the way who have helped shape me into who and what I am today. To them all, I will be forever grateful.

To all the Soldiers, leaders, and fellow service members who have impacted my military journey, kept me safe in times of strife, and kept me upright & moving forward in times of need, no words can express how I feel. May your road marches be on cool days, your flights be free of ground fire, and your cruises be on calm seas.

To my wife Mary, your enduring support and humor have kept me grounded. We have made a great life together and this accomplishment is as much yours as it is mine. I will always ‘just keep going’ because I love you.

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Chapter 1: Introduction

1.1: Pulmonary function: the essential operation for human life

Take a breath: your diaphragm descends, creating negative internal pressure, external air fills your lungs which expand to the extent your pleural cavity allows, oxygen exchange occurs between the alveoli and the capillaries that are in close physical association. This process is vital to feed the electron transport chain (ETC) responsible for generating the adenosine triphosphate which serves in many anabolic reactions required of regular metabolism, and without it life as we know it would not exist. Modern science still does not completely understand how this oxygen exchange occurs, only that an oxygen molecule associates with one of the four globin subunits of hemoglobin in the erythrocytes that flow through the capillaries in close proximity to the lung tissue. Once in the tissues this oxygen donates an electron to fuel the ETC on the inner membrane of the mitochondria. The resulting electron deficient oxygen molecule is repurposed in carbonic acid, which dissolved in your blood plasma returns to the lung releasing carbon dioxide to the alveoli for exhalation, completing the cycle.

How often do you think about that breath during the day? Land mammals typically rely on the autonomic nervous system to control this function subconsciously; however, seaborne mammals, such as whales and dolphins, must think about every breath. Our bodies contain a running engine that must be powered 24 hours a day. The breathing process is truly taken for granted because Mother Nature's efficient and reliable design serves us well, but what is often overlooked is that unlike the other internal organ systems the lungs are under constant siege. Just as your car motor is under the constant threat of corrosion and scoring damage from improperly compressed fossil fuels each one of our breaths allows a host of raucous pathogens inside of our bodies. With each expansion of our lungs scores of invaders ply the tools Mother Nature has in turn given them to overcome our natural immunological defenses and bring about damaging pathology to our precious lungs. Oxygen exchange is life, it is the most important

process our bodies perform yet it must be simultaneously executed in conjunction with a full-scale biological war.

A pulmonologist and a propulsion scientist in theory have very similar jobs, they both maintain the operation of a complex engine, that is fueled by oxygen, which mixes with a viscous liquid in a series of tubes, bringing about a chemical reaction creating the action potential that drives the forward movement of a greater machine. The major difference between the two is that the pulmonologist must diagnose and repair their engines while the machine is still running, but the similarity between the two is that the major issues in engine operation occur in the smaller tubing where impingements lead to malfunction. Bronchiolitis is an infection of the small airways (bronchioles) in infants and children which is typically caused by a virus (Altman, et al 2018; Sigurs, et al. 2010). This situation poses a major concern in that oxygen exchange capability must be maintained in the face of pathogen clearance, which can damage these small airways leading to inefficient remodeling. Just as our propulsion scientist would never hope to achieve proper propulsion dynamics with a damaged, maladapted fuel exchanging system, so too we cannot hope to properly conduct oxygen exchange with thickened, malfunctioning airways.

1.2: Characteristics of the innate immune response to viral pathogens in lung tissue

Leukocytes and epithelial cells have an array of pathogen receptors capable of sensing then signaling the presence of foreign bodies. The retinoic acid-inducible gene I (RIG-I) family detect viral nucleic acids in the cellular cytosol, after which they associate with the mitochondrial antiviral protein (MAVS) where they begin the initial generation of interferon-α (IFNα). The Toll-like receptors (TLRs) are a family of pathogen detectors with family members located inside cellular vesicles (primarily TLR3, TLR7, and TLR9) which detect nucleic acids and on the cell surface (primarily TLR2 and TLR4) which detect antigens (the F and G protein of RSV). TLR3 expression depends on the IFNα initially generated by RIG-I; TLR3 expression is vital for proper

antiviral immunity in both the epithelial cells and the alveolar macrophages (AM), which are the first immune cells to encounter virus in the lower airways (Liu, et al. 2007; Pribul, et al. 2008). RIG-I and TLR3 both detect double stranded RNA, which can only be generated by actively replicating virus, which makes vaccine manufacture difficult when popular opinion might be fervently against giving children a live virus (Tregoing, et al. 2010).

After the initial pathogen detection leukocytes have to cut out and destroy the infected cells. To gain access to and remove these infected cells myeloid cells have a series of cleavage capable enzymes, such as the matrix metalloproteinases (MMPs), the activity of which degrades extracellular matrix components such as Hyaluronan (HA). HA is a negatively charged, non-sulfated disaccharide polymer comprised of repeating N-acetyl glucosamine and glucuronic acid; it is the simplest of the glycosaminoglycans (GAG), a major component of the extracellular matrix (ECM) remodeling and forms stable tertiary structures in an aqueous environment (Harada, et al. 2007; Jiang, et al. 2011). During infection HA is typically cleaved from the ECM through enzymatic action or the release of ROS as emigrating leukocytes perform chemotaxis towards sites of inflammation to combat the invading pathogens, whom themselves are also capable of HA metabolism, as they occupy and disrupt the tissue scaffolding (Chang, et al. 2012; Jiang, et al. 2011). The impact the low molecular weight HA (LMWHA) generated by both infectious and mechanical sterile injury has on leukocytes and overall homeostasis has been well documented (Jiang, et al. 2011; Rayahin, et al. 2015; Scheibner, et al. 2006; Termeer, et al. 2000). The LMWHA stimulates dendritic cells (DCs), macrophages, and inflammatory monocytes (MO) (through TLR2 and TLR4) to release cytokines such as tumor necrosis factor (TNF), IL-1β, and IL-12 while releasing the chemokines: chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 1 (CXCL1), which are the primary stimulants for MO and polymorphic neutrophils (PMN) chemotaxis, respectively (Scheibner, et al. 2006; Termeer, et al. 2000). Further studies utilizing influenza virus as the infectious agent determined that MO were capable of performing unproductive HA recycling with the removal of

these MO resulting in the quelling of lung inflammation and normal HA kinetics (Bell, et al. 2018; Lin, et al. 2008).

Having to perform the crucial task of oxygen exchange while simultaneously defending against pathological invaders requires an intricate act by a practiced cast of characters. Bud Abbott and Lou Costello were a popular radio and television comedy duo during the 1940s and 50s. Abbott was the “straight man” who set the narrative and drove the story, while Costello would be “spastic” creating a series of gaffs that Abbott would have to address and resolve. There are such a pair of leukocytes who reside in the lung architecture who fit those roles rather well, with AMs playing the ‘Abbott’ to the PMNs ‘Costello.’ AMs maintain alveolar homeostasis tending the epithelial surface phagocytizing free surfactant and cell matrix materials, initiating an immune response through the release of CCLs and IFN-α (Goritzka, et al. 2015). PMNs patrol the pulmonary landscape often having to squeeze through tight capillaries and junctions as they scout for invaders, releasing a host of reactive oxidative species (ROS) and other defensins becoming easily excited when they encounter pathogens, which can damage the lung tissue itself if not kept in moderation (Wang, et al. 2017).

Just as Abbott keeps Costello in check and continuing with the narrative, the AMs must quell the PMN response as they increase IFN-α (which downregulates IL-17 production) and diminish CCL2 production (decreasing MO recruitment) posturing a return to homeostasis (Garofalo, et al. 2001; Moschen, et al. 2008). Without proper action and participation in playing their part of the scene, lung pathology ensues. Neutrophilia endures up to 72-hours in most infectious cycles, after which their recruitment trends back towards pre-infection population as the continued presence of PMNs leads to sepsis and pneumonia (Wang, et al. 2000). PMNs were implicated as a causative agent for unproductive tissue damage leading to the lung pathology observed in the children who perished in the previously attempted RSV vaccine trial (Kim, et al. 1969; Wang, et al. 2000). Both AMs and PMNs perform their functions to maintain

and protect the precious lung tissue while scouring out any pathogens which make it into the lower levels of the lungs.

Antigen presentation is the critical nexus where innate immunity pivots to adaptive immunity through T cell activity and antibody production. CD103+ DCs typically reside in the airway mucosa and adjacent to the capillaries where they can sample for antigens then traffic to draining lymph nodes for presentation to T cells (Jakubzick, et al. 2006). The production of neutralizing antibody is critical to mitigating the pathological effects of any invader, which hinges on proper DC function and trafficking that is seemingly dependent on the AM population in the lung space (Holt, 2000; Jakubzick, et al. 2006). As the quantity of AM decrease in the murine lung the antigen capture and lymph node trafficking increases; therefore, as AM phagocytize virus and begin the initial immune response they should then undergo apoptosis removing pathogen and the immunosuppressive signaling for proper adaptive immunity to occur (Jakubzick, et al. 2006.).

1.3: Respiratory syncytial virus; a ubiquitous yet nebulous pathogen

Respiratory Syncytial Virus (RSV) is a negative, single-stranded RNA virus of the pneumoviridae family with two antigenic subgroups, the A and B strains (the A-strain is the virulent form with Line 19 and A2 as the viruses most often used in research, the B strain produces symptoms equivalent to the common cold in humans) (Lukas, et al. 2010). RSV infects 37-million children worldwide annually, causing 59,000+ in-hospital deaths across the globe in children under the age of five, with RSV related bronchiolitis demonstrating the greatest risk ratio for allergic sensitization; by the age of 2 most people have antibodies to RSV with recently increasing morbidity seen in adult populations, especially the elderly and immunocompromised (Guvenel, et al. 2019; Kulkarni, et al. 2016; Sigurs, et al. 2010). Early life RSV infection has been linked to the progression to and onset of asthma. A Swedish patient record-based study conducted by Sigurs, et al. (2010) reported that 47% of the hospital

admissions due to RSV related bronchiolitis was associated with a later life diagnosis of asthma. Asthma is a chronic pulmonary disorder (CPD) characterized by airflow restriction and inflammation due to ECM remodeling that affects up to 10% of the population in the developed world, with decreasing worldwide air quality due to manmade and natural circumstances CPDs are becoming an ever-increasing healthcare burden (Becker, 2006; Cheng, et al. 2013; Reeves, et al. 2016; Sigurs, et al. 2010).

There is no RSV vaccine currently available. A previous trial conducted by Johns Hopkins University (Baltimore, MD) in 1969 utilized formalin attenuated virus; however, this resulted in two infant fatalities and over 30 children hospitalized due to vaccine enhanced disease when the children were naturally exposed to the virus (Kim, et al. 1969). Production of an RSV vaccine is difficult due to the immunological imbalance viral infection produces which is characterized by a mixed Th1/Th2 phenotype, a lapse in memory cell creation, DC production of interleukin (IL)-23 upon restimulation or allergen challenge, and the inability to generate a complete immune response to attenuated virus (Kallal, et al. 2011; Lukas, et al. 2010; Mukherjee, et al. 2010). Due to the increasing RSV related threat The World Health Organization has performed a pilot surveillance project, in line with their flu program, further placing an RSV vaccine on their priorities list (https://www.who.int/influenza/rsv/en/). Understanding the initial juvenile immune response is paramount to the manufacture of such a vaccine. Research needs to ascertain what leukocytes are involved, how they impact the processes leading to lung remodeling, which is critical to understanding how such a vaccine would provide safe and lasting RSV immunity (Garofalo, et al. 2000; Lee, et al. 2007; Morrison, et al. 2008; Tregoing, et al. 2010).

1.4: Respiratory Syncytial Virus infection and its impact on the human lung

Human based RSV studies utilize small doses of RSV (1×10^4 PFUs) given to willing volunteers to study the immunological qualities of infection; however, these volunteers are fully developed

adult's whose lung architecture has been established (Guvenel, et al. 2019). Animal based RSV infectious studies typically use higher doses of virus (1 x 10^6 PFUs or higher) generating more of a type 2 immune response with severe infection; however, in a recent study it was demonstrated that the typically RSV infected child exudes a 1 meter infectious perimeter around their person containing a viral concentration of 1-2x10^5 PFUs of virus (Kulkarni, et al. 2016).

What is also overlooked is RSV's wide range of normal immune evasion and if that evasion differs depending on the age of the organism. The non-structural protein 1 (NS1) and NS2 of RSV are the main pieces of RSV's immunosuppressive arsenal. NS1 attempts to block MAVS displacing RIG-I placement, while NS2 associates with RIG-I directly with both attempting to thwart the initial production of IFNα necessary for TLR3 expression leading to proper antiviral function (Goritzka, et al. 2015; Lindell, et al. 2011). The P protein of RSV has a 52% homology to the death domain of caspace-8, blocking its ability to serve in apoptosis initiation, while the F protein associates with the P53 complex restricting its ability to initiate apoptosis (RSV requires 18 hours to complete its replication cycle) (Schmidt and Vargas 2017; Sun and Lopez 2017).

In addition to immune evasion RSV infection elicits a sliding immunological response with differing observations including a mixed Th1 and Th2 response, a Th17 response, or a completely skewed type 2 immune response (Becker, 2006; Lee, et al. 2007; Lukacs, et al. 2010; Mukherjee, et al. 2011). DC production of IL-23 after secondary exposure to RSV makes the production of a viable vaccine difficult unless the production of IL-17 after initial RSV infection is considered (Lukacs, et al. 2010; Moschen, et al. 2008). A recent human based RSV infection trial pointed to innate lymphoid cell 2 (ILC2) production of IL-5 and IL-13 and not Th2 cells as the driver of type 2 immunity (Guvenel, et al. 2019). This sheds some light on RSVs immunological mystery giving scientists another research target while furthering the conundrum as ILC2s lack a T cell receptor and tack their reaction cues from local cell signals (Yu, et al. 2014).

The alteration of ECM kinetics during infectious processes has been well studied (Bell, et al. 2018; Chang, et al. 2014). The viral infection of epithelial cells and fibroblasts results in the decreased degradation & metabolism and increased expression, respectively, of HA in the two cell types (unpublished observation of S. Reeves). Infected animals generate increased TNF and IFN-β to insulate fibroblasts from viral infection while fibroblasts always express TLR3 on their surface, not requiring the activity of RIG-I to initiate viral sensing (Bartee, et al. 2009). The protection of fibroblasts is critical in that infected cells generate increased strands of HA, which absorb water, leading to pneumonia and leukocyte sequestration, which brings on the potential of airway remodeling in the absence of HA metabolism. MO are very plastic in the lung space and have been noted to contribute both tissue damage and increased ECM component production (Bell, et al. 2018; Chang, et al. 2014; Lin, et al. 2008; Soukup and Becker, 2003). An increase in myeloid recruitment during RSV infection would certainly result in that noted tissue destruction and an ECM kinetics imbalance enabling airway remodeling. (Soukup and Becker, 2003; Wang and Forsyth, 2000).

1.5: Outstanding questions

Current asthma related research focuses on the immunological posture of mice that are 6-to-8 weeks old (typically in the less inflammatory BALB/C line) (Han, et al. 2012; Kallal, et al. 2010; Lee, et al. 2012; Mukherjee, et al. 2011). The ECM components HA and versican have been well studied in regard to immunological stimulus, lung pathology, and their participation in asthma (Gill, et al. 2010; Kang, et al. 2017; Wight, et al. 2017). However, there are strikingly few studies that take into account the altered properties of the ECM during initial RSV infection, particularly in younger mice, and how the initial immunological response may impact the well characterized remodeling process necessary for the progression to asthma, particularly where HA is concerned. The use of older animals could be overlooking the age appropriate responses to early life RSV infection characteristic of a 1-3 year old human, which is better observed in 3-

week old mice due to the parallels in human and mouse lung development at these time points (Guvenel, et al. 2019; Faggian, et al. 2007; Schittny 2017; Woik and Kroll 2015). The deficiencies in the juvenile immune system are not completely understood; we sought to compare the juvenile to adult mice to glean any immunological “threads” to pull in order to decipher how the RSV infectious progression could potentiate airway remodeling priming the lung architecture for the induction of asthma when later challenged by another viral infection (namely rhinovirus) or environmental antigen (house dust mite or cockroach antigen) (Han, et al. 2012).

The ECM component versican has been specifically studied for its implication in virally induced inflammation and with asthmatic exacerbations (Andersson-Sjoland, et al. 2015; Evanko, et al. 2009; Kang, et al. 2017; Reeves, et al. 2016). Versican’s ability to sequester chemokines and MO during viral infections laid over the differing activity of the isoforms makes it a prime candidate as a therapeutic target (Potter-Prego, et al. 2010). We sought to remove versican from the epithelium in mice to study the RSV infectious progression in the absence of this ECM component. We wanted to test the hypothesis that the lack of versican in the epithelium would lead to a diminished immune response due to the body of previous literature implicating its negative effects during viral infections (Andersson-Sjoland, et al. 2015; Kang, et al. 2017; Reeves, et al. 2016). Versican is a necessary component for tissue repair as it stiffens the ECM influencing cellular growth down the newly laid matrix; however, is that activity context and situationally dependent (Wight, et al. 2014)? The ECM is a fluid environment, how it impacts immune cell phenotype and activity has been studied; however, due to the nature of infectious progression it is an unpredictable environment and impossible to keep track of the moment-to-moment cellular interactions with the tools currently available.

Chapter 2: Age-related differences to Respiratory Syncytial Virus infection

2.1: Introduction: The disparity of immunological responses during Respiratory Syncytial Virus infection

With the majority of scientific research focused on the mitigation and treatment of lung hyperresponsiveness there is a dearth of knowledge on what occurs during initial RSV infection, how that infection lends to the lung remodeling characteristic of the progression towards and onset of asthma, and what observable factors contribute to that remodeling. Since RSV infection has minimal pathological impact on the immunocompetent, we hypothesized that the initial immune profile of juvenile organisms during the acute phases of RSV infection accord intermediaries (myeloid cells) which potentiate ECM disruption (increased HA presence) establishing the foundation on which airway hyperresponsiveness is built. Myeloid recruitment is characteristic of asthma exacerbations with eosinophils (EO) and/or PMNs being recruited to the respiratory space, releasing their granular contents, driving airway inflammation causing the wheezing and shortness of breath characteristic of asthma (Lukas, et al. 2010; Mukherjee, et al. 2011; Trivedi and Llyod 2007). From this premise we sought to establish an immunological baseline during acute RSV infection with our eyes first turned to myeloid cells. The more inflammatory C57BL/6J mice were utilized instead of the less inflammatory BALB/C (which are more common in RSV research) comparing the initial myeloid profile of 3-week old (whose lung architecture resembles a 2 year old human (referred to as juvenile mice)) versus an 8 week old (whose lung architecture resembles an adult human (referred to as adult mice)) to glean what observable differences we could find (Schittny, 2017). We found that juvenile mice have enduring myeloid recruitment of MO and PMN, with an increase in the EO presence, at 72-hours post RSV infection, with the molecular expression of *Ccl2, Ccl3,* and *Ccl4* to support the recruitment of the myeloid populations.

Once we had established this baseline, found data which supported our initial hypothesis (or reflexed its revision), we pivoted to ascertaining what immunological factors could support

this myeloid recruitment, analyzed if RSV was capable of utilizing its series of immunological evasion tricks, and what the lung histology presented. Previous attempts at generating an RSV vaccine resulted in vaccine enhanced disease which was characterized by PMN infiltration and lung tissue damage (Kim, et al. 1969). Current attempts at RSV vaccine production hinge on myeloid recruitment, mitigation of lung pathology, and proper antigen presentation leading to neutralizing antibody production (Kallal, et al. 2010, Lindell, et al. 2011). In addition to the immune implications viral pathology includes ECM disruption, which itself furthers an immune response and complicates scientific analysis (Bollyky, et al. 2009; Scheibner, et al. 2006; Termeer, et al. 2000). We sought to examine the influence RSV infection had on the ECM, how that could modify the immune response, and what molecular ramifications infection presented. Once we complied the data we found that juvenile mice had increased myeloid recruitment, the chemokines to support that recruitment, an increased HA content in the lung parenchyma (which would further the immune response), and indicators as to why they had that increased HA signature (a lack of CD64 on MO to scavenge free HA and increased myofibroblast transition) (Hinz, et al. 2012; Lu, et al. 2008).

With MO as a continuing thread pulled from both the literature and our findings we utilized a human cell-based co-culture system to interrogate those cells to see what possible clues we could glean in furthering our knowledge of RSV pathology (Lin, et al. 2008; Soukup and Becker, 2003). We found that MO placed upon infected human cell cultures generated molecular transcripts for the same CCLs as those seen in the infected juvenile mice, matrix modifiers capable of disrupting ECM kinetics (*MMP7* and *MMP9*), and themselves were capable of generating RNA transcripts of ECM components (namely, *HAS3* and *TSG6*).

Taken as a whole, RSV infection facilitates increased myeloid recruitment with ECM modification and the potential for airway remodeling. These data observed in our murine infections translates into our human cell-based infection model lending to a greater understanding of how RSV drives lung pathology.

2.2: Results

2.2a: Initial immune profile of C57BL/6 mice during Respiratory Syncytial Virus infection

We determined the profile of myeloid cell recruitment into the airways and lung tissue following initial RSV infection of juvenile and adult mice. Both age groups displayed an acute increase in total leukocytes in the BAL 18-hours post infection; however, despite the significantly greater initial cellular presence in juveniles all test groups display a similar cellular presence by 72-hours (Fig. 2.1A). The classic neutrophilia (Fig. 2.1B) and MO recruitment (Fig. 2.1C) indicative of viral infection was observed in the BALF of both age groups at 18-hours post infection with a trend towards resolution of PMN and MO recruitment by 72-hours post infection (data not shown). Both age groups demonstrate leukocyte recruitment into the lung tissue itself at 18-hours which resolved by 72-hours in the adults, while juvenile leukocyte recruitment totals increase at the 72-hour mark (Fig. 2.1D). PMN and MO counts were similarly elevated in both infected age groups at 18-hours (data not shown); however, neutrophilia and MO recruitment endured in the juvenile lung at 72-hours (Fig. 2.1E and Fig. 2.1F, respectively) which we surmised was due to greater recruitment and not increased longevity (O'Connell, et al. 2015).

Myeloid recruitment has been linked to lung pathology in the face of viral infection (Lin, et al. 2008; Wang, et al. 2017). Annexin V staining was incorporated to determine if the observed differences between the juvenile and adult MO population was due to a lack of apoptotic activity; however, we found no demonstrated deficiency in that function for the juvenile MO population (Fig. 2.2A). As the juvenile mice demonstrated a continued overall leukocyte recruitment into the lung tissue at 72-hours we examined the expression of chemokines that recruit these cells. Quantitative PCR was performed on the RNA extracted from whole lung isolates of each age group; we found increased mRNA transcript expression for the classic myeloid chemokines *Ccl2*, *Ccl3*, and *Ccl4* in the juvenile mice at 72-hours post infection (Fig. 2.2B, Fig. 2.2C, and Fig. 2.2D, respectively).

Given the sustained myeloid cell infiltration in the lungs of RSV-infected juvenile mice we next examined whether RSV's wide range of normal immune evasion was involved (Goritzka, et al. 2015; Lindell, et al. 2011). We examined the RNA expression of the antiviral genes *Tlr3* and *Mx2* (which is downstream of IFNAR signaling), with no deficiency seen in the expression of *Tlr3* (data not shown) or *Mx2* in either age group (Fig. 2.2E), pointing towards RSV attempted association with MAVS failing to block the initial antiviral signaling through the generation of IFNα (Schmidt and Varga 2017; Sun and Lopez 2017). Sperm whales can hold their breath for extended periods due to the negative charge of the myoglobin in their tissues. AM are the first leukocytes to encounter virus in the pulmonary airway whereupon they initiate the immune response (through the generation of CCL2, CCL3, and CCL4). We observed no difference between the two age groups in the BALF or lung tissue at 18-hours (data not shown); however, the adult mice demonstrated a significant decrease in cellular residence in the lung tissue at 72-hours (Fig. 2.2F). These data suggested that this continued CCL expression most likely contributed to the continued myeloid recruitment the younger mice demonstrated at 72-hours post infection (Schmidt and Varga 2017; Sun and Lopez 2017).

2.2b: Viral infection potentiates a pathological immune response and airway remodeling

After engaging RSV, the AM would initiate the immune response after which the seemingly infected cells would undergo apoptosis; therefore, we incorporated Annexin V staining to determine if this delta in the infected AM cell populations was due to a deficiency in apoptotic activity. The juvenile mice demonstrated a significant difference compared to their age-related PBS controls for the apoptotic cell counts; however, that count was significantly less than the RSV infected adult mice (Fig. 2.3A), suggesting that any juvenile AM resilience is possibly due to RSV driven apoptotic evasion and not a cellular deficiency (Goritzka, et al. 2015; Steinwede, et al. 2012). The resilient AM presence would contribute to the continued myeloid recruitment seen in the juvenile mice. AMs have also been found to influence DC trafficking to

lymph nodes (Holt, 2000; Jakubzick, et al. 2006). RSV infected juvenile mice displayed a consistent CD103+DC compared to their respective PBS controls at 72 h post infection where the RSV infected adult mice have a significant decrease in CD103+ DCs presence, indicating a possible restriction in DC trafficking in the juvenile mice (Fig 2.3B).

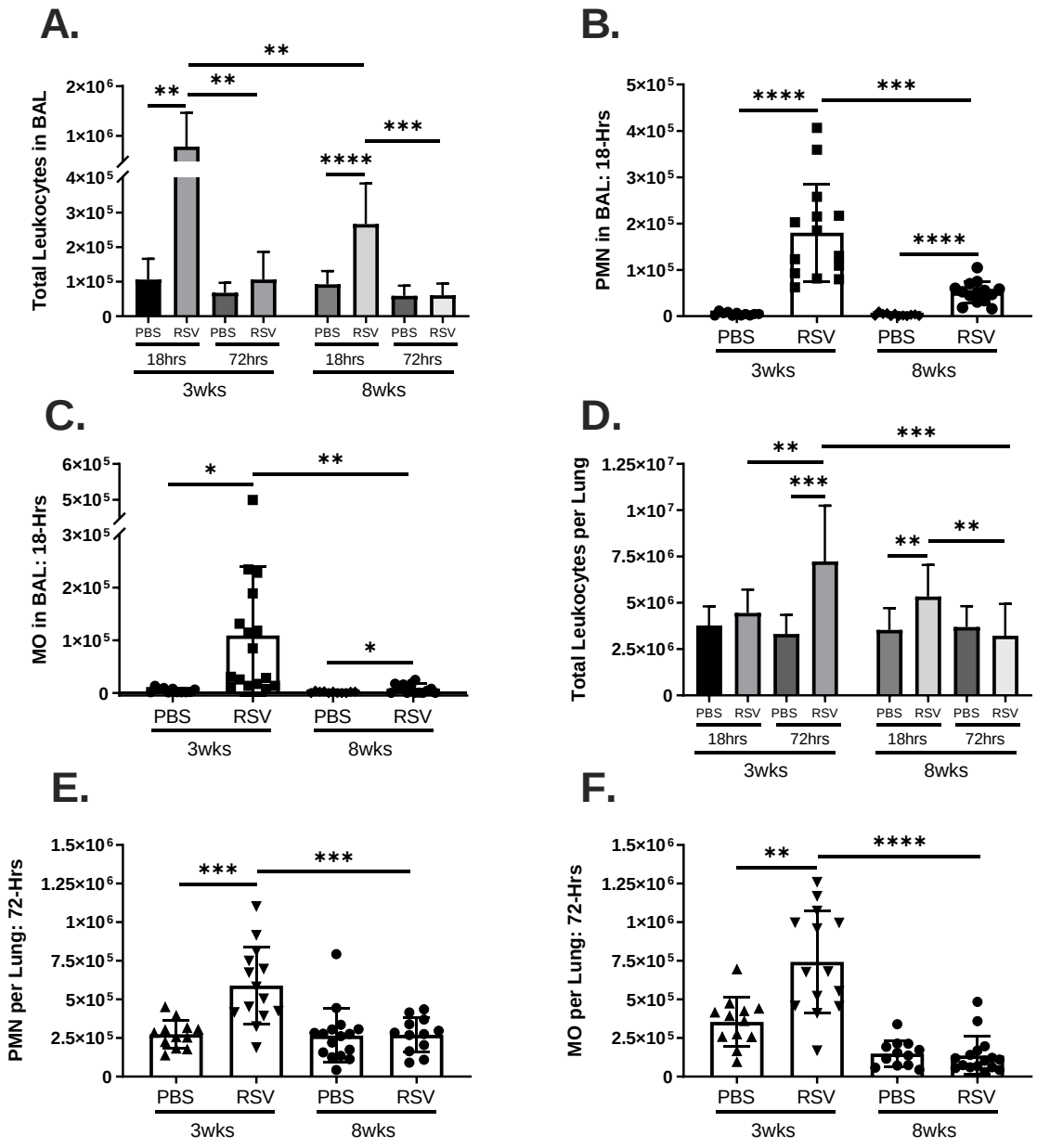

Figure 2.1: Juvenile mice have increased leukocyte recruitment in the BALF and Lung.

BAL was performed on RSV-infected and PBS control 3- and 8-week old mice at 18-hours and

72-hours with: (A) total leukocytes; (B) PMNs at 18-hours; and (C) MO at 18-hours. The right lung of each mouse was removed, minced, collagenase digested, stained with FACS antibodies, and then examined for: (A) total leukocytes; (B) PMNs at 72-hours; and (C) MO at 72-hours. Recruitment was measured by flow cytometry. (n = 11-16 mice per group combined from two separate experiments. Statistical comparisons depict the results of unpaired t tests with significance defined as: * indicated p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.)

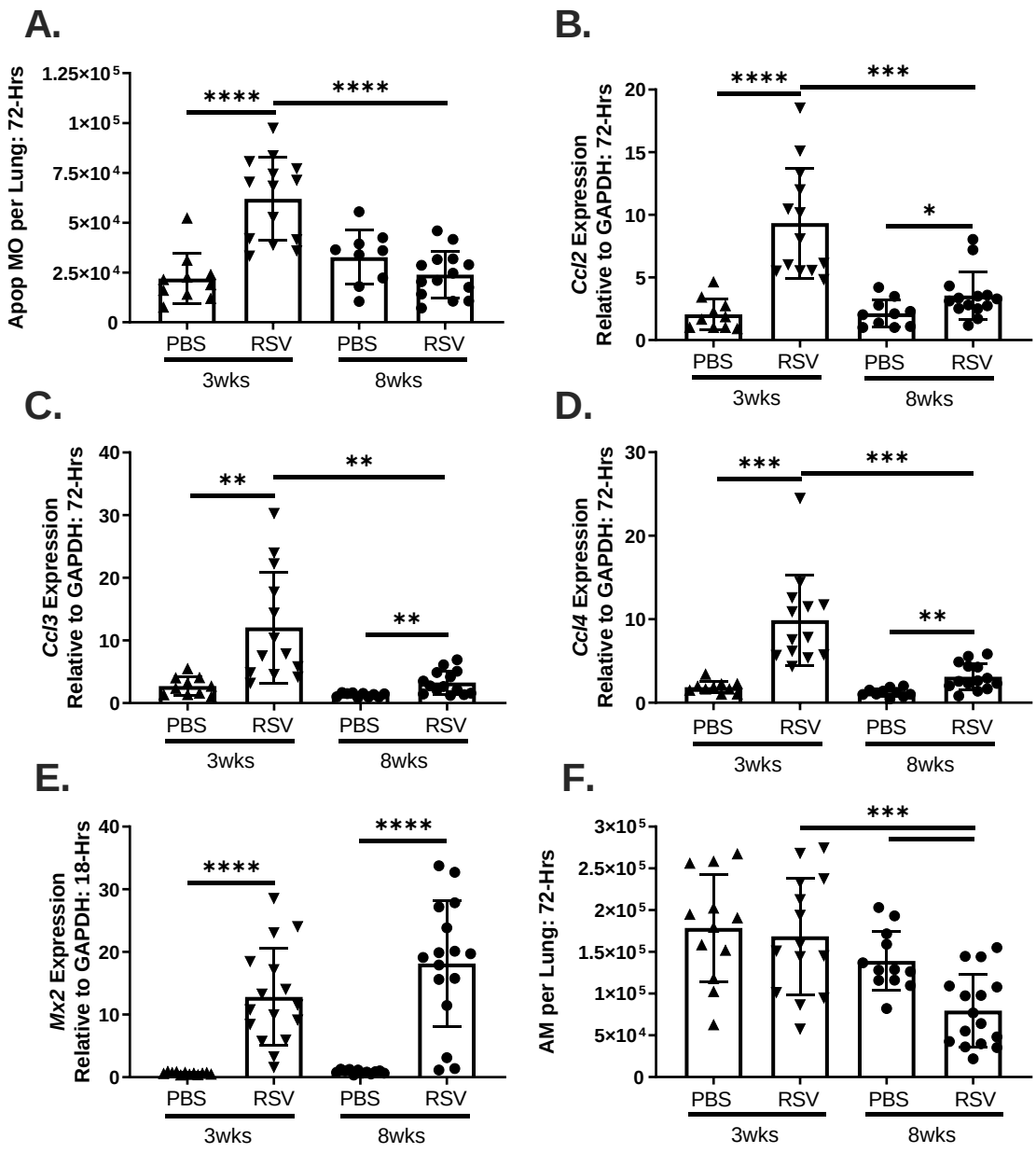

Figure 2.2: Factors determining an increased myeloid presence in juvenile mouse lungs at 72-hours post infection. Annexin V cell preparation & staining was incorporated to determine the apoptotic status of: **(A)** MO at 72-hours post infection. Gene expression of: **(B)** *Ccl2*; **(C)** *Ccl3*; **(D)** and *Ccl4* at 72-hours post infection and gene expression of: **(E)** *Mx2* at 18-hours post infection. Lungs were prepared as in Figure 2.1 then assessed via flow cytometry for: **(F)** AMs at 72-hours. (n = 10-16 per test group combined from two separate experiments. Statistical comparisons depict the results of unpaired t tests with significance defined as: * indicated $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.)

One aspect of the inflammatory response to respiratory virus infection is a remodeling of the extracellular matrix (ECM). Myeloid recruitment contributes to lung pathology not only through the direct modification of the ECM, but through an inability to properly scavenge debris naturally generated by pathogen clearance. MO in the lungs of RSV-infected juvenile mice showed reduced expression of CD64 at 18-hours post infection (Fig. 2.3C) which is a trend that continues at 72-hours post infection (Fig. 2.3D). CD64 is the Fc receptor that recognizes the liver-derived pentraxin serum amyloid-P (SAP), which coats cellular debris and free HA to be phagocytized by the MOs. In the absence of CD64 reduced debris “clean-up” occurs, leading to increased HA accumulation in the lung spaces (Cox, et al. 2014; Lu, et al. 2008; Mold, et al. 2001).

Recent studies have shown that the architecture and composition of a 3-week old mouse lung typically resembles a 2-year old human lung where an 8-week old mouse lung typically resembles that of an adult human lung (Faggian, et al. 2007; Schittny, et al. 2017; Woik, et al. 2015). The PBS treated adult B6 mice display similar HA staining when compared to the juvenile mice (Figs. 2.4A and 2.4B); however, the RSV treated juvenile mice show increased HA deposition in the parenchyma (Fig. 2.4C) when compared to the adult mice (Fig. 2.4D). Increased parenchymal HA could further contribute to continuing unproductive inflammation;

lower molecular weight HA fragments stimulate DCs further driving myeloid recruitment, effecting more ECM modification, feeding a cycle which could perpetuate the remodeling that primes the lung landscape for hyperresponsiveness (Jiang, et al. 2011; Rayahin, et al. 2015; Scheibner, et al. 2006; Termeer, et al. 2000).

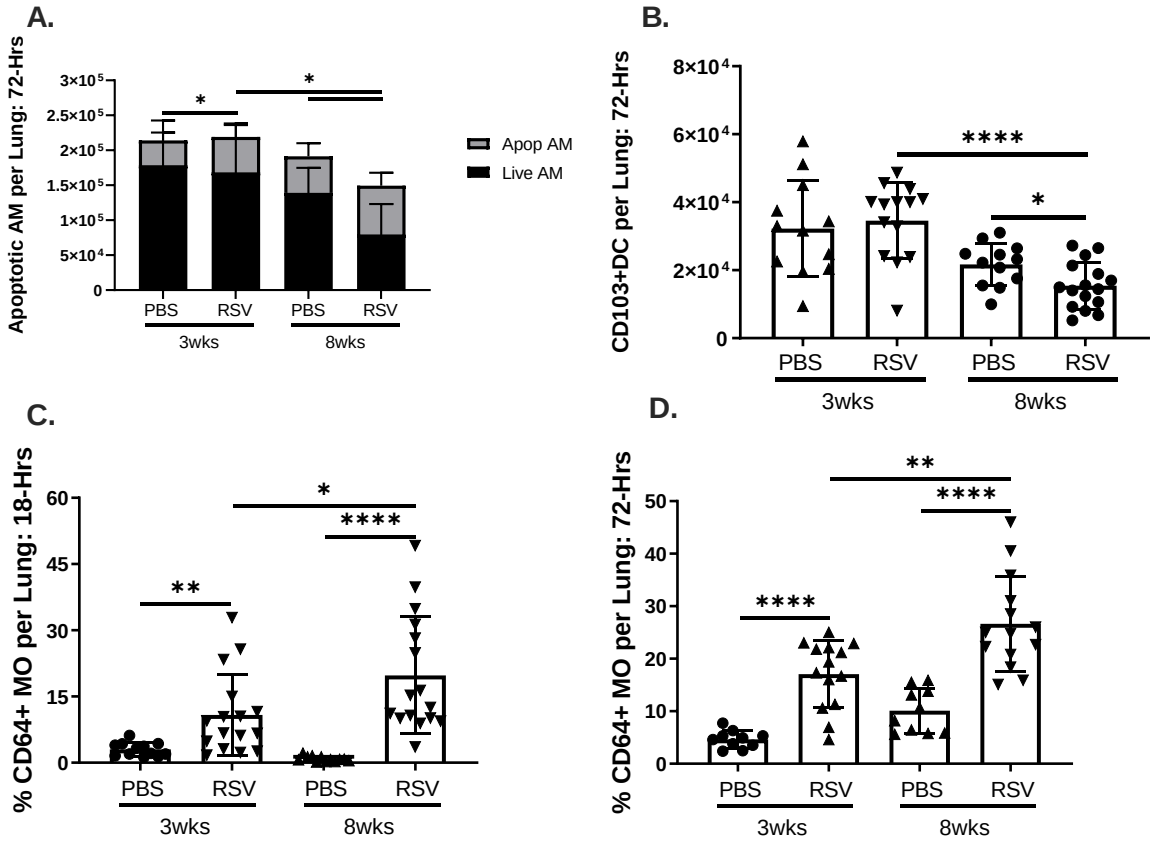

Figure 2.3: Lungs from RSV-infected juvenile mice have determinants leading to increased ECM components. Annexin V staining was incorporated to determine apoptosis by flow cytometry: **(A)** Annexin V+ (grey) and Annexin V- (black) AMs for each test group (n = 10-14 mice per group combined from two separate experiments). **(B)** CD103+DC presence in lung tissue at 72-hours post infection. **(C)** Frequency of CD64+ MO at 18-hours post infection and **(D)** 72-hours post-infection in juvenile and adult lungs. (n = 12-16 per test group combined from two separate experiments. Statistical comparisons depict the results of unpaired t tests with significance defined as: * indicated $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

To analyze differences in parenchymal HA content between the juvenile and adult mice the ImageJ software package was utilized to compare the pixel area intensity area amongst the age groups. No difference was observed when the airway staining was compared (Fig. 2.4E); however, the juvenile mice demonstrated a statistically significant increase in parenchymal staining (Fig. 2.4F). Infected lungs from juvenile mice also show a significant increase in *αSma* expression at 72-hours, indicating myofibroblast transition (Fig. 2.4G) (Hinz, et al. 2012). This increased myofibroblast transition can further increase deposition of HA and other ECM components, such as versican, which has been implicated in asthma (Gill, et al. 2010; Wight, et al. 2014).

2.2c: Infected human cell culture systems allude relevant monocyte derived factors

Due to their plasticity and elevated recruitment in the juvenile mice in our mouse model we further investigated the role of MO activity during RSV infection examining the response to RSV infection in an ex vivo human airway epithelial (HAE) and human lung fibroblast (HLF) cell model system co-cultured with a human monocyte cell line (U937). The cultures were infected for 24 hours before the U937 cells were added simulating a cell that had been recruited to the already infected lungscape. The U937 cells were then harvested 24 or 72 hours later with expression of chemokine and matrix remodeling genes later assessed. U937 cells displayed elevated expression of *CCL2*, *CCL3*, and *CCL4* (Figs. 2.5A, 2.5B, and 2.5C, respectively) which would contribute to myeloid recruitment into the infected space, consistent with what was seen in the lungs of infected juvenile mice. U937 cells also showed virus-driven expression of matrix metalloproteinase-7 (*MMP7*) and *MMP9* (Figs. 2.6A and 2.6B, respectively), with *MMP7* expression increasing at 72-hours and *MMP9* production remaining constant; both of these agents have been implicated in ECM pathology and increased myeloid recruitment (Kang, et al. 2018; Trivedi, et al. 2007).

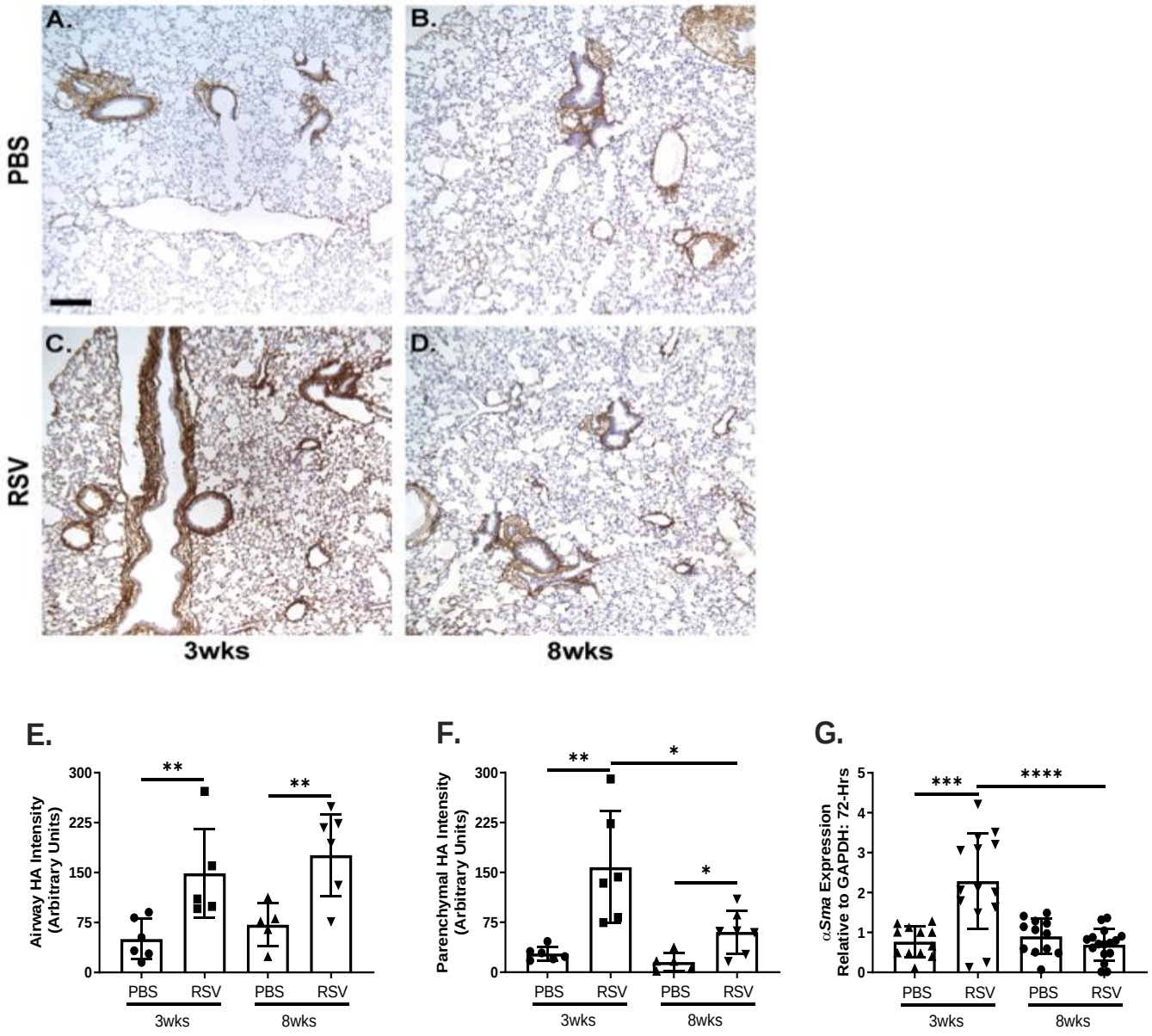

Figure 2.4: RSV infected juvenile mice display increased HA deposition in the lung parenchyma. Formalin-fixed paraffin embedded (FFPE) lungs from juvenile and adult mice were sectioned and stained for HA deposition. Sections from PBS-treated (**A**) juvenile or (**B**) adult lungs. Sections from (**C**) juvenile or (**D**) adult RSV-infected lungs. Scale bar in Fig. 2.4A represents 100μM. ImageJ software was utilized to quantify the pixilation of the HA stained images with **(E)** depicting airway staining and **(F)** parenchymal staining. (n= 5-7 images per test group.) (**G**) αSma gene expression in lungs of juvenile and adult lungs 72 hours post infection.

(n = 10-16 per test group combined from two separate experiments.) Statistical comparisons depict the results of paired t tests with significance defined as: * indicated $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

In addition to making RNA transcripts for myeloid recruitment factors and direct matrix modifiers, human U937 MO generate transcripts for matrix components that would directly impact continuing inflammation and the status of the ECM. Expression of tumor necrosis factor stimulated gene-6 (*TSG6*) was elevated at 24 hours from U937 cells (Fig. 2.6C). TSG-6 is an HA-binding protein with the capability to sequester chemokines, cross-link the increased HA content, and initiate PMN apoptosis while filling the space that would otherwise include the more inflammatory chondroitin sulfate versican; however, production of TSG-6 returns to the levels expressed by the PBS controls at 72-hours, which could contribute to continuing inflammation (Swaidani, et al. 2013). The MO initially generate transcripts for hyaluronan synthase 3 (HAS3) (Fig. 2.6D), which is the HAS capable of generating a lower molecular weight HA product, which in itself could also antagonize myeloid cells, further contributing to an inflammatory state (Jiang, et al. 2011; Rayahin, et al. 2015; Scheibner, et al. 2006; Termeer, et al. 2000). To address the increase in HA content the MO generate hyaluronidase 1 (HYAL1) (Fig. 2.6E), which is responsible for the cell's internal metabolism of phagocytized HA (Jiang, et al. 2011). The increased expression of *HYAL1* could be in response to the increased HA generated in the RSV infected co-cultures. Neither the PBS nor RSV inoculated cultures experienced a significant increase in HA production at 24 hours; however, both the PBS and RSV treated cultures had an increase in production at the 72 hours, with the RSV treated being significantly greater than the PBS cultures. (Fig. 2.6F).

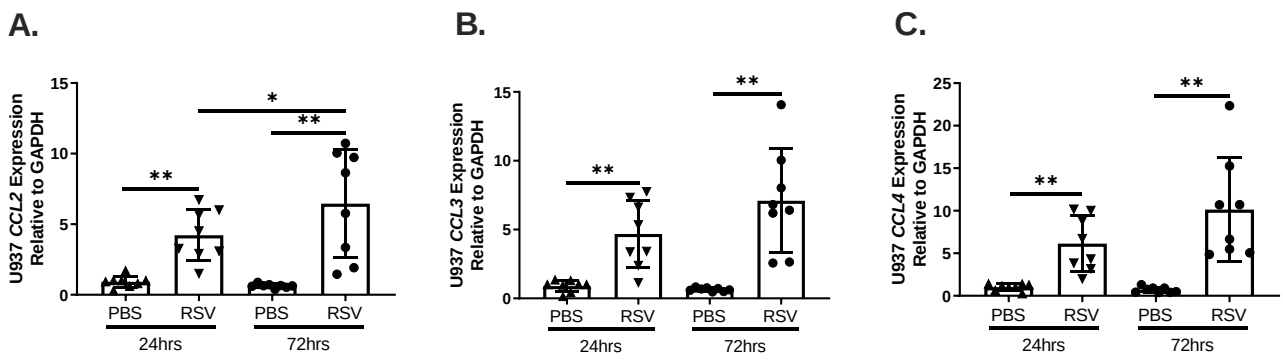

Figure 2.5: U937 MO co-cultured with differentiated primary human airway epithelial cells (AECs) and human lung fibroblasts (HLFs) express *CCL2*, *CCL3*, and *CCL4*. U937 MOs were added to the HLF co-culture 24-hours after RSV infection of human epithelium in an Ex Vivo human co-culture system, and then assayed 24 or 72 hours later for expression of: **(A)** *CCL2* **(B)** *CCL3*; and **(C)** *CCL4*. (n = 8 cultures per test group combined from two separate experiments.) Statistical comparisons depict the results of paired t tests with significance defined as: * indicated p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001)

2.3: Discussion

To my knowledge this is the first study examining the age-related immune response in mice utilizing an environmentally relevant dose of RSV, then using those initial findings as an examination template to interrogate a human cell-based ex vivo lung model (using airway epithelial cells from children) showing that increased myeloid recruitment potentiates ECM remodeling, supporting the concept that RSV infection may contribute to the development of asthma.

We have demonstrated that juvenile mice exhibit a distinct myeloid recruitment pattern in response to RSV infection as compared to adult mice, including exacerbating factors which support this recruitment, with a resultant impact on lung ECM which could contribute to remodeling. In an ex vivo HAE – HLF – U937 MO co-culture model system we then confirmed

the presence of the chemokines responsible for myeloid recruitment and certain ECM impactors generated by MOs which may drive airway remodeling. Therefore, our age appropriate mice utilizing a lower, more environmentally relevant dose of RSV provides a measuring stick against which to compare the juvenile (in 3-week old B6 mice) with the immune response of adults (in 8-week old B6 mice) to elucidate why a virus that is so globally confluent has such an impact in early childhood.

The sustained myeloid response and CCL gene expression observed in the juvenile mouse lung at 72-hours should be addressed to better enable the production of a viable vaccine; enduring neutrophilia has been implicated in the vaccine enhanced disease associated with the previously released RSV vaccine and MO are noted to exacerbate lung pathology during viral infection (Kim, et al. 1969; Lin, et al. 2008; Mukherjee, et al. 2011). Both infected age groups had an initial recruitment into the airways; however, by 72-hours that recruitment had resolved as it appears the complications caused by RSV infection transitions to the lung tissue itself. The enduring CCL production in the juvenile mice could be attributed to the persistent AM and increased MO presence in the infected mice compared to the adults (Goritzka, et al. 2015). The immune evasion of RSV has been linked to the blockage of initial RIG-I activity (disrupting IFNα production) and subsequent IFNAR signaling; however, our B6 mice do not appear to have overall difficulty as demonstrated by the upregulation of *Mx2*; however, there was an apparent lack of AM apoptosis in the juvenile mice which could be caused by the cell populations RSV infected, making it a possible viral reservoir to infect the parenchymal tissue further driving unproductive pathology (Goritzka, et al. 2015; Reeves, et al. 2018; Schmidt and Varga 2017; Sun and Lopez 2017). A second possible explanation for the AM endurance in the juvenile mice is that nature has performed a calculated risk and found it more beneficial to allow the AM survival where they would act to quell any overzealous epithelial reactions while serving to phagocytize the free SAP covered HA & cellular debris the CD64

lacking MO cannot (AM have a high density of CD64 on their surface to scavenge free particles maintaining alveolar homeostasis) (Mold, et al. 2001).

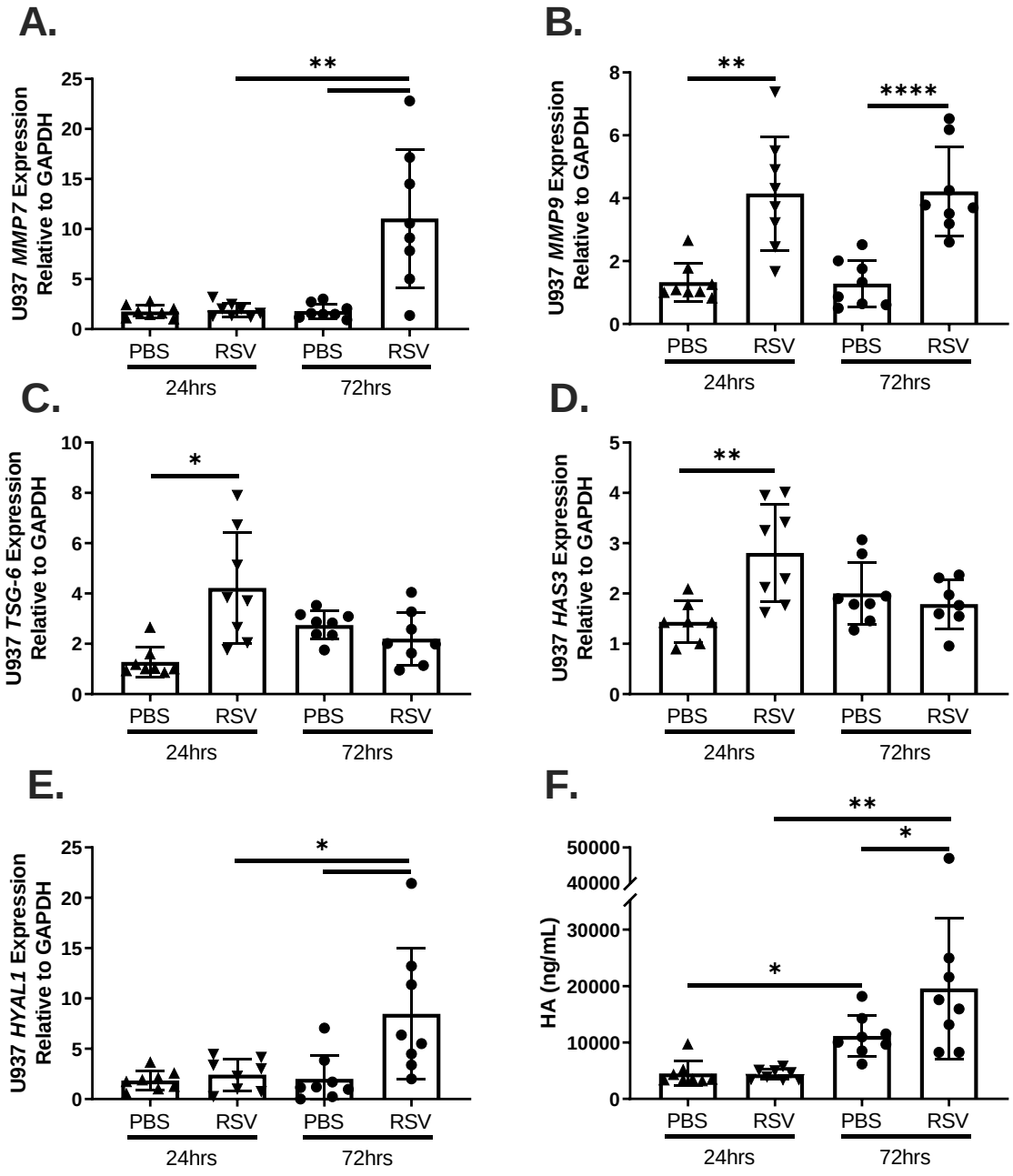

Figure 2.6: U937 MO in RSV infected co-cultures express EMC-modifying genes. U937 cells were co-cultured with human AECs and HLFs as described and analyzed at 24 and 72-hours for expression of: **(A)** *MMP7*; **(B)** *MMP9*; **(C)** *TSG-6*; **(D)** *HAS3*; and **(E)** *HYAL1*. The HA

content of the culture supernatants was measured **(F)** HA content in the culture media was analyzed through an Enzyme-Linked Immunosorbent Assay (ELISA) from R&D Systems. (n = 8 cultures per test group combined from two separate experiments.) Statistical comparisons depict the results of paired t tests with significance defined as: * indicated $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

Free low molecular weight HA has consistently been linked to DC activation, inflammation, and further myeloid recruitment (Rayahin, et al. 2015; Scheibner, et al. 2006). CD64 activity through SAP is the natural process by which free HA and other cellular debris is eliminated that would otherwise be contributing to inflammation causing further lung pathology; is the apparent lack of MO function through CD64 in the younger mice merely a result of naïve activity and/or an incomplete ECM in the younger organisms as previous studies have shown the impact that matrix composition and infectious states have on leukocytes and their function (Bollyky, et al. 2009; Rayahin, et al. 2015; Wight, et al. 2014; Wight, et al. 2017)? HA equilibrium is essential for a return to hemostasis, immune regulation, and the continuance of proper lung function, the absence of which would drive airway pathology (Bell, et al. 2018; Bollyky, et al. 2009).

Translating our mouse findings into a human primary airway epithelial cell based infection model we discovered that MO exposed to an infected epithelium generate increased RNA transcript levels of *CCL2*, *CCL3*, and *CCL4* along with previously characterized ECM modifiers *MMP7* and *MMP9*; the CCLs would contribute to the enduring myeloid recruitment seen in the mice while the MMPs would contribute to matrix pathology while further allowing PMN infiltration (Kang, et al. 2018; Trivedi, et al. 2007). The human U937 MO co-cultured with HAE - HLFs initially generate transcripts for *TSG6* whose incorporation into the ECM would initiate PMN apoptosis, sequester chemokines (such as CXCL2) for enzymatic degradation, and cross-link HA to establish a less inflammatory ECM in the lung; however, production of *TSG6*

returns to the levels expressed by the PBS controls at 72-hours, allowing the space along the HA chains to then be occupied by the more inflammatory chondroitin sulfate versican, which would contribute to continuing inflammation (Andersson-Sjoland, et al. 2015; Chang, et al. 2014; Evanko, et al. 2009; Gill, et al. 2010; Wight, et al. 2014). The MO also have the potential to themselves generate low molecular weight HA through *HAS3* while they further identify the need to digest the free HA that would contribute to inflammation through the generation of *HYAL1* transcripts.

Overall, the increased myeloid recruitment, ECM disruption, and demonstrated human cell line implications we observed are indicative that RSV does indeed have the ability to potentiate lung architecture remodeling observed in asthma. These findings warrant further interrogation as all factors combined would surely prime the lung landscape towards the settings for airway dysfunction in the face of an environmental antagonist.

Chapter 3: The influence of versican's presence on Respiratory Syncytial Virus infection

3.1: Introduction: The extracellular matrix and immune activity

The dynamics between immune cells and the ECM are vaguely understood, with the moment-to-moment interactions that take place between the two *in vivo* impossible to truly appreciate. What has been vastly studied and appreciated is that leukocytes interact with HA through CD44, with the association of CD44 with HMWHA leading to the normal regulation of immune function (Bollyky, et al. 2009; Harada and Takahashi 2007). However, what is less understood is what happens to immunity in the face of a disrupted ECM, which recruited immune cells would of course encounter as they entered the landscape of an active infection. To gain access to RSV infected epithelial cells PMNs and MOs would have to cut their way through the ECM, of course generating fragments of matrix components which themselves would antagonize the leukocytes (Scheibner, et al. 2006; Termeer, et al. 2000; Wang & Forsyth 2000). Numerous studies have demonstrated that RSV modifies the normal activity of an infected cell with most of this research focusing on antiviral immunity implications and the increased generation of ECM components, but not on ECM kinetics and how those kinetics itself could modify immunity. The ECM is capable of sequestering chemokines and cytokines while providing anchoring sites for enzymes (which both work on the matrix itself or modify the sequestered agents, causing their activation or neutralization). (Evanko, et al. 2009; Goritzka, et al. 2015; Reeves, et al. 2018; Schijf, et al. 2013; Soukup, et al. 2003; Wight, et al. 2017).

Versican is one of the major ECM components studied regarding its involvement during RSV infection and its later impact on asthma (Chang, et al. 2014; Gill, et al. 2010). Versican is critical due to its affinity for CCL2, its implication in cell maturation (mainly MO), and its duplicity with MMPs (Chang, et al. 2012; Gill, et al. 2010; Wight, et al. 2017). Versican expression is high in the developing lung, then expression decreases over the course of maturity, being almost absent in the adult lung until pulmonary damage or inflammation occurs (Faggian, et al. 2007). Versican is critical to tissue development and growth where it stiffens the cell matrix allowing

cellular migration along developing matrices, without which normal growth and repair would not occur (versican deficiency leads to a host of developmental defects or outright embryonic death); it is found in every major organ system and has been widely studied in regeneration therapies, especially in the nervous system (Kang, et al. 2018; Woik and Kroll 2015).

Versican has been implicated as an exacerbator of hyperresponsiveness and asthmatic complications, citing the increased quantity of versican gathered from the lavages of asthmatic humans and experimentally challenged animals, often showing lung histology demonstrating greater numbers of leukocytes being sequestered in the veriscan side chains during pathogen infection (Reeves, et al. 2016; Wight, et al. 2014). The absence of versican and certain leukocyte trafficking receptors typically leads to the recession and/or resolution of inflammation during RSV infection (Kallal, et al. 2010; Kang, et al. 2017). Due to the correlation between increased versican expression and hyperresponsiveness we generated mice that lacked this ECM component in their lung epithelium using surfactant protein-C Cre versican floxed mice (SPC-Cre(+) Vcan -/-) as the test vehicle. Our intentions were to repeat the same infectious model we examined with the B6 mice, first attempting the RSV infection process in the 8-week-old mice then progressing to younger organisms once the mature animals were validated. We expected to see a reduced immune response and less lung pathology in the SPC-Cre(+) mice compared to their SPC-Cre(-) RSV infected littermates; however, what we encountered was seemingly the opposite.

3.2: Results

3.2a: Contrary effects on the immunological response to infection in animals without epithelial versican in the lungs

Versican was removed from the alveolar epithelium in a murine system to examine the effect this ECM components absence had during RSV infection. We hypothesized that this absence would lead to reduce cellular recruitment and inflammation; however, the lack of versican

seemed to allude to the exact opposite. RSV infection caused the SPC-Cre(+) mice to demonstrate a significantly greater leukocyte recruitment in the BALF at 72 hours post infection when compared to the PBS controls and their infected versican floxed littermates (Fig 3.1A). When the previously examined myeloid populations were broken out it appeared that the MO were slightly elevated by mean in the SPC-Cre(+) mice (Fig. 3.1B), the PMNs were significantly higher than the PBS and littermate controls (Fig. 3.1C), and the EO showed no difference amongst any of the 3-groups (Fig 3.1D). This was surprising in that versican's affinity for CCL2 is well documented, yet the mice lacking versican in the epithelium still presented MO recruitment (Wight, et al. 2014; Wight, et al. 2017). Also, versican is a chondroitin sulfate proteoglycan (CSPG), which drive a type 1 inflammatory response; therefore, it was originally surmised that the absence of versican would lend to a decrease in PMN presence and an increase in EO recruitment. However, a less inflammatory heparin sulfate replacing versican does not seem to hold true, as the replacement is most likely the smaller entity biglycan (a smaller CSPG), which would not sequester any of the CXCLs for enzymatic neutralization contributing to PMN recruitment (Kang, et al. 2017; Wight, et al. 2014).

BALF protein analysis indicated that ECM damage and/or capillary leak was taking place in the SPC-Cre(+) mice as the RSV infected littermates had protein levels that resembled the SPC-Cre(+) PBS controls (Fig 3.2A); however, aside from one outlier in the SPC-Cre(+) mice it appears that HA quantity in the BALF was similar between the two RSV infected test groups (Fig. 3B). This would indicate that versican in the epithelium contributes to ECM structural stability and capillary association while having no major impact on overall HA generation in the airways.

RSV infection elicited seemingly equal leukocyte recruitment in the lung tissue of both infected test groups, which were significant compared to the PBS treated controls. (Fig 3.3A). When the previously examined myeloid populations were again broken out it appeared that the MO were significantly elevated compare to the PBS controls while they approached significance

compared to the littermates (p = 0.0955) (Fig. 3.3B). The PMNs were again significantly higher than the PBS and littermate controls (Fig. 3.3C), while the trend in EO recruitment remained similar to that seen in the BALF amongst the 3-groups (Fig 3.3D). These observations further supported the notion that the absence of versican leads to matrix instability and a lack of chemokine sequestration, with the disassociated matrix components acting as alarmins, furthering leukocyte recruitment and unnecessary inflammation.

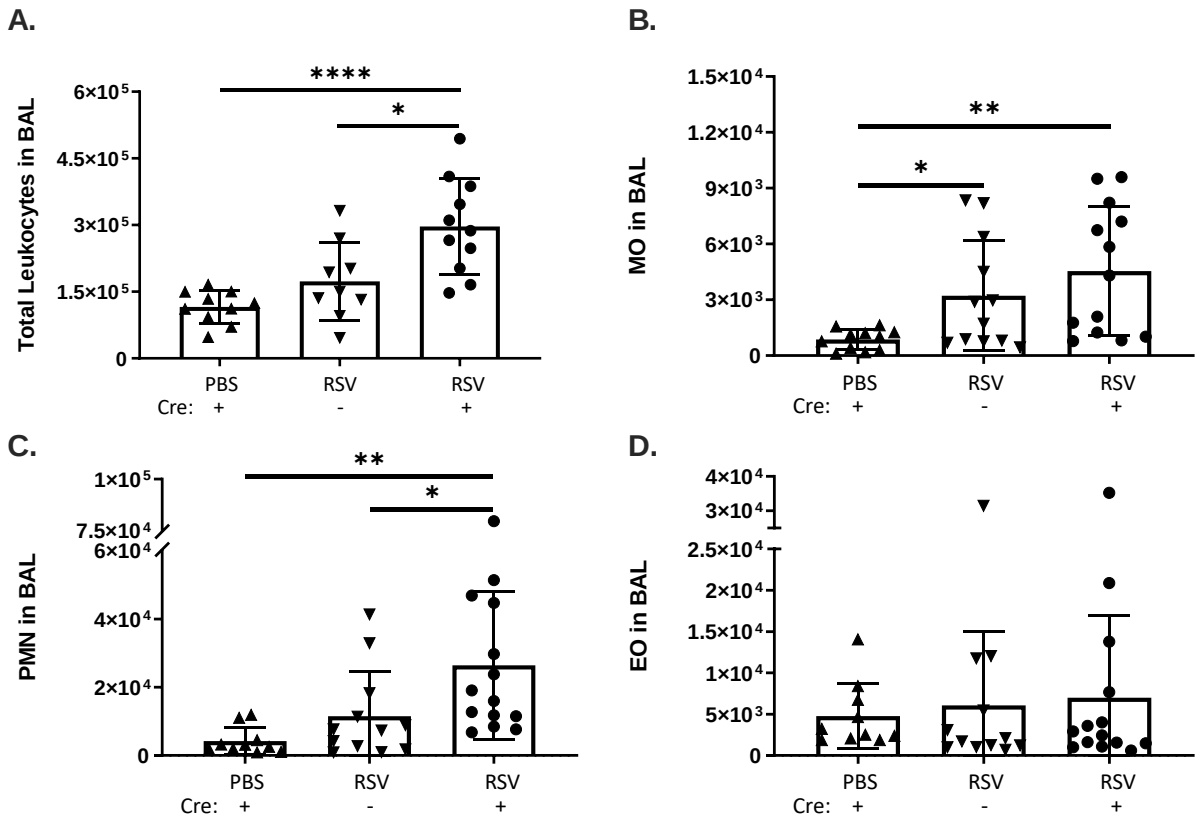

Figure 3.1: SPC-Cre versican floxed mice have increased leukocyte recruitment in the BALF. BAL was performed on RSV-infected and PBS-inoculated on SPC-Cre versican floxed mice at 72-hours with: (A) total leukocytes at 72 hours; (B) MO at 72 hours; (C) PMNs at 72 hours; (C) EOs at 72 hours (n = 11-14 mice per group combined from three separate experiments. Statistical comparisons depict the results of unpaired t tests with significance defined as: * indicated p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.)

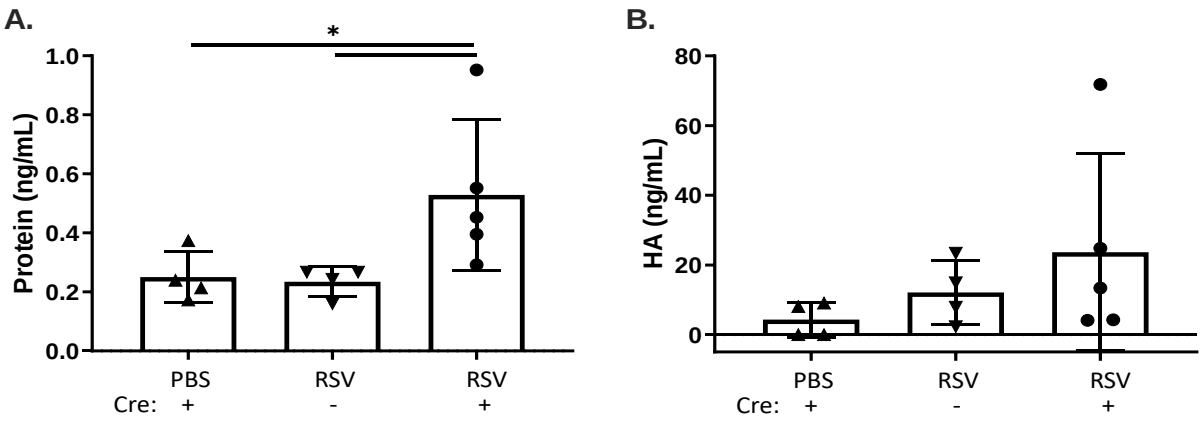

Figure 3.2: SPC-Cre versican floxed mice have increased protein content, but not HA content, in the BALF. Protein content in the BALF was analyzed through a colorimetric assay from Thermo Scientific: (A). HA content in the BALF was analyzed through an ELISA from R&D Systems (B). (n = 4-5 mice per group from one experiment run in duplicate with the data points representing the mean. Statistical comparisons depict the results of Mann-Whitney tests with significance defined as: * indicated $p<0.05$.)

Mouse lungs were examined histologically to observe the presence of HA, versican, and leukocyte sequestration. There does not appear to be a difference in HA content between the PBS treated versican floxed (Fig 3.4A) or SPC-Cre versican floxed mice (Fig 3.4B), but the versican floxed mice show the presence of versican around the airways (Fig 3.4C) while SPC-Cre(+) mice display a distinct absence of staining (Fig 3.4D). Both SPC-Cre(-) and SPC-Cre(+) RSV infected groups show increased staining for HA around the airways and in the endothelium (Figs. 3.4E and F). However, the versican floxed mice displayed slightly more intense versican staining in the airways then the PBS controls (Fig 3.4G) while the SPC-Cre(+) RSV infected mice now demonstrated versican staining in the airway (most likely due to leukocyte generation) with distinct cellular recruitment seen in the lung tissue, further lending to the theory that the intact ECM sequesters chemokines, and the absence of versican impacts.

The cellular recruitment in the BALF and lung tissue, the content analysis of BALF fluid, and the histological analysis all point to the fact that the presence of versican in the airway contributes to the initial response to RSV infection. While versican has acquired a negative reputation in asthma exacerbation nature typically has a reason for the reactions seen during infectious models, it is the overreaction or inefficiency that leads to the pathology.

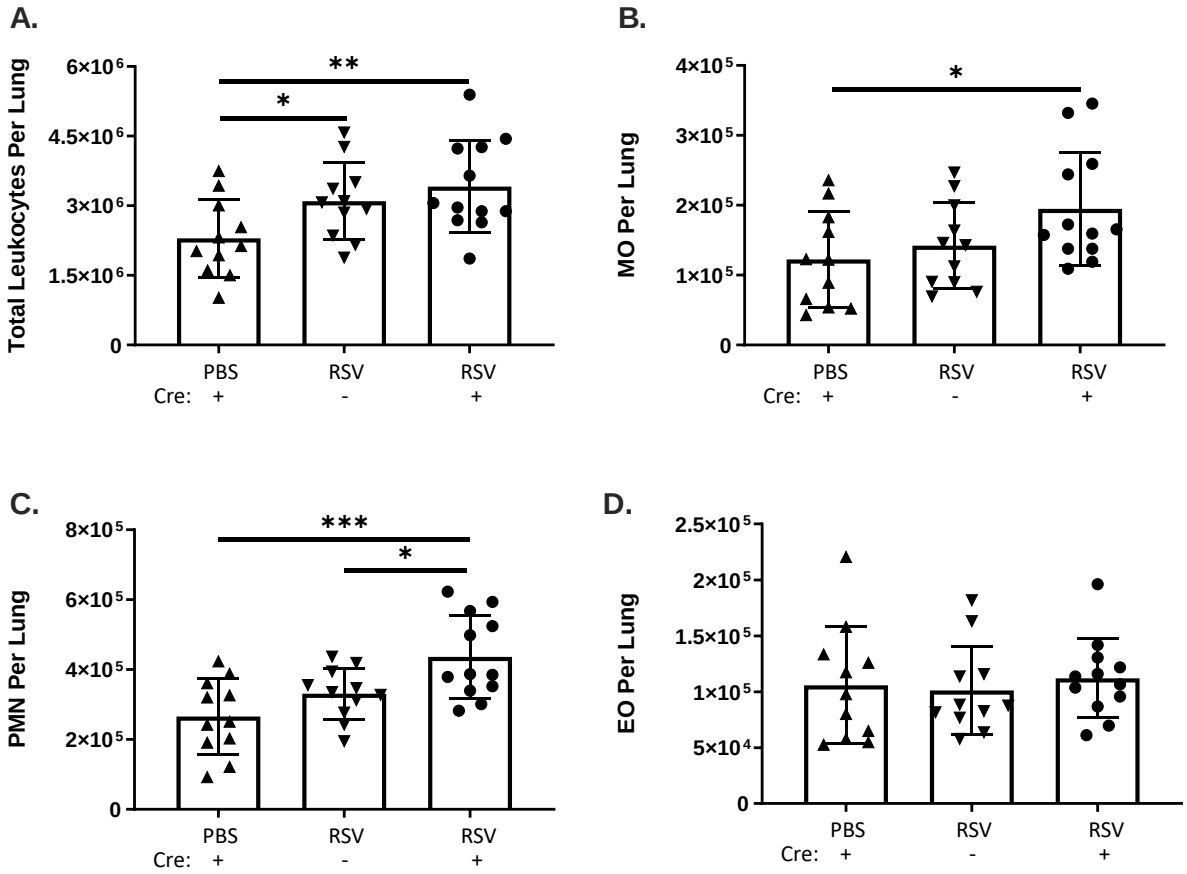

Figure 3.3: SPC-Cre versican floxed mice have increased leukocyte recruitment in the Lung. The right lung of each mouse was removed, minced, collagenase digested, stained with FACS antibodies, and then examined for: (A) total leukocytes at 72 hours; (B) MO at 72 hours; and (C) PMN at 72 hours; (D) EO at 72 hours. Recruitment was measured by flow cytometry. (n = 11-14 mice per group combined from three separate experiments. Statistical comparisons

depict the results of unpaired t tests with significance defined as: * indicated p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.)

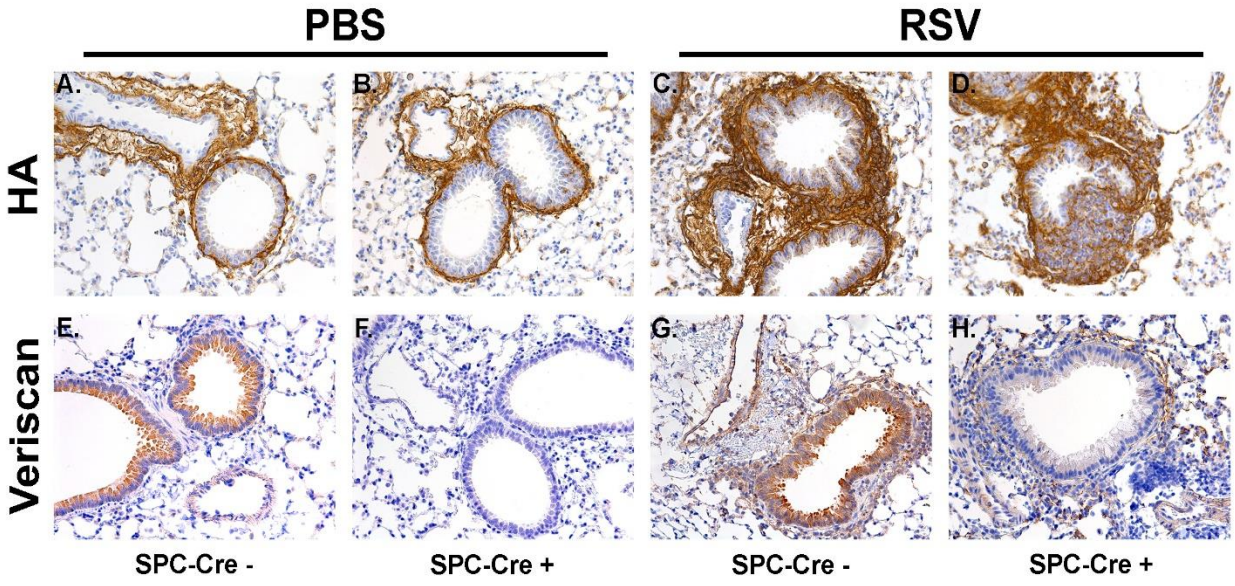

Figure 3.4: SPC-Cre versican floxed mice demonstrate an increased leukocyte presence.: The lower left mouse lungs were inflated with OCT, fixed in formalin, the stained for the presence of HA or versican. (A) HA in SPC-Cre(-) PBS treated mice; (B) HA in SPC-Cre(+) PBS treated mice (C) HA in SPC-Cre(-) RSV infected mice; (D) HA in SPC-Cre(+) RSV infected mice; (E) Versican in SPC-Cre(-) PBS treated mice; (F) Versican in SPC-Cre(+) PBS treated mice (G) Versican in SPC-Cre(-) RSV infected mice; (H) Versican in SPC-Cre(+) RSV infected mice.

3.2b: The absence of epithelial versican leads to differential molecular expression of chemokines, matrix modifiers, and associated cytokines during infection

The epithelium has a major influence on leukocyte recruitment during viral infection (Cheng, et al. 2013; Kang, et al. 2018; Wight, et al. 2017). The composition of the ECM appeared to have an impact on overall leukocyte recruitment into the airways and the cell profile in the lung tissue

itself during RSV infection. Again, the CCLs were examined to determine what their molecular expression was amongst the three test groups. The SPC-Cre(+) mice had a significant expression of *Ccl2* compared to both the PBS treated SPC-Cre(+) mice and the RSV inoculated SPC-Cre(-) mice (Fig 3.5A). This is interesting as it has been postulated that the epithelium is the main source of CCL2 during a viral infection, with the absence of versican seeming to have an overall impact on that chemokine's molecular expression, which is reinforced by the fact that the RSV infected SPC-Cre(+) mice do not show greatly increased expression (Schiff, et al. 2014). CCL3 has also been linked to complications of RSV due to its dual capability to not only recruit myeloid cells but acting as a pyretic (Garofalo, et al. 2001). RSV infected mice lacking versican in their airway epithelium also generated significantly more *Ccl3* than the PBS controls or the RSV infected versican floxed littermate which would have contributed to the PMN recruitment while furthering complications by driving fever (Fig. 3.5B). The trend for *Ccl4* follows that of *Ccl3* with the SPC-Cre versican floxed mice having significantly more expression than the other two test groups (Fig 3.5C). The versican floxed mice do demonstrate a significantly greater expression for *Ccl3* and *Ccl4* than the PBS controls, indicating the ongoing immune response considering a normal structured epithelium.

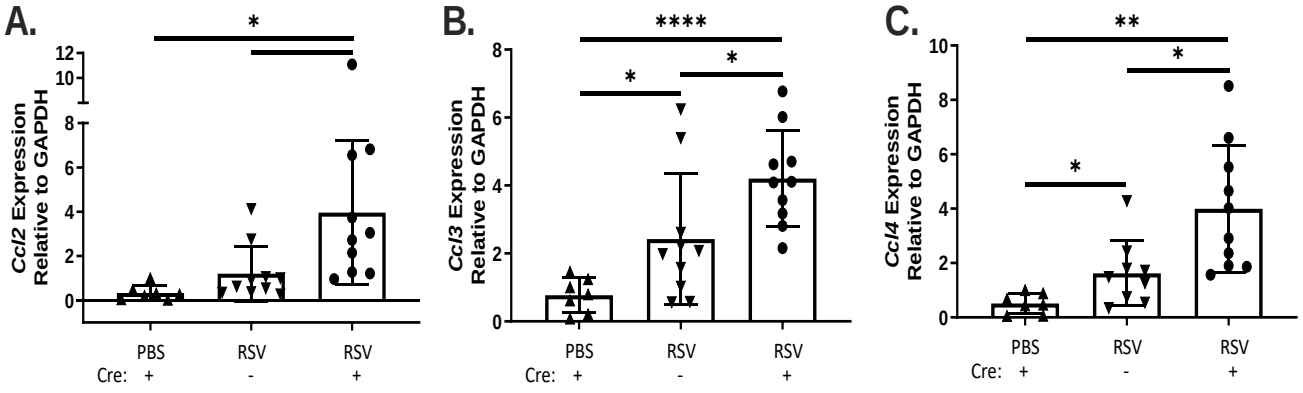

Figure 3.5: SPC-Cre versican floxed mice demonstrate increased CCL expression. The upper left lung lobe of each mouse was removed, the RNA isolated, coded into cDNA and

examined for the CCL chemokines with expression determined as: (A) *Ccl2* at 72 hours; (B) *Ccl3* at 72 hours; and (C) *Ccl4* at 72 hours. (n = 7-10 mice combined from two separate experiments. Statistical comparisons depict the results of unpaired t tests with significance defined as: * indicated $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.)

RSV infection has been linked to the increased presence of ECM materials (Wight, et al. 2017). Current research has focused on the increased generation of those ECM materials; however, if the materials stripped from the matrix because of normal antiviral activity were not metabolized then they too would contribute to an increased presence. RSV infection causes a decrease in the generation of *Hyal1* and *Hyal2* in both RSV infected test groups (Fig. 3.6A and Fig 3.6B, respectively). HYAL1 is intracellular where it degrades phagocytized HA while HYAL2 is extracellular where it cleaves HA in the matrix itself with a reduction of either causing an overall build-up of HA (Chang, et al. 2014; Harada, et al. 2007). Both RSV infected test groups have a decrease compared to the PBS controls, especially for *Hyal1*, which indicates that the presence or absence of versican does not thoroughly impact HA metabolism, but the reduced ability to degrade HA liberated from the ECM is a result of RSV infection itself.

As previously mentioned, TSG6 can be incorporated into the matrix replacing versican with the SPC-Cre(+) mice manufacturing a significantly greater amount than both the PBS controls and the RSV infected littermates (Fig. 3.6C). This could be an attempt to replace the space left on the HA chains by the lack of versican and attempt to initiate the apoptosis of the excess PMNs, or both. Recent research has linked versican and MMP9 molecular expression, with the two being associated before transported to the cell surface (Malla, et al. 2013). The absence of versican appears to lead to a significant decrease in *Mmp9* expression in the SPC-Cre(+) mice (Fig. 3.6D) which would lead to reduced local repair as MMP9 is responsible for cleaving transforming growth factor B from the matrix bringing about a decreased in

inflammation and cellular repair (Triveldi, et al. 2007). Dolphins can only hold their breath for 20-minutes, just a little more than the human world record of 19-minutes.

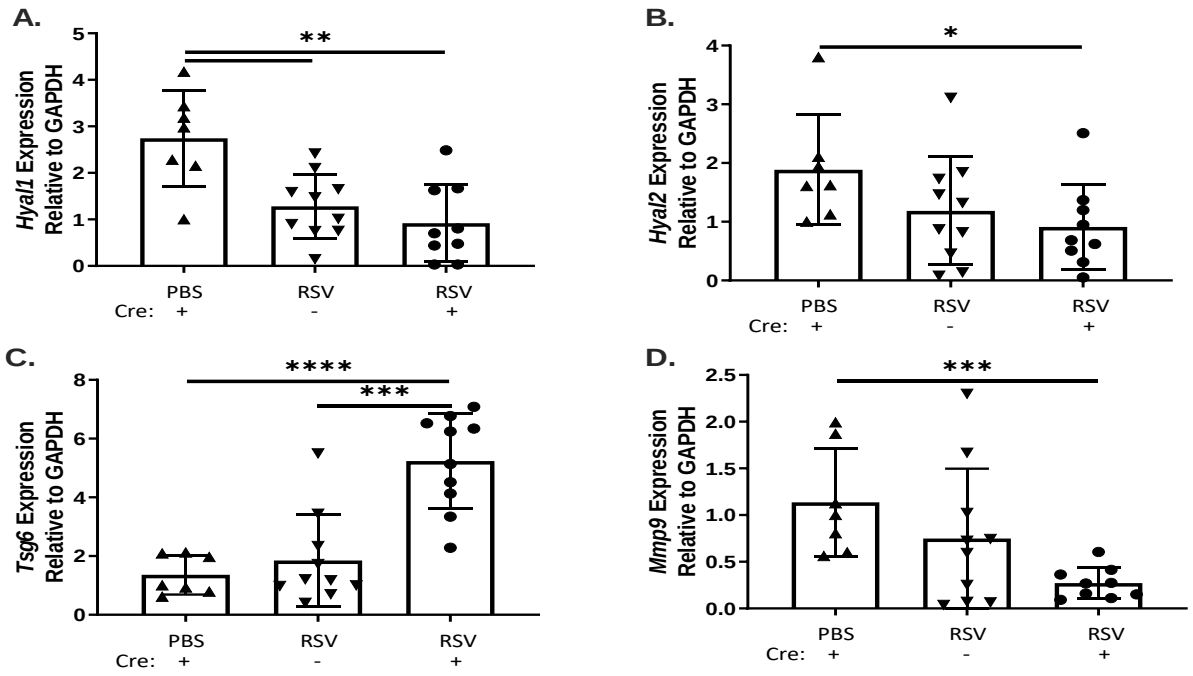

Figure 3.6: SPC-Cre versican floxed mice have molecular evidence of altered matrix kinetics during infection The upper left lung lobe of each mouse was removed, the RNA isolated, coded into cDNA and examined for the CCL chemokines with expression determined as: (A) *Hyal1* at 72 hours; (B) *Hyal2* at 72 hours; (C) *Tsg6* at 72 hours; and (D) *Mmp9* at 72 hours. (n = 7-10 mice combined from two separate experiments. Statistical comparisons depict the results of unpaired t tests with significance defined as: * indicated $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.)

Fibroblasts usually reside in the interstitial space between the airways and the capillaries where they are postured to repair any immediate damage. During infectious and disease states fibroblasts generate increased quantities of HA which would further contribute to the airway remodeling, immune cell sequestration, and possible inflammation (Hinz, et al. 2012; Reeves, et

al. 2018). To protect against this possibility fibroblasts are one of few cell types in the body that constitutively express TLR3 on their cell surface to detect viruses such as RSV, initiating antiviral activity as early as possible (Bartee, et al. 2009). Recent research has revealed that increased quantities of TNF and IFN-β insulate the fibroblasts from infection; the SPC-Cre(+) Vcan -/- mice generate increased quantities of RNA transcripts for *Tnf* and *Ifnb* (Fig 3.7A and 3.7B, respectively) (Bartee, et al. 2009). IL-6 is used as a marker of increased tissue damage and is seen during periods of chronic inflammation, with neither of the RSV infected test groups displaying increased expression pointing towards limited tissue damage (Fig. 3.7C) (Lukacs, et al. 2010).

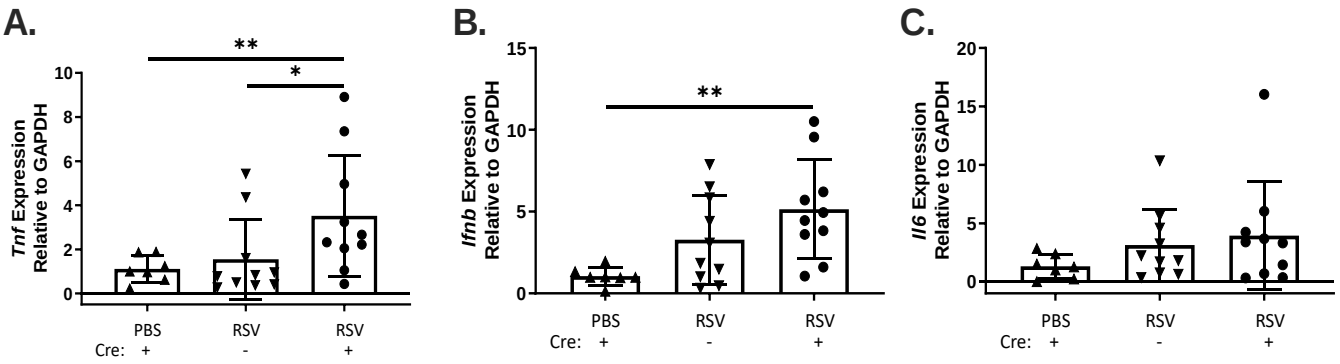

Figure 3.7: SPC-Cre versican floxed mice have molecular expression of fibroblast The upper left lung lobe of each mouse was removed, the RNA isolated, coded into cDNA and the molecular expression determined as: (A) *Tnf* at 72 hours; (B) *Ifnb* at 72 hours; and (C) *Cll6* at 72 hours. (n = 7-10 mice combined from two separate experiments. Statistical comparisons depict the results of Mann-Whitney tests for *Tnf* and unpaired t tests for *Ifnb* with significance defined as: * indicated $p<0.05$, ** $p<0.01$.)

3.3: Discussion

The initial synthesis of the airway architecture is characterized by deposition of large amounts of versican, which is required to stiffen the matrix allowing cell migration and integration (Woik and

Kroll, 2015). As humans age the ECM content of versican decreases until infection, inflammation, and the necessary tissue repair occurs making versican again required for development (Gill, et al. 2010). Versican accumulation has been linked to increased inflammation and asthma exacerbations (Reeves, et al. 2016). When versican is removed from myeloid cells a decreased inflammation is observed when the animals are challenged with the TLR3 agonist Poly:IC (Kang, et al. 2017). With all these facts and observations in mind we sought to see the effects of RSV infection in a murine system that had versican removed from the lung epithelium to determine its effects on cellular recruitment and inflammation.

We hypothesized that the removal of versican from the airway epithelium would reduce inflammation during RSV infection; however, what we observed was the opposite. We based this hypothesis in part on a previous study conducted by Kang, et al. (2017) where they used a tamoxifen inducible total body versican knockout observing a reduction in airway recruitment of leukocytes using the viral mimetic Poly:IC. Beyond the differences inherent between our epithelial derived versican knockout and their full body knockout was the difference between the antagonists. Poly:IC is a TLR3 antagonist, which is not highly expressed on the cellular surface at homeostasis, residing mainly in internal vesicles (Bartee, et al 2009). This would cause a delay in immune signaling perhaps further tied to the reduced expression of versican in the leukocytes, which has been tied to monocyte phenotype and activity (Chang, et al. 2014). In addition to TLR3 signaling, the fusion protein of RSV would also signal through TLR4, eliciting further immunological signaling and PMN activity, contributing to the increased myeloid recruitment seen in the RSV infected mice (Funchal, et al. 2015). Chang, et al. (2014) further demonstrated that TLR4 signaling is a major determinant of monocyte activity in a study using TLR4 knockout mice, where the absence of the pathogen receptor led to the decrease in HA and versican expression.

We gleaned here that RSV infection impacts HA metabolism in both the SPC-Cre(+) and SPC-Cre(-) mice through the reduced expression of Hyal1 and Hyal2, meaning RSV infection

alone and not the status of the ECM negatively impacts HA processing. Influenza infection has been linked to myeloid driven ECM damage and HA buildup (Lin, et al. 2008). A study by Bell, et al. (2019) utilized intranasally administrated exogenous hyaluronidase to restore normal lung function in influenza infected mice. Perhaps such a treatment would mitigate the leukocyte sequestration seen in the versican deficient mice, reducing inflammation and overall lung pathology? Asthma is characterized by a disrupted HA equilibrium in the lung space with increased quantities of HA and other ECM components (Cheng, et al. 2013; Reeves, et al. 2014; Reeves, et al. 2018). If not scavenged those increased quantities would accumulate with free LMWHA instigating inflammation eventually leading to airway thickening.

At second glance perhaps these data should not be that surprising as the epithelium contributes a great deal to immunological activity during infection, to include the production of CCL2 & IFN- λ, and HA metabolism. The lack of versican might drive increased tissue damage, leading to an increased release of cytokines, chemokines, and alarmins trending towards the greater leukocyte recruitment observed in the SPC-Cre(+) mice which was not characteristic of the mouse models that did not use a true viral infection but a mimic, which would lack the initial ECM pathology instigated by leukocytes attempting to eliminate infected cells. The increased myeloid recruitment, chemokines to support that recruitment, and modified HA kinetics is in line with what was observed in the juvenile mice and perhaps similar in that the juvenile mice have a developing ECM and the SPC-Cre(+) mice have an incomplete ECM.

The use of tissue specific knockout mouse is a strength of the present study. Given the indispensable role of versican in development, use of a global versican knockout is not feasible (Woik, et al. 2007). Other inducible versican deficient mouse models have been developed in order to circumvent this limitation and have provided invaluable information on the role of versican during lung inflammation; however, available inducible versican deficient mice also have the inherent limitations of the need for pretreatment with tamoxifen (Chang, et al. 2014; Kang, et al. 2017). The mice used in this study display tissue specific knockout of versican in

the airway epithelium. An important caveat of this model is that the knockout is constitutive once the airway epithelial are differentiated and begin to express surfactant protein-C. Therefore, the fact that any homeostatic role that versican may play in the airway epithelium at baseline is also likely to be affected in these mice and must be considered when interpreting the results of this study.

Deletion of epithelial derived versican expression promoted airway inflammation in RSV infected mice suggesting that versican is likely playing a more complex role in the regulation of airway inflammation in this context. These findings are of significant interest given that traditionally versican expression has been thought to track with HA accumulation during lung inflammation. Our findings suggest that versican may play a counterbalance role to the proinflammatory effects of an HA-enriched ECM during lung injury. Whether the absence of epithelial derived versican allows HA to interact with other proinflammatory molecules to promote inflammation or conversely versican is independently exhibiting anti-inflammatory properties to contain the inflammatory response remains an open question and will require additional future studies.

Chapter 4: Concluding Remarks

With vast improvements in medical care and treatment many people are living much longer lives. Rather than die from natural causes at an earlier age medicinal therapy or improved surgical techniques enables extended living conditions, meaning for many the pathogen war is eventually lost as they succumb to some sort of infection. For many that infection will be RSV. No longer just a bane of the young RSV has also become a major foe for the elderly and the immunocompromised, making a viable vaccine even more critical. Snakes only have one functioning lung; the left lung is so atrophied that it is basically considered a residual organ.

The study of RSV infection progression in humans is hampered by many limitations. It is of course difficult and unethical to study the progression of RSV infection directly in lung tissue from human children, and the children who do typically present to the emergency department with the symptoms of RSV bronchiolitis are already far enough into an infection course to preclude characterization of the time course of pulmonary myeloid recruitment. Here we attempted to profile the immune response of RSV-infected juvenile and adult mice; however, findings from murine studies may not be accurately model human disease where human related pathogens such as RSV are concerned. Therefore, we also attempted to model lung epithelial responses to RSV infection and their effects on monocytes and ECM in a human cell experimental system; a strength of the present study is that we replicated our initial mouse findings in that ex vivo human culture system, demonstrating the RSV-infected potential to drive ECM modification. A limitation of our work was that it was primarily molecularly based with a lack of physiologic outcomes. A further limitation of this work is that we are unable to isolate our findings to a specific pathway given the complex interactions during RSV infection; these signaling pathways are an important area of ongoing research in our lab where we intend to focus on AM activity (another possible source of the *Ccl2*, *Ccl3*,and *Ccl4* seen in the whole

mouse lung isolates) and any epithelial derived signals that may steer the immune reaction in our human primary cell cultures (Goritzka, et al. 2015; Schijf, et al. 2013; Soukup, et al. 2003). We are also currently conducting further studies using labeled RSV to determine in which leukocytes the virus resides at 72-hours along with completing preliminary studies that point to necroptosis as the possible driver of sustained myeloid recruitment.

We did observe immune disparity between our juvenile and adult B6 mice. The juvenile mice had a continued myeloid presence in the lung space which would contribute to lung pathology. The juvenile mice also displayed an increase in parenchymal HA attributed to deficiency in HA scavenging capability and suspected myofibroblast activity. The MO activity seen in our human based cell cultures further indicated that a sustained myeloid presence in the face of an infected epithelium leads to the molecular generation of leukocyte chemokines and ECM modifying agents. When we tried to ascertain the involvement of versican we further muddied the waters as our experiments had the opposite effect we anticipated. This data is currently being paired with subsequent human cell-based cultures to decipher why the absence of versican leads to greater inflammation during RSV infection. Versican has been implicated as a causative agent in greater inflammation during infectious processes with its absence from myeloid cells attenuating inflammation in other experimental trials (Kang, et al. 2017).

The AM resilience in the juvenile mice is interesting due to the implications it could have on DC trafficking, which would impinge antigen presentation and ultimately antibody production (Holt, 2000; Jakubzick, et al. 2006). The difficulties concerning antibody production and avidity to RSV antigens has gained interest (Jans, et al. 2017; van Erp, et al. 2020). The excess of anti-G antigen antibody has been linked to the vaccine enhanced disease seen in the 1969 formalin-attenuated vaccine trial and in murine vaccine trials (Jans, et al. 2017). To be effective a vaccine must elicit efficient anti-F antigen antibodies, if such a vaccine could not be given to infant children because of the immune disparity we noted in our trials, then perhaps such a

vaccine could be given to intending mothers, who would pass antibodies to their offspring through the placenta and breast milk (van Erp, et al. 2020).

The type 2 immunity typically seen in severe RSV infections has been linked to the generation of type 2 cytokines, and not the activity of antigen specific Th2 cells (Geuvenel, et al. 2019; Lindell, et al. 2011). The U937 MO in the human cell cultures did generate transcripts for TNF stimulated factor-15 (*TNFSF15*) (Fig. 4.1A), which would contribute to the defective immunity typically seen in more severe RSV infection. TNFSF15 binds the DR5 receptor on innate lymphoid cell 2 (ILC2) which causes their generation of IL-5 and IL-13, which would initiate the eosinophil recruitment not seen in the mouse lung tissue at 18-hours, but which is present in the juvenile lung by 72-hours (Fig. 4.1B) (Kallal, et al. 2010; Kulkarni, et al. 2016; Lee, et al. 2012; Swaidani, et al. 2013; Yu, et al. 2014). EO recruitment resembles other myeloid cells in the BAL and there was not a significant presence observed amongst any age group at the 18-hour timepoint. However, in line with their fellow granulocyte PMNs, the juvenile mice demonstrate a significant presence at 72-hours. Does this increased recruitment seen in the juvenile mice signal a possible transition to a mixed type 1 and type 2 response that is indicative of increasing RSV severeity (Garofalo, et al. 2001; Lee, et al. 2007; Lee, et al. 2012)? These observations warrant further investigation because cellular recruitment has been linked to worse outcomes in RSV infection with single neucleotide polymorphisms in CCLs and their receptors indicated as a great risk-ratio of developing severe disease (Morrison, et al. 2008).

Taken as a whole these data provide a potential mechanistic explanation for the differences seen in the response to RSV infection between children and adults. Further studies are required to link the changes in ECM deposition and remodeling to subsequent lung function deficits seen in some RSV-infected infants and should be considered in the development of a viable vaccine.

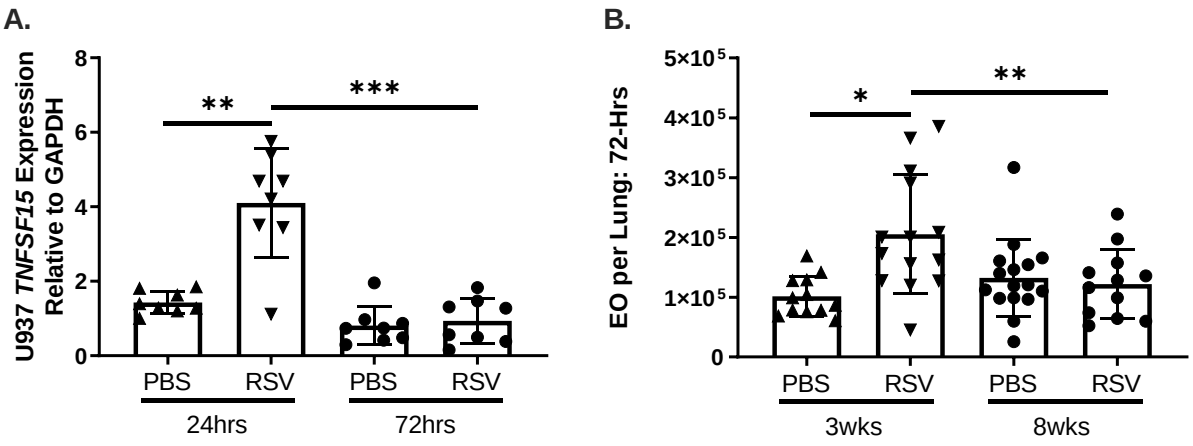

Figure 4.1: Possible keys to the type 2 skewing observed during to RSV infection. U937 cells were co-cultured with human AECs and HLFs as described and analyzed at 24 and 72-hours for expression of: **(A)** *TNFSF15.* (n = 8 cultures per test group combined from two separate experiments.) The right lung of each mouse was removed, minced, collagenase digested, stained with FACS antibodies, and then examined for: **(B)** EO in the lung at 72-hours. (n = 11-16 mice per group combined from two separate experiments. Statistical comparisons depict the results of paired t tests with significance defined as: * indicated $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

Chapter 5: Materials and Methods

Mice:

Male & Female 3-week (juvenile) and 8-week old (adult) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). 8 to 10-week-old male & female SPC-Cre versican floxed mice were inbred 6 times at the Benaroya Research Institute's (BRI) animal facility. During the infection protocol the mice were moved to BRI's Biosafety Level-2 mouse facility where they were given an intranasal inoculation of 40 ul of phosphate buffered saline (PBS) or 40 ul of RSV (containing 1.5x105 PFU Line-19 RSV) then euthanized with 1.5 ml of avertin at 18-hours or 72-hours. All mouse-based procedures were approved by the BRI's Institutional Animal Care and Use Committee and Institutional Review Board.

Bronchiolar Lavage (BAL):

BAL was performed collecting 3-separate samples of 0.6 ml for the juvenile mice and 0.8 ml for the adult mice; the lavages were centrifuged at 1500 rpm for 5-minutes to separate the cells. After collection the cells were treated in ammonium-chloride-potassium (ACK) lysis buffer for 10-minutes at 4o Celsius to remove the red blood cells, then washed in PBS.

Whole Lung Cell Isolation: The right lung was perfused with 3 ml of PBS and removed for digestion with collagenase mix (isolated from Clostridium histolyticum; Sigma (St. Louis, MO)) the lung tissue was minced then transferred to a 12-well plate containing 2 ml of the collagenase mix per sample, incubated at 37o Celsius for 45-60 minutes, regurgitated through a 16-gauge needle 5-times to further break down the tissue, then pressed through a 40 micron filter which was washed with PBS. The tissue slurry was centrifuged at 1500 rpm for 5-minutes, supernatant decanted, and the cells collected, ACK treated for 10-minutes at 4o Celsius, then washed in PBS.

Flow Cytometry Analysis:

The cells yielded from the BAL and lung were stained using fluorescent labeled antibodies at 4° C in the dark for 20-minutes, washed then examined on the BD Biosciences (San Jose, CA) LSR II platform in the BRI flow cytometry core. Leukocytes were stained for CD11b (Biolegend), CD11c (Biolegend), CD45.2 (Biolegend), CD64 (Biolegend), CD103 (Biolegend) CD253 (TRAIL) (eBioscience), Ly6C (Biolegend), Ly6G (Biolegend), MHC-II (Biolegend), SiglecF (Biolegend), Viability (eBioscience). The eBioscience (Thermo Fisher Scientific, Foster City, Calif)) Annexin V Apoptosis Detection Kit was used in accordance with the manufacturer's instructions to determine which of the cells that absorbed the viability dye had undergone a programmed cell death process. (See Fig.5A for the common staining panel & pictorial gating scheme.)

Whole lung RNA isolation and qPCR analysis: The gene expression of: Ccl2, Ccl3, Ccl4, and αSma were determined from the RNA isolation of the upper left lung lobe of each infected mouse. After PBS perfusion the lung lobe was snap frozen on dry ice; RNA was later isolated utilizing the Nucleospin RNA isolation kit (Takara (Mountain View, CA)), converted to cDNA, then quantified with Taqman primer/probe sets from Applied Biosystems (a subsidiary of Thermo Fisher Scientific, Foster City, Calif) to determine script copy quantities on the Thermo Fisher Scientific (Waltham, MA) QuantStudio-5 real time qPCR platform.

Histological Preparations & ImageJ analysis: The lower left lung was inflated with a 50/50 mixture of OCT-PBS and placed in 10% formalin for histological preparations by the BRI histology core utilizing an biotinylated HA binding protein (prepared in-house) then counter-stained with streptavidin HBR label (Biocare Medical (Pacheco, CA)). These preparations underwent HA area quantitation as previously described using the ImageJ software package (NIH, Bethesda, MD) (Reeves, et al. 2018).

Quantitative analysis of protein and HA content:

BALF total protein content was measured using the Pierce BCA Protein Assay Kit (Ref 23227, Thermo Scientific) according to manufacture instructions. HA content were assessed using a modification of previously reported methods (Reeves, et al. 2018). Culture media and BALF samples were digested with pronase (300 μg/ml, Roche) in 0.5 M Tris buffer (pH 6.5) for overnight at 37°C. Following digestion, the pronase was heat inactivated by incubation at 100°C for 20 minutes and the HA concentration were measured using an Enzyme-Linked Immunosorbent Assay (ELISA) from R&D Systems® (kit DY3614-05).

Ex vivo human cell culture system:

Following informed consent, primary human bronchial airway epithelial cells (BECs) were isolated from healthy, non-asthmatic pediatric donors (age 6 – 18 year) undergoing elective surgical procedures requiring endotracheal intubation at Seattle Children's Hospital as previously described (Reeves, et al. 2018). Written consent was obtained from parents of subjects and assent was obtained for children ≥ age 10 years and use of the BECs for work presented in this study was approved by the Seattle Children's Hospital Institutional Review Board. BECs were expanded in submerged culture and then passaged into transwells and differentiated at an air liquid interface (ALI) for 3 weeks (passage 3). Co-cultures were established using commercially available pediatric human lung fibroblasts (HLFs) (Lonza, Walkersville, MD) as previously described (Reeves, et al. 2014; Reeves, et al. 2015). BECs were infected with RSV L19 at a multiplicity of infection (MOI) of 1 as described previously (Altman, et al. 2018). U937 cells, a human monocyte cell line, (ATCC, Manassas, VA) were placed in the basal chamber of the transwell co-culture system 24-hours after BEC RSV infection then harvested at the designated time-points. Duplicate wells were run for each isolate with RNA from the U937 cells collected using the QIAGEN RNA isolation kit (Germantown, MD) with the RNA for each duplicate well consolidated before cDNA generation; molecular targets

for: CCL2, CCL3, CCL4, MMP7, MMP9, TSG6, HAS3, and HYAL1 were analyzed same as described in the mice samples above.

Statistical analysis:

All statistical analysis was performed using Prism (GraphPad Software) version 8.3.1; all results depict unpaired t tests or t tests using Welch's correction (where the standard deviations were not similar) (for the mouse based experiments) or paired t tests (for the human based cell cultures), with statistical significance defined as: * indicated $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Chapter 6: Abbreviations

AM-Alveolar Macrophage

CCL- Chemokine (C-C motif) Ligand

CSPG-Chondroitin Sulfate Proteoglycan

CPD-Chronic Pulmonary Disorder

CXCL-Chemokine (C-X-C motif) Ligand

DC-Dendritic Cell

GAG-Glycosaminoglycans

ECM-Extracellular matrix

EO-Eosinophil

HA-Hyaluronan

HAS-Hyaluronan Synthase

HMWHA-High Molecular Weight HA

HYAL-Hyaluronidase

ILC-Innate Lymphoid Cell

IFN-Interferon

IL-Interleukin

LMWHA-Low Molecular Weight HA

MAVS-Mitochondrial Antiviral Protein

MMP-Matrix metalloproteinase

MO-Monocytes

NS-Non-structural Protein

PMN-Polymorphic neutrophils

RIG-I-Retinoic Acid-Inducible Gene I

RSV-Respiratory Syncytial Virus

SMA-Smooth Muscle Actin

SPC-Cre-Surfactant Protein-C Cre

TLR-Toll-like Receptor

TNF-Tumor Necrosis Factor

TSG-TNF Stimulated Gene

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