THE ROLE OF MAST CELLS

DURING INFLUENZA A

VIRUS INFECTION

by

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DEDICATION

For my family and friends who were with me throughout this adventure.

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ABSTRACT

Influenza A virus (IAV) is a major cause of seasonal viral respiratory infections and causes ~36,000 deaths and ~1.7 million hospitalizations each year in the United States alone. Moreover, IAV has the potential to cause global pandemics, which have significantly greater morbidity and mortality. Morbidity and mortality associated with IAV infections is thought to be the result of significant pulmonary immunopathology from the inflammatory response rather than viral replication. The initial lines of defense against pathogens in the lungs include alveolar epithelial cells, endothelial cells, tissue resident alveolar macrophages, dendritic cells, and mast cells. Additionally, recruitment of neutrophils and macrophages is required for optimal clearance of IAV. Recent global analysis of lungs from mice infected with highly pathological IAV strains demonstrated enrichment of a mast cell transcriptional response, but the role of mast cells during severe pulmonary viral infections has been under studied. We found that A/WSN/33 causes significant immunopathology in C57Bl/6 mice and viral-induced pathology is mast cell-dependent. A/WSN/33 is able to directly activate bone marrow cultured mast cells (BMCMC) to produce histamine, leukotrienes, inflammatory cytokines, and anti-viral chemokines. Moreover, human H1N1, H3N2, and influenza B virus isolates can activate murine BMCMC *in vitro* suggesting this pathway could play a role during human infections. BMCMC activation requires infection of mast cells by IAV, which is dependent on the viral hemagglutinin specificity for α2,6-linked sialic acids. Cytokine and chemokine production from BMCMC occurs in a RIG-I-dependent fashion that requires the *de novo* production of vRNA. Conversely, degranulation occurs through a RIG-I-independent mechanism. Reconstitution of mast cell deficient mice with RIG-I$^{-/-}$ BMCMC generates lung pathology similar to wild-type BMCMC, suggesting that mast cell degranulation, rather than production of cytokines, causes A/WSN/33 induced lung pathology. Using recombinant A/WSN/33 strains, we found an association between binding of the A/WSN/33 hemagglutinin to α2,6-sialic acids and subsequent interactions with neuraminidase is important for degranulation. Thus, we have identified a unique inflammatory cascade that could be therapeutically targeted to limit morbidity following infection with IAV.

CHAPTER ONE

INTRODUCTION

Influenza A Virus

Influenza A virus (IAV) is one of the most common seasonal respiratory infections. Seasonal outbreaks typically lead to around 36,000 influenza-related deaths and around 1.7 million hospitalizations each year in the United States (1, 2). However, novel strains of influenza virus can cause major global pandemics and increase mortality in all age groups. Pandemic IAV strains result in robust inflammatory cellular infiltrate into the lungs and generate a dramatic 'cytokine storm', resulting in significant lung pathology. Therefore, understanding early inflammatory events can provide directions for development of new therapies.

IAV belongs to the family *Orthomyxoviridae*, which also includes influenza B and influenza C viruses, as well as Isavirus, Thogotovirus, and Quaranjavirus. IAV is an enveloped virus containing a segmented negative-sense RNA genome that encodes for ten essential viral proteins and several accessory proteins that can interact with the host immune response. As a pleomorphic virus, IAV can be spherical or filamentous (3) and its shape is genetically linked to its matrix proteins (M1 and M2) (4, 5). Three IAV proteins (hemagglutinin (HA), neuraminidase (NA), and M2) are located in the viral envelope. IAV is classified based on two of the surface proteins embedded in the viral envelope, the HA and NA proteins. Currently, there are 18 known HA proteins and 11 known NA proteins (6, 7). IAV subtypes HA1-16 and NA1-9 are found in the host

reservoir of birds, where it is typically asymptomatic and replicates in the respiratory and gastrointestinal tract (6, 8, 9). Newly discovered H17N10 and H18N11 IAV strains have been found in New World bats in South America (10, 11). IAV has a wide range of infectivity in mammals, including humans, pigs, horses, dogs, seals, whales, minks, and anteaters (9, 12). Of the IAV subtypes, only H1, H2, and H3 are known to be actively transmissible between humans and to cause pandemics. However, other subtypes, such as avian H5N1 and H7N9, can infect and cause illness in humans, but typically are zoonotic spillover events (13-16).

One characteristic of IAV is its ability to mutate and produce newly emerging strains that are transmissible. As a RNA virus it contains a RNA-dependent RNA polymerase that has no proofreading mechanism (17). This polymerase creates a high rate of insertions, deletions, and mutations, producing many defective particles, with only a fraction of these particles infectious (17). Seasonal strains of IAV are maintained in humans due to the virus' ability to undergo antigenic drift, where slight mutations change the already circulating virus such that it can evade neutralizing antibodies. This is why yearly vaccines are required for adequate protection. The World Health Organization (WHO) predicts the IAV strains that will most likely circulate 6-9 months ahead of the influenza season, and is usually fairly accurately (18, 19). However, IAV can undergo enough antigenic drift that the seasonal vaccine is not able to effectively protect against the circulating IAV strain, making the vaccine less effective, as is currently happening during the 2014-2015 influenza season (20). Subtle mutations that occur with antigenic drift change the antibody binding sites to evade the immune system (18, 21). There are four antigenic sites on the viral HA protein recognized by monoclonal antibodies that can

neutralize the virus: Sa, Sb, Ca, and Cb (21, 22). These are the sites where mutations usually occur. However, mutations at these sites do not change the protein conformation, only allowing the virus to escape immune detection and antibody recognition (18, 21). The HA can also become glycosylated during the viral replication cycle, which acts as another escape mechanism to avoid the immune system by 'shielding' antibody-binding sites (23).

IAV pandemics usually occur due to the virus undergoing antigenic shift, which involves reassortment to create novel strains of IAV (19). Some hosts, such as pigs, can be infected with both avian and human IAV (8). Dual infection of host with IAV strains allows the segmented RNA to randomly 'mix' to create a new strain of IAV. When antigenic shifts happen, global pandemics have the potential to arise, as the human immune response may not be sensitized to this newly created virus. Generally, subsequent mutations in the HA gene are required to alter binding to sialic acids on the surface of cells and allow human infection. However, some avian strains can infect humans directly without any changes in this envelope protein, suggesting that mutations in other viral genes, such as NS-1 and PB2, can play a role in infectivity (3).

Pandemics greatly increase morbidity and mortality associated with IAV and have been occurring since the 16th century (24). In the 20th century, three major pandemics occurred: the 1918 'Spanish' influenza, the 1957 'Asian' outbreak, and an emergence in 1968 known as the 'Hong Kong' flu. These pandemics were caused by IAV strains H1N1, H2N2, and H3N2, respectively (19). In the early 21st century, there was a reemergence of a novel H1N1 IAV strain that subsequently caused a pandemic in 2009, which is being referred to as the 'Mexican' flu, 'North America' flu, or 2009 H1N1pdm

(19, 25). The 1918 Spanish influenza is considered the most deadly, with ~500 million infections and between 20-100 million deaths, with the best estimate being ~50 million deaths. In addition to causing deaths in the young (< 5 years old) and elderly (> 65 years old), there was a significant portion of young adults (18-35 years old), who succumbed to infection during the 1918 Spanish influenza (26). This 1918 H1N1 strain was able to infect deep in the lungs, compared to seasonal strains that infect the upper respiratory tract. Furthermore, the 1918 Spanish influenza virus had unique PB1-F2 and NA proteins that allowed for faster replication and enhanced immune response, which led to higher lung damage and secondary infections (26, 27). The 2009 H1N1pdm appeared to be following a similar trend as the 1918 Spanish influenza, as young adults appeared to be at a higher risk of infection (28), but the 2009 H1N1pdm did not develop a high fatality rate for unknown reasons. Novel IAV strains that can cause pandemics are impossible to predict (7); therefore, researchers are trying to develop vaccines that target conserved epitopes of IAV, or are trying to find ways to manipulate the host immune response to lessen morbidity and mortality associated with these pandemic IAV outbreaks.

There are two main classes of drugs approved by the Food and Drug Administration (FDA) to treat IAV infected patients. These drugs work by targeting and inhibiting viral proteins M2 and NA (19, 29, 30). M2 is a viral protein that is important in the replication process, and two drugs are available that inhibit the function of this protein, amantadine and rimantadine (29-32). While these drugs that target the M2 protein are available, they are not recommended for use due to widespread resistance in the seasonal IAV strains. The other class of prescriptions for IAV infections is the NA inhibitors. Inhibiting NA prevents spread of newly replicated virus, and in the United

States, two NA inhibitors are readily available for use: oseltamivir and zanamivir (19), although a third, peramivir, became available in 2014 (FDA.gov). These drugs work by preventing NA activity, reducing sialic acid removal from the host cell membrane, therefore preventing the release of newly formed virions and minimizing IAV spread (33). While these drugs can moderate the severity of illness, they again have limitations. Due to IAV's high mutation rate, drugs can quickly become ineffective (19, 29, 30, 34-40); therefore, other anti-viral drugs are required to fight the spread of the virus. Many drugs are in development that target viral entry, viral transcription, and even host factors that IAV utilizes, but the effectiveness of these drugs against IAV remains to be seen (30).

IAV Hemagglutinin, Sialic Acid, and the Viral Replication Cycle

During IAV infection the HA binds to sialic acids located on the cell surface, initiating endocytosis of the virus into the cell. Sialic acids are N-acetylneuraminic acids that are added to the terminal sugar of glycoproteins and glycolipids. They are expressed on most tissues and play a large role in cell surface biology. These sialic acids act as signaling molecules for a wide variety of pathogens, including viruses, bacteria, and toxins, as well as the immune system, such as C-type lectins and the sialic acid binding Ig-like lectins (Siglec) family of receptors both of which are important for innate and adaptive signaling (41-44). Once the virus has gained entry into the endosome, acidification of the endosome occurs, which causes the HA to undergo a conformation change and fuse the viral envelope with the endosomal membrane. The lower pH triggers M2 proteins to open up ion channels and acidify the viral core. This accomplishes two

things 1) the HA mediated fusion event and 2) release of the M1 matrix and the viral nuclear protein (vRNP) complex into the cell cytoplasm. The vRNP complex is composed of the single stranded RNA (ssRNA) and IAV proteins nucleoprotein (NP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), and polymerase basic protein 2 (PB2), and each of these proteins have nuclear localization signals to help transport the vRNP to the nucleus (45). The replication cycle of IAV also has several accessory proteins that are involved in host defenses. NS-1 is known to suppress the host immune response (46) and PB1-F2 contributes to pathogenesis of IAV infections by interfering with mitochondrial anti-viral signaling protein (MAVS) (47, 48), thereby interfering with downstream anti-viral cellular responses.

Fusion of the viral envelope and the endosome, mediated by the HA and M2, allow the release of the IAV segments into the cytoplasm of the cell. The vRNP then moves to the nucleus for transcription (45). Once in the nucleus, the negative sense ssRNA is converted to mRNA with viral RNA-dependent RNA polymerase and then the mRNA is transported to the cytoplasm for translation. The proteins HA, NA, and M2, which will eventually be embedded in the envelope, are translated by ribosomes near the endoplasmic reticulum and then enter the host's secretory pathway to be inserted into the plasma membrane. All other proteins are translated by ribosomes in the cytoplasm, but they are then translocated into the nucleus to aid in replication or to generate new vRNPs for incorporation in newly forming virions. Assembly of newly produced vRNP occurs at the cell membrane, into which HA, NA, and M2 have been incorporated. Once assembly of proteins is complete, the virus will bud from the cell, becoming fully released when

NA cleaves the virus from sialic acid residues and releases the new virions into the environment (17, 45, 49).

Important mechanisms for IAV infectivity include sialic acid binding and subsequent entry of the virus into the cell. Since sialic acids are vital to IAV's entry into the host cell, understanding the basic chemistry involved in their synthesis is crucial. Sialic acids are linked to glycoproteins by sialyltransferases in the Golgi apparatus and can be attached to galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) or another sialic acid (44, 50). There are 20 known sialyltransferases, conserved between species, which are expressed in a cell and species-specific manner (42, 44, 50, 51). In the Golgi apparatus, the sialyltransferase uses a common donor, CMP-Neu5AC, and transfers the sialic acid directly onto the sugar. If attaching to another sialic acid, they will attach in an α2,8-linkage to form polymers of sialic acids (51). When attaching to galactose, they can attach to the 6-OH or the 3-OH to form a α2,6- or α2,3-linkage, respectively. There are two enzyme sialyltransferases that can form the α2,6-linkage, ST6Gal I and II, while the enzyme family that forms the α2,3-linkages is larger, consisting of ST3Gal I-VI (50, 51). IAV HA preferably binds to α2,3- or α2,6-linked sialic acids (52, 53) and binding preference is IAV strain dependent. Avian IAV preferably bind to α2,3-linked sialic acids, as avian IAV replicates mainly in the gastrointestinal tract that expresses these linkages (52-54). Human and swine IAV preferably bind to α2,6-sialic acid linkages (52, 53). Humans express higher amounts of α2,6-sialic acid linkages in their upper respiratory tract; however, deeper in the lungs, there is higher amounts of α2,3-sialic acid linkages (55). This differential expression of linkages in humans could be the explanation of how avian IAV can infect humans,

causing severe lower respiratory tract pneumonia, and prevent the ability to spread human-to-human without adaptation. The 1918 Spanish influenza, hypothesized to be of avian origin, was able to replicate in the lower respiratory tract (26), suggesting that it was able to bind to α2,3-sialic acid linkages as well as α2,6-sialic acid linkages (56). Humans who work in close contact with infected birds can inhale the virus deep into their lungs and become infected, but these avian viruses are not yet transmissible to other humans. Pigs express both α2,3- and α2,6-sialic acids allowing both avian, human, and swine IAV to infect these animals and also supplying a 'mixing vessel' for antigenic shift to occur, which could potentially give rise to the next pandemic strain of IAV.

Viral HA is a homotrimeric protein located in the viral envelope that binds to sialic acids to initiate entry of the virus into the cell. Each HA monomer is synthesized as a single polypeptide, called HA0, which is then cleaved into HA1 and HA2 subunits by host proteases that have been sequestered to the virus by NA (51, 57). IAV HA actually binds with low affinity to the sialic acid linkages on the cell, but because of the sheer abundance of available targets on cell, the virus is able to bind and enter efficiently (51).

Once bound, the IAV is endocytosed within the cell. IAV can enter the cell by different but redundant mechanisms, either by clathrin mediated endocytosis (CME) or by non-clathrin or caveolin mediated endocytosis, including macropinocytosis (non-CME) (58). CME is the most characterized mechanism of IAV uptake and is based on the concentration of clathrin receptors on the surface of the cell. Using a real time single-virus tracking approach, it was found that clathrin-coated pits formed *de novo* around IAV after binding. Once IAV binds to the cell, roughly three minutes later, the formation of the clathrin-coated pit begins to form, but the pit only lasted briefly before it began to

disassemble (59, 60). This method of viral entry requires epsin-1 and epsin-15, which are essential components of these clathrin-coated pits. Once the pit is formed around the virus, it is 'pinched' off by dynamin to form clathrin-coated vesicles (61), which then fuses with other endocytic vesicles or endosomes. However, it was found that cells lacking epsin-15 still were able to mediate viral entry, implying that this method was not the only way for IAV to enter the cell. In contrast, Semliki Forest virus, used as a control, can only use clathrin-coated pits for entry and was not internalized in these epsin-15 deficient cells (59, 60, 62).

Non-CME of IAV includes macropinocytosis and occurs independently of dynamin (61). This mechanism can be induced by serum (58, 61). Filamentous IAV mainly uses this route of entry (3), where it then degrades into smaller spherical like fragments when exposed to a lower pH of 5.5 (3). Once pinocytosed, the vacuole fuses with an endolysosomal system as described with CME, similar to Ebola (3, 49).

Once in the endosome, the pH drops to ~5, which triggers fusion of the HA to the endosome allowing the genome to be delivered to the cytoplasm. Low pH allows 1) HA to undergo a conformational change to expose its' fusion peptide and 2) uncoating of the vRNPs. This second step occurs as the M2 ion channels acidifies the interior of the virus that occurs around pH of 6. Endosomal trafficking occurs no matter which internalization route transpires (63) and Rab proteins are important; Rab5 interacts with the early endosome and Rab7 with late endosomes. Dominant negative proteins of each Rab inhibited influenza infectivity, unlike Semliki Forest Virus and vesicular stomatitis virus (VSV), which only go through early endosomes (63).

IAV is a common pathogen that typically has low morbidity and mortality every year. This virus binds to sialic acids on cells to initiate entry by CME or non-CME mechanisms. Once in the cells, the virus genome is replicated in the host nucleus and proteins are translated and assembled at the cell membrane for budding. New virions are released from the cell via NA cleavage of SA. While typically a seasonal pathogen, IAV has the potential to create novel strains that can cause global pandemics. Understanding the pulmonary immune response is viral for reducing susceptibility to this pathogen.

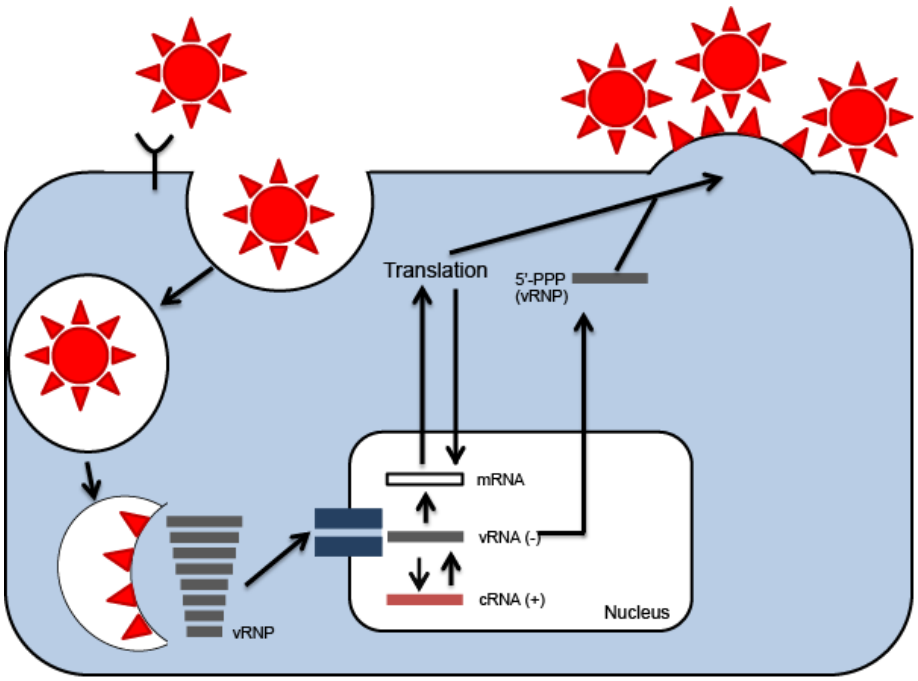

Figure 1.1. IAV replication cycle. IAV HA binds to α2,3- or α2,6-linked sialic acids on the cell surface to initiate infection. Once bound, IAV enters the cell through clathrin mediated or non-clathrin mediated endocytosis into an endosome. Via M2, the protons will enter the virion and HA will fuse to the endosome, allowing the vRNP to enter the cytoplasm. The vRNP will translocate to the nucleus, where the negative sense single stranded RNA will transcribe to positive sense cRNA or mRNA. cRNA is used to create more copies of vRNA, while mRNA will move back into the cytoplasm for translation. Envelope proteins will be embedded into the plasma membrane, while other proteins traffic back to the nucleus to aid transcription. Once replication is complete, the vRNP will collect at the membrane for assembly and subsequent budding as fully formed virions.

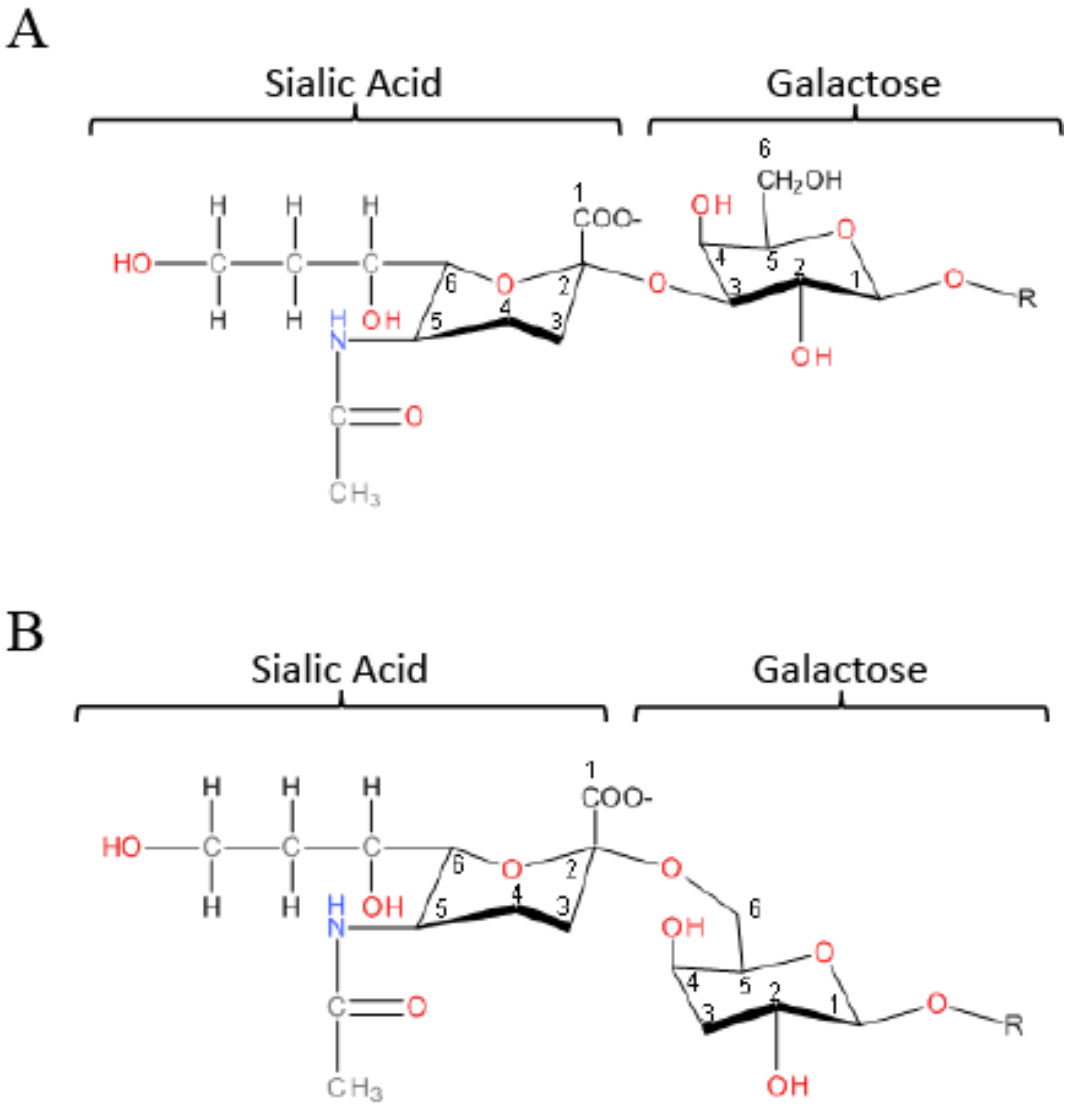

Figure 1.2. IAV HA binds to α2,3- or α2,6-linked sialic acid linkages to initiate entry into host cells. Sialic acids are 9 carbon N-acetylneuraminic acids that are attached to glycolipids or glycoproteins in the Golgi apparatus and then translocated to the surface of the cell. Avian IAV preferably bind to galactose bound α2,3-linked sialic acids (A) while human IAV preferably bind to α2,6-sialic acid linkages (B). Once bound, IAV will then enter the cell to begin the infection process. Models designed by Kim M. Hilmer.

Pulmonary Response to IAV

IAV is a respiratory virus, causing symptoms that include rhinorrhea, fever, cough, sore throat, fatigue, and head and body aches. IAV is spread by coughing or sneezing (airborne transmission) and infects the lungs of humans. After infection, IAV usually has an incubation time of 2-5 days before symptoms begin to appear (64). The first barrier that IAV encounters is the mucus in the respiratory tract. The mucus membrane can trap small particles, including viruses, and the beating motion of the epithelial moves these particles out of the respiratory tract. Once in the throat, these particles are usually swallowed, where the high acid content in the stomach digests them. Mucus contains soluble glycoproteins rich in sialic acids, which were once thought to act as 'decoys' for IAV and prevent the virus from reaching epithelial cells (65). In a recent study, it was found that IAV does bind sialic acids in the mucus layer of frozen human tracheal and bronchial tissue. NA on the virus is important for getting through this mucus layer, as it can cleave these glycoproteins to allow the virus access to epithelial cells, their primary source of replication (65). Mucin added to cells in culture lowered viral infection of these cells, and if the virus was pre-treated with the NA-inhibitor oseltamivir (Tamiflu), there was increased inhibition of infection of these cells (65). This data indicates that while the mucus layer in the upper respiratory tract is important for inhibiting IAV infection, the virus is able to penetrate it and reach epithelial cells (66, 67). The lung is protected by alveolar epithelial cells, endothelial cells, tissue resident alveolar macrophages, dendritic cells, and mast cells, which can respond immediately to

infection by pathogens. During IAV infection, it is these cells that are first infected, and first able to initiate the innate immune response before the adaptive response kicks in.

Alveolar macrophages are phagocytic cells that reside deep in the lung airways. These cells can phagocytize pathogens and travel to draining lymph nodes to act as antigen presenting cells (APC) and prime the adaptive immune response (68). Within the lung parenchyma, dendritic cells (DC) are major APC. There are two types found in the respiratory tract: conventional DC and plasmacytoid DC. Conventional DC continually sample the lung environment to capture antigens, travel to lymph nodes, and then present the antigen to $CD4^+$ or $CD8^+$ T cells (69). Plasmacytoid DC do not efficiently mediate antigen uptake and presentation, but are major contributors to type I interferon (IFN-I) production (70, 71), and therefore play a large part in the early immune response against IAV.

Pattern recognition receptors (PRR) play a major function in detecting IAV and initiating an anti-viral response. PRR play an essential role in innate immunity by detecting conserved pathogen associated molecular patterns. These receptors are located both on the cell surface to detect extracellular pathogens, as well as located within a cell to sense intracellular invading pathogens (72). Immune recognition of IAV is mediated by numerous PRR, including toll-like receptors (TLR)-3, TLR-4, TLR-7, Nod-like receptors (NLR) NLRP3, C-type lectins receptors (CLR) and retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) (73-85). These PRR are expressed on dendritic cells (DC), alveolar macrophages (AM), epithelial cells, endothelial cells, and mast cells. TLR are a family of PRR that can detect a large range of pathogen associated molecular patterns. For IAV, TLR-3 is an important PRR that is constitutively expressed in

epithelial cells and detects ssRNA (86). In mice, it can lead to production of cytokines during IAV infection, but it also contributes to pathology. TLR3$^{-/-}$ mice survive longer during lethal influenza infections, even though the mice maintain higher viral titers, and have less damage to the lungs during infection (75). Priming of TLR-4 with LPS resulted in increased survival during IAV infection (84). TLR-7 is an endosomal PRR that recognizes viral ssRNA, and after detection of IAV, stimulates an IFN-I response (77).

RIG-I is a cytosolic protein that detects single stranded RNA, and has previously been shown to become activated by detecting the nuclear exportation of genomic IAV RNA (79, 81, 87). Once RIG-I detects intracelluar RNA, it undergoes a conformational change and binds to MAVS. To enhance signaling, MAVS can interact with STING, located on the endoplasmic reticulum, and induce the production of downstream signalling molecules (82). IAV detection by RIG-I leads to IFN-I production (75, 79). Dixit et al (88) published that MAVS is associated with both the mitochondria and peroxisome membrane. MAVS at both locations can respond to viruses, including IAV and reovirus; however, the signaling networks activated by each sub-cellular compartment, dependent on the C-terminal tail motif, resulted in different effective anti-viral events (88). Peroxisome-associated MAVS triggered rapid expression of interferon stimulating genes and IL-6, CCL2, and CCL4 from virus infected fibroblasts, while mitochondrial-associated MAVS had delayed response consisting of type I IFN expression (88).

IAV can also be detected by NLR, which can result in the formation of the inflammasome complex (73, 74, 76). Mice lacking the NLRP3 inflammasome lead to increased mortality with lower cellular recruitment during infection (73, 74).

Inflammasome complexes are important in maturation of cytokines IL-1β, and IL-18 (89). IL-1β, along with IL-1α, signals through the IL-1R1 to initiate signal transduction (90). IL-1α production is regulated through S1P receptors, as blocking these receptors can lead to a decrease in cytokine production including IL-1α (91). While mice lacking IL-1R1 had increased mortality during IAV infections, there were no differences in viral titers (90), indicating that IL-1R1 is important in the immune response to IAV. In fact, IL-1R1$^{-/-}$ mice had decreased acute pulmonary inflammation, with decreased neutrophil recruitment during infection with IAV (90). Thus, IL-1 signaling is important during IAV infection.

IFN-I are usually the first cytokines produced during IAV infections and can be produced by epithelial cells, alveolar macrophages, and DC. Detection of IAV ssRNA by plasmacytoid DC results in a type I interferon (IFN-I) response (77, 78). IFN-I signaling occurs through the IFN-α receptor (IFNAR), and IFNAR$^{-/-}$ mice have increased susceptibility to IAV (92, 93). These cytokines induce neighboring cells to enter an anti-viral state that includes production of many interferon-stimulated genes (ISG) (94). Of these ISGs, MX genes are known to be important to block IAV replication, but have different roles in mice and humans. MX1 in mice block viral transport into the virus, whereas MXA in humans inhibit nuclear transcription (95, 96). Other ISGs include IFN-inducible transmembrane (IFITM) proteins, which block IAV membrane fusion (97). The OAS family and ribonuclease L (RNase L) degrade RNA in the cytosol (98). PKR are serine/threonine kinases that bind to dsRNA and inhibit translation by binding to the α-subunit of eukaryotic translation initiation factor 2α. This activates the NF-κB pathway and stabilizes *IFNα* and *IFNβ* mRNA (99). IFN-I activation through NF-κB can lead to

the production of other cytokines and chemokines including IL-1β, IL-6, IL-8, TNF-α, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL10 (IP-10), and co-stimulatory molecules CD40, CD80, and CD86 (66, 80, 81). These proteins are important for local and systemic inflammation, fever, and instructing the adaptive immune response. Most importantly they will recruit additional cells, such as monocytes, neutrophils, and natural killer (NK) cells to help mediate the clearance of infected cells (66, 67, 100).

During IAV infections, monocytic cells and neutrophils infiltrate the lungs (66, 100, 101). Monocytes are recruited from the bone marrow and follow CCR2 receptor-mediated signaling to the lungs. Recently, the role of IFN-I has been defined in the regulation of monocytes and neutrophil recruitment (101). In the presence of IFN-I signaling, $Ly6C^{hi}$ monocytes amplify monocyte recruitment by enhancing the production of CCL2 (MCP-1), which acts on the CCR2 receptor. Conversely, in the absence of IFN-I, in $IFNAR^{-/-}$ mice, higher amounts of the neutrophil recruiting chemokine CXCL1 (KC) are produced and there is a greater influx of neutrophils into the lungs during infection (101). The role of neutrophils during IAV infection is controversial. When neutrophils are present at high numbers, there is greater damage observed within the lungs (100). However, the absence of neutrophils resulted in higher viral titers and decreased survival (102-104). Only when neutrophils are partially depleted during infection was there increased survival (100). Therefore, inflammatory cellular recruitment during IAV infection needs to be tightly regulated to control infection but limit host damage.

The adaptive immune response is important for clearance of the virus by T cells, whereas B cell production of antibodies has the potential to protect against reinfection.

DC are the main antigen-presenting cell for initiating the adaptive immune response in the draining lymph nodes. T cells will proliferate, and primed effector T cells will exit the lymph node and travel back to the lung for viral clearance (105). $CD8^+$ T cells directly lyse IAV infected cells by 1) exocytosis of granules containing perforin and granzyme, 2) inducing apoptosis through FAS or TNF-related apoptosis-inducing ligand (TRAIL) signaling, or 3) production of proinflammatory cytokines (105-108). $CD4^+$ T cells also have cytotoxic activity during IAV infections, although the effect *in vivo* is low (109). $CD4^+$ T cells largely aid in activation, differentiation, and antibody class switching of B cells (105).

Mice lacking $CD8^+$ or $CD4^+$ T cells will survival IAV infection. However, mice deficient in B cells do not survive (110, 111). B cells start producing antibodies by day 3 of influenza infection (112). In the lung, IgM is the first antibody produced and offers initial protection against IAV before antibody class switching occurs (112, 113). IgG and IgM are the main antibodies detected in the serum, while in the upper respiratory tract mucosal IgA is the main antibody in the serum (112, 113). Antibody producing memory B cells are long-lived and provide lasting protection (114).

The immune response is important for controlling and clearing IAV infections. However, a robust immune response can also cause damage during infection. The influx of immune cells into the lung induces more production of cytokines, and can start a dangerous forward feedback loop, termed the ‘cytokine storm’, that has been blamed as one of the causes of the high number of deaths seen during pandemics (115). The term ‘cytokine storm’ was first coined in 1993 in response to graft-vs-host disease, and has since been used in literature to describe infectious diseases (116, 117). Even during the

most recent pandemic in 2009, histopathological studies show 2009 H1N1pdm virus infection had extensive diffuse alveolar damage and higher levels of pro-inflammatory cytokines, including TNF-α, IL-6, IL-8, CCL2 (MCP-1), CCL4 (MIP1-β), and IP-10, than in seasonal IAV infections (115). There is evidence that this uncontrolled inflammatory response and 'cytokine storm' causes excessive damage to the lungs during infection (66, 118-120). IFN-I production is an early immune response against IAV and plasmacytoid DC produce a dose-dependent response of IFN-I during IAV. Low doses induce a robust response of the cytokine, while at high titers triggering plasmacytoid DC apoptosis. This cell death mechanism is hypothesized to be a way the immune response controls the inflammatory environment during IAV infections (71). Recently it has been demonstrated that if you inhibit the S1P receptor, found on lung endothelial cells, then this 'cytokine storm' was diminished during IAV infection resulting in a decrease in morbidity and mortality (91). Thus the 'cytokine storm' appears to be a factor in the pulmonary damage observed during IAV infection and by targeting this excess inflammation, we can decrease IAV-induced disease.

The 1918 Spanish IAV pandemic and the more recent spillover infections with H5N1 and H7N9 viruses have been peculiar in that they cause high lung damage in young adult populations (121, 122). Research has shown that highly pathogenic viruses such as these have increased cellularity during infection compared to low pathogenic viruses (66, 104). Compared to IAV with lower pathogenicity, highly pathogenic viruses induce increased infiltration of macrophages and neutrophils to the lung during infection (66, 100, 102, 104). Neutrophil recruitment in particular has been extensively studied. Neutrophils have been detected in the upper and lower respiratory tracts by eight hours

post-infection with a highly pathogenic H1N1 strain (102). The importance of neutrophils during IAV infection strongly correlated with the pathogenicity. BJx109 IAV is an H3N2 strain that has low virulence during infection and depletion of neutrophils did not affect its pathogenicity (104). In contrast, neutrophils are critical in controlling high virulence H1N1 strain A/PR/8/34 infection (104). Moreover, lethal IAV responses had a different early innate response compared to non-lethal IAV infections that recruited neutrophils that released additional cytokines to attract more neutrophils (100). A recent pulmonary genetic comparison was made in mice infected with a 2009 H1N1 isolate A/California/04/2009 (CA/04) and its mouse-adapted strain (MA-CA/04). The MA-CA/04 has only 5 genetic differences compared to its parent strain (three in the HA and one each in the PB2 and NP IAV proteins), and causes higher disease severity in mice due to disregulation of the immune response with a higher macrophage and neutrophil signature (123). Thus, while inflammatory cell recruitment is required to control IAV infections, too much of this response is detrimental and can affect the outcome of infected individuals.

As stated, infection with IAV can damage the lung architecture, and those infected have a higher chance of becoming infected with secondary bacterial infections (124-126). This is one of the major causes of the high mortality observed during the 1918 Spanish influenza pandemic (24). Depletion of alveolar macrophages during IAV infections has been implicated in allowing secondary bacterial infections to occur (127). These infections can develop 7-21 days after IAV has been cleared. Major causes of these infections include *Staphylococcus aureus*, *Streptococcus pneumonia*, and *Hemophilus influenza* (9, 124, 126).

The immune response to IAV is important for host survival. However, an excessive inflammatory response can be detrimental and cause pulmonary damage during infection. Understanding the mechanism behind the immune response to IAV is important for tailoring therapies that will limit IAV-associated morbidity and mortality. Interestingly, there was an enhanced mast cell transcriptional signature in the lungs of MA-CA/04 infected mice, indicating that mast cells are present within the lungs of IAV infected mice (123). However, the role that mast cells play during IAV is only beginning to be explored.

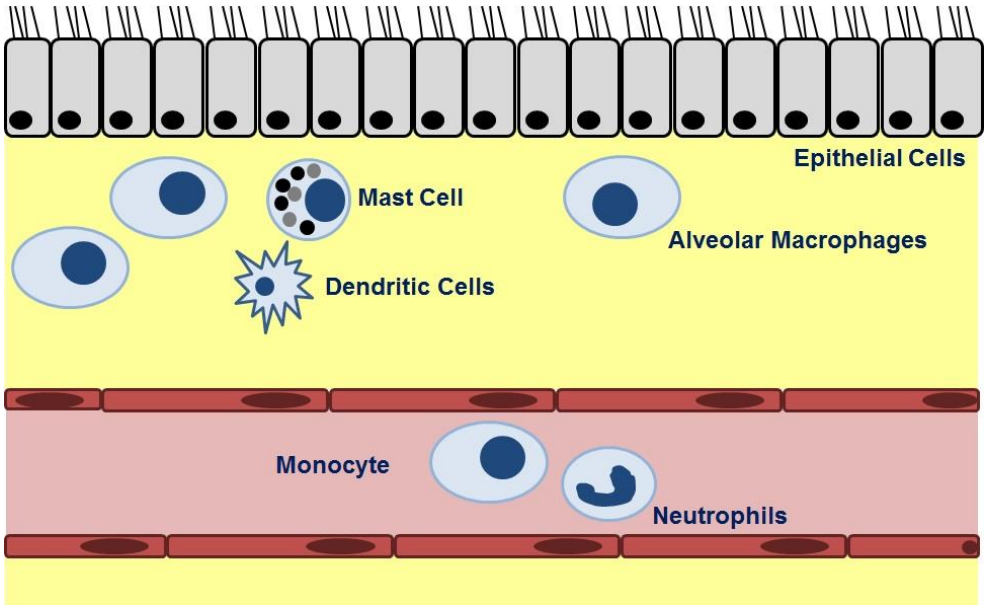

Figure 1.3. Biology of IAV infection. Alveolar epithelial cells, endothelial cells, tissue-resident alveolar macrophages, tissue-resident dendritic cells, and mast cells are all ideally positioned to be the first responders to pathogens invading the lung. Alveolar epithelial cells are the primary replication sites for IAV, although the virus can replicate in immune and non-immune cells. Pattern recognition receptors in infected cells recognize IAV and initiate an anti-viral response, which includes the production of pro-inflammatory cytokines and chemokines. Neutrophils and macrophages are recruited into the lungs from the blood to help clear infections. Mast cells are present in the lungs during IAV infection; however, the role mast cells play during IAV infection remains understudied.

Unique Role of Mast Cells

Paul Ehrlich first described mast cells in 1878. He originally called them "mastzellen", meaning 'well-fed' cells, due to their appearance of being full of granules (128). Mast cells are a heterogeneous cell population of hematopoietic origin, typically enriched in tissues that are exposed to the environment, including the skin, gastrointestinal tract, and respiratory tract (129-133). This location allows them to quickly respond to invading pathogens. Extensively studied for their involvement in allergic and asthmatic reaction, where they play a role in airway hyperresponsiveness and tissue remodeling, and during parasitic and bacterial infections, their participation during viral infections is only now being elucidated (129-131).

Mast cells originate from the hematopoietic stem cell population in the bone marrow (134) and require IL-3 and stem cell factor (SCF) for differentiation and maturation (128). SCF signals through its receptor, the tyrosine kinase receptor c-kit (also known as CD117), which is a highly conserved transmembrane glycoprotein (135). The extracellular domain is divided into five immunoglobulin-like domains (D1-D5). D1-D3 bind SCF, while D4-D5 dimerize with another c-kit, initiating autophosphorylation to regulate gene expression for proliferation, cell growth, and differentiation (135). In humans, it is the $CD34^{+}/CD117^{+}$ pluripotent progenitor cells that differentiate into mast cells. In mice, while similar, mast cells do not require the SCF ligand for full differentiation (136). However, mutations in c-kit or SCF result in mast cell deficient mouse strains (133). Mast cells do not fully mature while in the bone marrow. Instead, they exit the bone marrow as mast cell progenitors (MCp) and traffic to tissues where

they will complete differentiation under the influence of tissue specific cytokines (137). In mice, MCp express $CD117^{+}$ but contain fewer granules than mature mast cells (137). Full MCp characterization has yet to be determined (136). However, once in the tissue, differentiated mast cells are long-lived and radiation resistant (138). While they can undergo cell division, increase in cell number during infection is usually due to influx of MCp (139).

A major characteristic of mature mast cells is the high numbers of electron-dense secretory granules that are easily visualized with metachromatic stains, such as toluidine blue (140). There are two subsets of mast cells in mice: connective tissue mast cells (CTMC) and mucosal mast cells (MMC) (133). CTMC are defined as the 'classical' mast cell, as these are the cells originally identified by Paul Ehrlich and can be identified by all cationic dyes. MMC on the other hand are more weakly stained by these metachromatic stains due to the lower amounts of heparin in their secretory molecules (133). Other differences between the types of mast cells include the location and types of proteases each mast cell stores within its granules. CTMC are located mainly in the skin and in the tissue parenchyma around blood vessels, while MMC are, as the name suggests, in the mucosal compartments of the lung and gastrointestinal tract (133). In the lung, CTMC produce a wide range of proteases, including chymase (in mice, mouse mast cell protease (mMCP)-1 and mMCP-2), tryptase (mMCP-6 and mMCP-7) and carboxypeptidase A (CPA3), while MMC only produces mMCP-1, mMCP-6, and mMCP-7 (141). Even though there are differences between the two types of mast cells, both play a role in allergic reactions and can be activated through the same mechanisms (132).

Mast Cell Activation

Mast cells are a heterogeneous cell population that can alter their reaction to pathogens and tailor their responses and the subsequent inflammatory response to the type of invading pathogen (128). Mast cells can be activated in response to stimuli such as chemical substances including toxins or venoms (128, 142, 143), through PRR, or by antigens, as in the case with allergies (129, 144). Immune recognition of IAV has been shown to be mediated by numerous PRR: TLR3, TLR7, CLR, NLRP3, Nod2, MAVS, and RIG-I (73-85). Each of these PRR has been shown to be expressed and functional in mast cells (145-148). Mast cell activation is biphasic and can be divided into two responses: 1) immediate degranulation of pre-synthesized stored mediators, and 2) delayed secretion of secondary *de novo* mediators (129, 149, 150). Pre-synthesized and stored mediators include amines, proteoglycans, proteases, lipid inflammatory mediators, histamine, β-hexoaminidase, tryptases, chymases, serotonin, antimicrobial peptides, and accumulated TNF-α, which are stored in granules located in the mast cell cytoplasm (149, 151). *De novo* mediators include a wide variety of cytokines, chemokines, growth factors, and eicosanoids.

These bioactive products contained in granules are released from granules within minutes of stimulation. Mast cell granules are secretory lysosomes and express the lysosomal markers LAMP-1 (CD107a), LAMP-2 (CD107b), and CD63, which can be used for detection of mast cell degranulation (152-154). Early granule units are formed by budding off of the Golgi apparatus and mature by fusing with other immature granules or mature granules (155, 156). Some mediators, such as proteases, are packaged in these immature granules from the onset (157). Biological amines, histamines and serotonin, are

transported into granules from the cytoplasm in a vesicular monoamine transporter-2 (VMAT-2) dependent mechanism (158). Cytokines that are stored in granules, including TNF-α, are produced in the endoplasmic reticulum and are usually transiently expressed on the surface of mast cells, before being endocytosed into a lysosome that fuses with granules adding these cytokines to the stored mediators (159). These mediators are able to remain stable due to the heparin and chondroitin sulfate that composes the granules and gives them an anionic nature (160). Mice that lack heparin sulfate (N-deacetylase/N-sulfotransferase-2 (NDST-2)) cannot form stable mast cell granules containing histamine and proteases, and therefore have lower histamine and protease storage, and degranulation potential (161, 162).

Mast cell degranulation takes place immediately upon stimulation. Once stimulated, mast cells can either release all or part of their contents as the granule fuses with the membrane of the cell to form a pore, or release a full granule into the extracellular space (163). Degranulation requires actin rearrangement of the cell and the use of Rab GTPases and SNARES (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) (160). Thirty Rab GTPases have been identified as modulators in this process (163). Rabs are important in transport of granules from the endoplasmic reticulum to the plasma membrane. Once at the plasma membrane, vesicle associated membrane protein (VAMP) and SNARE proteins are involved in the fusion of the granule membrane to the plasma membrane (164). VAMP proteins are important vesicle fusing proteins, and mast cells from mice lacking VAMP-8 had reduced serotonin and cathepsin D release (164).

Once activated, the granules can release their stored mediators into the immediate environment or intact granules can travel through the bloodstream and lymphatics, acting as a signaling mechanism to recruit other cells (165, 166). Mast cell degranulation induces bronchoconstriction, mucus secretion, vasodilation, and edema, and begins the inflammatory response by recruiting other cells (such as neutrophils, macrophages, and lymphocytes) to the site of mast cell activation (167). One key characteristic of mast cells is that they are long lived, and once they degranulate, are able to reform their granules, a process that can take days or weeks (168).

The secondary *de novo* response from mast cells is delayed as these mediators require enzymatic synthesis or transcriptional and translation up-regulation. Lipid mediators, such as prostaglandins and eicosanoids, are released from mast cells after activation due to enzymatic changes within the mast cell (169). Arachidonic acid is the precursor to both prostaglandins and leukotrienes and is synthesized from cell membrane phospholipids by $cPLA_{2\alpha}$ (170). Arachidonic acid is then converted via cyclooxygenase or 5-lipoxygenase to PGH2 and LTA4, respectively, to initiate the synthesis of biologically active prostaglandins or leukotrienes (171). These mediators can cause vascular leakage and tissue edema by breaking down the barriers between cells and also serve in neutrophil chemotaxis (172, 173). Leukotriene B_4 from mast cells aids in early neutrophil response and bacterial clearance during *Escherichia coli in vivo* (174).

Other mediators, including cytokines and chemokines, must be transcriptionally and translationally produced before being secreted, and are usually released hours post exposure to a pathogen or allergen. Mast cells are capable of producing an extensive list of cytokines and chemokines, including TNF-α, GM-CSF, SCF, IL-3, IL-4, IL-5, IL-6,

IL-10, IL-13, IL-14, IL-16, type I IFN, type III IFN, CCL3 (MIP1-α), and CCL4 (MIP1-β) (167, 175, 176). These mediators recruit other effector cells to sites of mast cell activation and subsequently cause activation of these available cells. Mast cell IL-6 was shown to be important in enhancing neutrophil killing for clearance of *Klebsiella pneumoniae* infection, and IL-4 promotes macrophage killing of *Francisella tularensis* (177, 178).

The most well characterized signaling pathway for mast cell activation is the recognition of antigens through the IgE Fc receptor (FcεRI) resulting in a dependent activation type I hypersensitivity response (128). Mast cells express a large array of Fc receptors. Most notably, mast cells express high levels of FcεR1 that interact with high-affinity IgE receptors. The antigen-binding portion F(ab') faces out, free to bind the antigen (allergen), of which it has been previously sensitized. Once re-exposed to the allergen, the IgE will become cross-linked and trigger mast cell activation. One characteristic of asthma patients is a higher number of mast cells in the lungs compared to non-asthmatic patients, and these mast cells are more likely to degranulate (179). As mast cells are a common participant in the allergic asthmatic response, there are already drugs approved by the FDA to address mast cell activation. Cromolyn sulfate is a mast cell stabilizer that can be aerosolized (180). Montelukast (Singulair®) is a leukotriene receptor antagonist and improves chronic asthma parameters (airway obstruction, patient-reported end-points, and asthma outcomes) in a dose-dependent manner (181). Diphenyhydramine (Benadryl®) is an anti-H1 histamine receptor that blocks histamine production (182). Thus, the stabilization of mast cells by pharmacological inhibitors can be used to decrease pathological effects of mast cell activation.

Mast cells are hematopoietic cells that have a large role in allergic and asthmatic reactions. Additionally, mast cells play a critical role in controlling certain parasitic and bacterial infections. During these infections, mast cells are able to tailor their response to quickly control infections (129). However, their role in virus infection is only now being elucidated. Understanding how mast cells respond to viruses is important because viruses are common triggers of asthma exacerbations (183-187). A full understanding of the interactions between mast cells and viruses will help limit the severity these infections cause.

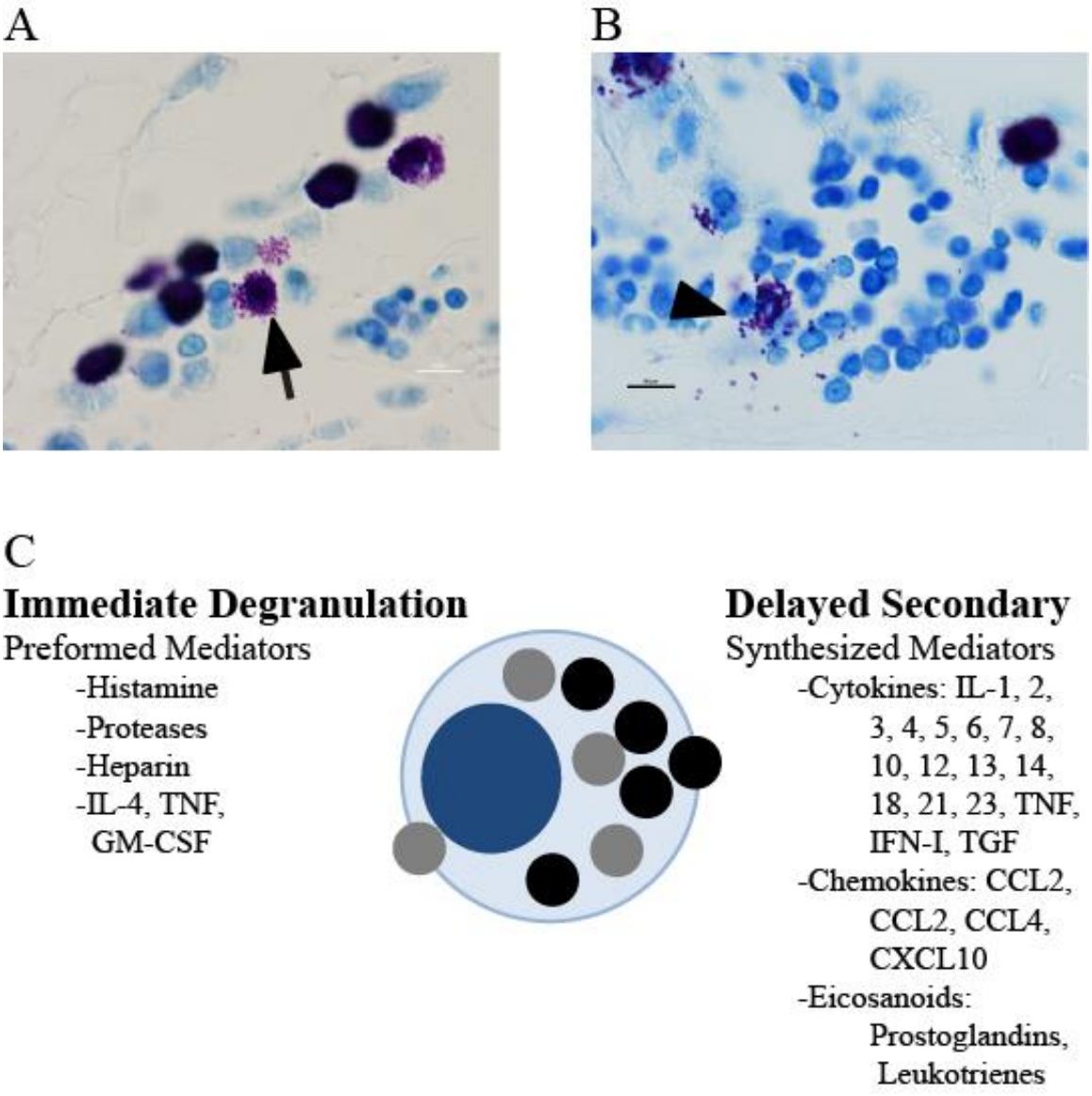

Figure 1.4. Mast cell response to stimuli includes degranulation or production of *de novo* mediators. Mast cells are granular cells enriched in tissues peripheral to the environment including the lung. Formalin-fixed lungs were paraffin embedded. 5μM sections were stained with toluidine blue for analysis by microscopy. Mast cells are stained dark purple in the lungs (A). Mast cells degranulate and release granules when activated. Arrows point to cells that have clearly defined granules within the section. Arrowheads are pointing to granules that have been released into surrounding lung tissue. (B) Mast cell can immediately degranulate when activate, releasing preformed mediators, or newly synthesize mediators, including cytokines, chemokines, and eicosanoids, which are released hours after activation. These mediators contribute to pro-inflammatory inflammation and host defense (C).

Research Goals

Predicting the next strain of IAV that will cause a global pandemic is impossible due to the random mutations IAV can undergo. During pandemics, such as the 'Spanish' influenza pandemic of 1918, one of the reasons for high mortality was a robust immune response, which is why this IAV affected young adults (18-35 years old) so severely (24). Illness during IAV infections often follow a damage-response framework, where damage during pathogen infections is not always the result from the pathogen alone but can be due to a host's immune response (188). Having no immune response allows a pathogen to replicate out of control and cause elevated damage within a host, which is directly caused by the pathogen. However, having too strong of an immune response can also cause damage induced by the host immune response, such as with the cytokine storm that is observed during severe IAV infections (91). As discussed in chapter 1, there is a delicate balance that must be struck by the host in controlling viral replication. Therefore, one way to limit morbidity and mortality during severe IAV infections is to tune the immune response to a manageable level. The research conducted in this thesis aimed to examine novel immune response mechanisms, which initiate the pathological inflammatory response during IAV infection and could be targeted to modulate influenza damage.

While the crucial role of mast cells in bacterial and parasitic infections has been investigated, studies examining their role during viral infections are limited (129-131) Mast cells respond *in vitro* to viruses such as VSV, Sendai virus, Hantavirus, dengue virus, and reovirus (189-192), but the role mast cells play during IAV infection is not well understood. A recent transcriptome analysis of mice infected with a mouse-adapted

2009 pandemic strain of IAV or a recombinant 1918 IAV strain demonstrated an enrichment of transcripts associated with mast cells; furthermore, enrichment of mast cell associated genes positively correlated with IAV-induced pathology (123). The immediate and delayed activation of mast cells can lead to the induction of a pro-inflammatory response, which could potentially cause tissue damage within the lung. Interestingly, humans experimentally infected with influenza have elevated histamine levels, a mediator immediately released from mast cells upon activation, which correlated with the onset of symptoms (193, 194). Taken together, these studies suggest that mast cells may be playing a role in IAV infections in both humans and mice.

For my thesis, we hypothesized that mast cells are actively infected with IAV, resulting in their activation, which culminates in their contribution to the amplification of the pathological inflammatory response induced by IAV infection. We have addressed this hypothesis with the following two specific aims: 1) Identify the receptors mast cells utilize to initiate IAV-induced inflammatory disease, and 2) Determine the key characteristics of the hemagglutinin protein necessary for mast cell activation.

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CHAPTER TWO

MATERIALS AND METHODS

Viral Strains

H1N1 A/Wilson-Smith Nucleus/33 virus in allantoic fluid was originally obtained from Dr. David Topham (University of Rochester) and A/Puerto Rico/8/34 (A/PR/8/34; H1N1) was originally purchased from Charles River. A/WSN/33 NA130 was originally obtained from Dr. Peter Palese (Mount Sinai School of Medicine). All viruses except human isolates and UV inactivated virus were subsequently grown in embryonic chicken eggs. Human influenza virus isolates were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH.

Constructing A/WSN/33 D225G

A/WSN/33 D225G recombinant virus was created by an eight-plasmid transfection system using reverse genetics system (1). Eight plasmids containing the cDNA of A/WSN/33 [pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, and pHW188-NS] on the pHW2000 plasmid backbone were gifts from Dr. Robert Webster (St. Jude Medical Research Center). Plasmid containing IAV HA cDNA with specific mutation D225G that changes sialic acid binding preference (2) was a gift from Dr. Leo Poon (University of Hong Kong). All IAV plasmids were grown and over-expressed in *Escherichia coli* (NEB5α or NEB10β; New England Biolabs) and purified via Qiagen plasmid purification kit. For transfections,

$4x10^5$ 293T cells/well were plated in 6-well plates in DMEM supplemented with 10% FBS and 1X Pen/Strep. The next day, the cells were transfected with the 8 bidirectional IAV plasmids using *Trans*IT®-293 Transfection Reagent (Mirus Bio LLC) per the manufacturer's protocol. Roughly, 0.8μg of each of the 8 plasmids were combined with serum-free DMEM and allowed to incubate at RT for 45 minutes with 2 μL/μg of TansIT-293T transfection reagent (Mirus Bio LLC). This mixture was then added drop-wise to the cultured cells and moved to a 37°C 5% CO_2 incubator for 72 hours. The transfected cells and supernatant were then transferred to a confluent flask of MDCK cells in low-serum media (DMEM high glucose, 1.4% (w/v) BSA. 0.14% (v/v) sodium bicarbonate) for 72 hours to amplify the recombinant viruses. Virus-containing supernatants were cleared by centrifugation for 10 minutes at 400 x *g*. Supernatant was aliquoted and stored at -80°C. Virus was grown in eggs and titered before use in experiments.

UV Virus Inactivation

To UV inactivate A/WSN/33, egg-grown virus was placed in a 6 cm petri dish in a thin layer. This was placed on ice ~10cm from a 254 -W UV light for 60 minutes. As a control, A/WSN/33 was placed in a petri dish and kept on ice without being exposed to UV light. The virus was aliquoted and stored at -80°C until use. The virus was titered using IAV plaque assay to confirm inactivation of the virus. UV inactivated virus had decreased PFU compared to control A/WSN/33. To further confirm UV inactivation, cytokine and chemokine production from infected mast cells was assessed, because their production requires the production of newly synthesized vRNP (36). Mast cells were

treated with equivalent numbers of infectious particles of A/WSN/33 for these assays based on the starting PFU values.

Mouse Strains and Infection Protocol

B6.Cg-*kit*$^{W\text{-}sh}$ mice were originally purchased from Jackson Laboratories and subsequently bred in house. C57Bl/6J mice were bred in house or purchased from Charles River. Specific knock-out bone marrow was kindly provided by multiple investigators: RIG-I by Dr. Michael Gale (University of Washington) (3), MAVS by Dr. Mathias Schnell (Thomas Jefferson University)(4), MYPS/STING by Dr. John Cambier (National Jewish Health)(5), CARD9 by Dr. Tobias Hohl (Fred Hutchinson Cancer Center)(6), STAT6 by Dr. Daniel Campbell (Benoyra Institute)(7), MyD88 by Dr. Mark Jutila (Montana State University) (8), TRIF by Dr. Brent Berwin (Geisel School of Medicine at Dartmouth College; originally from Jackson Laboratories), MyD88/TRIF by Dr. Kate Fitzgerald (University of Massachusetts Medical School)(9), Par2 (Jackson Laboratories), Dap12 by Dr. Lewis Lainer (UCSF)(10), FcεRIγ (Taconic), ASC by Dr. Vishva Dixit (Genentech)(11), and NLRP3, NOD2, and RIP2 by Gabriel Nuñez (University of Michigan)(12, 13).

Mice were intranasally infected with 1000-1500 plaque forming units (PFU) of A/WSN/33, A/PR/8/34, A/WSN/33 NA130, or A/WSN/33 D225G, as indicated in the text, under 2,2,2-tribromoethanol (Avertin) anesthesia. At the indicated times after IAV infection, mice were given a lethal overdose of pentobarbital. Broncheoalveolar lavage fluid (BALF) was collected by washing the lungs with 2 ml of PBS containing 50 mM EDTA. Lungs were stored at -80°C for viral titers or paraffin embedded for histological

analysis. BALF was pelleted by centrifugation. Cells were resuspended in 200uL PBS containing 50mM EDTA and analyzed for total cell numbers or for cellular recruitment by cytospins. Clarified supernatant was analyzed using a lactate dehydrogenase kit (Promega), BCA assay (Thermo Scientific), or luminex assay for cytokine and chemokine analysis (Millipore). For weight loss studies, mice were infected as previously described and weighed daily. When mice lost 25% of their initial body weight, they were humanely euthanized. All animal protocols were approved by the Montana State University Institutional Animal Care and Use Committee.

IAV Propagation in Eggs

Fertilized eggs (Charles River) were placed in an egg incubator at 37.5°C with a wet bulb temperature for humidity at 31°C for ten days with rotation. Infection took place 11-12 days post-fertilization. Eggs were first candled to check viability and to mark the air sac. After candling, eggs were disinfected with 70% EtOH and 100 PFU/egg of virus in 100μL PBS was injected into the allantoic fluid, avoiding blood vessels, using a 21G needle and the hole was sealed using glue (Elmer's). Eggs were put back into the egg incubator for 55 hours, followed by a 4°C incubation for at least 4 hours to kill the embryos. To harvest the allantoic fluid, eggs were disinfected with 70% EtOH and allantoic fluid was collected, pooled, frozen in liquid nitrogen, and stored at -80 for titering.

IAV Plaque Assay

MDCK cells (ATCC Cat. No. CRL-2939) were grown to confluence in a 6-well plate in a 37°C incubator with 5% CO_2. On the day of titering, 2x Dulbecco's Modified Eagle Medium (DMEM) [20g/L DMEM low glucose + sodium pyruvate and l-glutamine (HyClone), 0.4% (w/v) Probumin (Millipore), 10mL/L 100x Pen-Strep (HyClone), pH 7.4 with 1M sodium biocarbonate] was equilibrated to 37°C in a water bath and 1.6% SeaKem® agarose (Lonza) was warmed to 48°C in a water bath. Viruses were serial diluted 10-fold in serum free DMEM and placed in a 35°C 5% CO_2 incubator. The MDCK cells were washed with PBS and placed in serum free DMEM for serum starvation for 30 minutes. After serum starvation, media was removed and the virus dilutions were added to the cells in duplicate wells and placed in the 35°C 5% CO_2 incubator for one hour, tipping the plates every 10 minutes to ensure the monolayer remained covered and did not dry out.

After one hour, 2x DMEM and 1.6% agarose were mixed in a 1:1 ratio and 0.5µg/mL TPCK-treated trypsin (Worthington Biochemical Corporation). The virus was removed by aspiration and 3mL of the DMEM/agarose/trypsin mixture was added to the monolayer. Once the agarose had set, the plates were incubated in a 35°C 5% CO_2 incubator for 3-4 days. Viruses that preferably bind to α2,3-sialic acid linkages tend to form clear plaques by 4 days, while viruses that preferably bind to α2,6-sialic acid linkages had plaques at 3 days.

To fix the cells, 3mL of a 1:1 methanol:acetone solution was added to the wells and incubated overnight. The next day, the agarose overlays were removed with a spatula

and monolayers were stained with enough 0.1% crystal violet dissolved in water to cover the monolayer (~500uL). The crystal violet was washed off by repeatedly dunking the 6-well plates in warm water and the cell monolayers were left to dry. Titers were calculated using the equation:

$$\frac{\text{\# of plaques}}{(\text{dilution} * \text{volume of virus in mL})}$$

IAV viral titers in the lungs were quantified using a standard plaque assay. Briefly, lungs were homogenized in 2 ml of DMEM using a dounce homogenizer. The lung tissue was centrifuged for 10 seconds to pellet lung debris. Clarified supernatants were diluted as described above and added to MDCK cells for titering.

Histological Analysis of Lungs

Lungs were inflated with 10% buffered formalin phosphate, placed in the same solution for at least 24 hours, then paraffin embedded. Sections of 5μm were deparaffinized with clearite, rehydrated, and then stained with hematoxylin and eosin (H&E) to assess lung inflammatory cell infiltration or toluidine blue (T-blue) to detect mast cells. Stained lung sections were visualized on an upright 80i eclipse microscope (Nikon) and images were captured with a DS Ri1 color camera (Nikon).

H&E

Tissue was placed in hematoxylin for one minute 30 seconds and then washed three times in running water for one minute. Prior to eosin counterstain, tissue samples were placed in bluing solution, clarifying solution, and 70% EtOH for one minute, with a

one minute rinse in running water between each solution. After one minute in eosin, sections were dehydrated through an alcohol gradient, placed in xylene to remove residual alcohol, and mounted with a thin cover glass for microscopic examination.

Toluidine Blue

T-blue stock solution was made by adding 1% (w/v) of Toluidine Blue O (Sigma-Aldrich) into 70% EtOH. A 10% working solution into 1% (w/v) sodium chloride was used to stain lung sections. Deparaffinized and hydrated samples were placed in T-blue working solution for one minute 30 seconds with slight agitation, then rinsed under running water three times for one minute each. Samples were dehydrated through an alcohol gradient and cover-slipped.

Growth of Bone Marrow Cultured Mast Cells (BMCMC)

BMCMC were grown as previously described (14). Briefly, femurs from 4-8 week old mice were removed and bone marrow cells were collected by centrifugation for 30 seconds at 2348 x g. Cells were resuspended in RPMI supplemented with 1% non-essential amino acids, 1mM sodium pyruvate, 1% HGPG, 10 mM HEPES, 10% fetal bovine serum, and 0.1% 2-mercaptoethanol. For the first three weeks, the cells were incubated with 10 ng/mL recombinant murine IL-3 (Peprotech). In subsequent weeks, 25 ng/mL recombinant murine stem cell factor (Peprotech) was added along with IL-3. After five weeks, the purity of the population was >90% mast cells as determined by flow cytometry analysis using anti-FcεR1α (BioLegend) and anti-CD117 (BioLegend). Five and six week cultures were used for experiments.

Generation of Mast Cell Knock-in Mice

BMCMC 'knock-in' mice were prepared as described previously (15-17). Briefly, BMCMC were generated as described above. B6.Cg-*kit*$^{W\text{-}sh}$ Mice 3-4 weeks of age were reconstituted with 5x10^6 BMCMC via intravenous injection and rested for ~8-10 weeks before use.

In Vitro Mast Cell Activation Assay

For activation assays, 5 2.5x10^5 BMCMC were plated per well in a 96-well U-bottom plate. Plates were centrifuged and supernatant was discarded. Virus was added at an MOI of ~0.4-1 in a final volume of 100μL media or PBS, as indicated in text, and incubated for 30-60 minutes for degranulation studies and 4-6 hours for assessment of secondary mediator activation. As positive controls, BMCMC were stimulated with LPS (5 μg/mL; List Biological Laboratory) for cytokine/chemokine release or the calcium ionophore A23187 (40 nM; Fisher Scientific) for leukotriene synthesis and mast cells degranulation.

For inhibition of viral entry pathways, the same procedure was followed. Each compound was diluted into media or PBS before being added onto the cells. Compounds in these experiments were bafilomycin A (200nM; DMSO; Tocris Bioscience cat. No. 1334), cytochalasin D (20μM; DMSO; Sigma-Aldrich D7283), Dynasore hydrate (120μM; DMSO; Sigma-Aldrich D7693), 5-(N-Ethyl-N-isopropyl) amiloride (EIPA; 120μM; DMSO; Sigma-Aldrich A3085), and genistein (50μM; DMSO; Sigma-Aldrich G6649). BMCMC were pre-incubated with the indicated compound for one hour, and each compound was included with the activation at the same concentration.

Assay for Cytokine and Chemokine Secretion

In vitro BMCMC activation assay supernatants from 4-6 h post-treatment and BALF from the indicated time points were analyzed for cytokine and chemokines using custom Milliplex® plates (Millipore) or by IL-6 ELISA (Biolegend). Murine IFN-α were measured using Procarta® 2-plex assay (Affymetrix). Plates were read using a BioPlex® 200 (Bio-Rad) or a SpectraMax Paradigm plate reader (Molecular Devices).

Histamine and Leukotriene B_4 Enzyme Immunoassays (EIA)

Histamine and leukotriene B_4 EIA assays were conducted following the manufacturer's instructions (Cayman Chemical). Briefly, the samples were incubated with an acetylcholinesterase linked histamine or leukotriene B_4 tracer for 24 hours or overnight, respectively. Plates were washed and Ellman's Reagent was added to detect the tracer labeled histamine or leukotriene B_4. Obtained results are inversely proportional to free histamine or leukotriene B_4 present in the well.

Annexin V Binding for Mast Cell Degranulation

Annexin V binding was used to measure degranulation of mast cells as previously described (18). BMCMC were activated for 30 minutes as stated above. Activated BMCMC were then washed with cold PBS. Cells were stained with 5μL/well Annexin V (BioLegend) in 100μL Annexin V binding buffer (10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$, pH 7.4) for 30 minutes at room temperature. Immediately after incubation, cells were moved directly into 100μL Annexin V binding buffer. Annexin V binding was

measured with a LSRII (BD Biosciences) and data was analyzed via FlowJo software (Tree Star).

Intracellular Staining for Viral Proteins

To measure virus infectivity of mast cells, we stained BMCMC for intracellular expression of the IAV NS-1 protein. Briefly, $2.5x10^5$ BMCMC were plated per well in a 96-well U-bottom plate. Virus was added at MOI ~0.4-1 in a final volume of 100 μL and incubated for 4-6 hours. The cells were then fixed with 100 μL BD Cytofix/Cytoperm (BD Biosciences) for 30 minutes. Next, cells were stained with a mouse monoclonal antibody against the IAV NS1 protein (NS1-1A7; BEI Resources) for 30 minutes in BD Perm-wash buffer (BD Biosciences). Subsequently, the cells were washed with BD Perm-wash buffer and then stained with PE-labeled goat anti-mouse IgG F(ab') fragments (Jackson ImmunoResearch Laboratories). Cells were then washed with BD Perm-wash buffer and resuspended in FACS Buffer. Data was collected using a LSRII (BD Biosciences) and data was analyzed via FlowJo software (Tree Star).

Statistical Analysis

Statistical significance was determined by using a Mann-Whitney U-test or one-way ANOVA using Prism 5 (Graphpad Software).

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CHAPTER THREE

MAST CELLS AND INFLUENZA A VIRUS: ASSOCIATION WITH ALLERGIC RESPONSES AND BEYOND

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MAST CELLS AND INFLUENZA A VIRUS: ASSOCIATION WITH ALLERGIC RESPONSES AND BEYOND

The following work has been officially submitted to a peer-review journal for publication

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Abstract

Influenza A virus (IAV) is a widespread infectious agent commonly found in mammalian and avian species. In humans, IAV is a respiratory pathogen that causes seasonal infections associated with significant morbidity in young and elderly populations and has a large economic impact. Moreover, IAV has the potential to cause both zoonotic spillover infection and global pandemics, which have significantly greater morbidity and mortality across all ages. The pathology associated with these pandemic and spillover infections appear to be the result of an excessive inflammatory response leading to severe lung damage, which likely predisposes the lungs for secondary bacterial infections. The lung is protected from pathogens by alveolar epithelial cells, endothelial cells, tissue resident alveolar macrophages, dendritic cells, and mast cells. The importance of mast cells during bacterial and parasitic infections has been extensively studied, yet the role of these hematopoietic cells during viral infections is only beginning to emerge. Recently, it has been shown that mast cells can be directly activated in response to IAV, releasing mediators such histamine, proteases, leukotrienes, inflammatory cytokines, and antiviral chemokines, which participate in the excessive

inflammatory and pathological response observed during IAV infections. In this review, we will examine the relationship between mast cells and IAV, and discuss the role of mast cells as a potential drug target during highly pathological IAV infections. Finally, we proposed an emerging role for mast cells in other viral infections associated with significant host pathology.

Introduction

Influenza A virus (IAV) is a common human respiratory pathogen which causes annual seasonal infections with a low frequency of morbidity and mortality, usually limited to the young (<5 years) and the elderly (>65 years) populations. Importantly, IAV has the potential to cause global pandemics, which can significantly increase morbidity and mortality throughout the entire population (1). In the past century, there have been four major IAV pandemics: the 1918 H1N1 'Spanish' influenza, the H2N2 'Asian' influenza in 1957, the H3N2 'Hong Kong' influenza in 1968, and more recently, the reemergence of a pandemic H1N1 (H1N1pdm) influenza in 2009 (2). Moreover, significant spillover infections from the zoonotic avian reservoir of IAV continue to have an impact on the human population, this includes the current avian H5N1 and H7N9 IAV outbreaks in Southeast Asia (3). To date these H5N1 and H7N9 outbreaks have remained a spillover event, but the potential of these novel avian IAV strains to develop the ability to efficiently transmit human-to-human through aerosol droplets exists (3-5); thus increasing the threat of new global pandemics.

As an RNA virus that lacks proofreading capabilities, IAV has a high mutation rate, resulting in significant antigenic drift in the immunodominant hemagglutinin (HA)

and neuraminidase (NA) proteins. Furthermore, owing to its segmented genome, IAV can undergo genetic reassortment (antigenic shifts), resulting in novel IAV strains with the potential to rapidly transmit between humans to cause a new pandemic. Given these factors, the next pandemic IAV strain is nearly impossible to predict, leading to many challenges in vaccine development. Current vaccine strategies take approximately 6 months for production. During the 2009 H1N1 pandemic, this delay resulted in no effective vaccine being available for the first wave of the pandemic (2). Thus, it is necessary to find alternative ways to alleviate and treat IAV-induced disease during the early wave(s) of a novel pandemic IAV outbreak.

Antiviral drugs are an obvious front line of defense against the emergence of novel IAV strains. Currently, two main classes of antiviral drugs are approved to treat IAV-infected patients. The first class of antiviral drugs target the M2 ion channel (amantadanes), which is important for virus uncoating. However, amantadanes are no longer recommended for prophylaxis or treatment of IAV due to widespread resistance among current human seasonal H1N1 and H3N2 isolates (6-8). The second class of antiviral drugs targets the enzymatic active site of the viral NA. The viral NA is a sialidase capable of hydrolyzing terminal sialic acid residues from glycoproteins and glycolipids. The NA is crucial in allowing the IAV to traverse the glycan rich soluble mucins in the respiratory tract, as well as allowing newly formed virions to be released from host cells, to be shed into the extracellular space for dissemination within a host and transmission between hosts. NA inhibitors are becoming of limited efficacy as well, due to emerging resistance among IAV isolates found in humans and the requirement for early administration (within 48 hours of the presentation of symptoms) for maximal

effectiveness (2, 7, 9-12). Therefore, additional antiviral drugs are required to limit IAV-induced disease and fight the spread of IAV. Numerous drugs are currently in development which target viral entry, viral transcription, or host factors necessary for IAV replication (9). However, the effectiveness of these drugs against IAV in the clinical setting is unknown.

An alternative front line defense against the emergence of novel IAV strains is to target the inflammatory pathways that lead to lung damage and loss of function (13, 14). Alveolar epithelial cells, endothelial cells, tissue-resident alveolar macrophages, dendritic cells, and mast cells protect the lungs, as these cells are readily able to respond to invading pathogens. Pandemic strains of IAV, including the 1918 ‘Spanish’ influenza and the 2009 H1N1pdm influenza, and spillover infections with avian IAV isolates, can produce excessive tissue damage and pathological changes to the lung architecture (1, 15, 16). Current evidence suggests the lung injury induced during IAV infection is the result of excessive leukocyte infiltration and an exaggerated inflammatory cytokine response that is disproportionately high relative to the level of viral replication, which has been termed a ‘cytokine storm’ (16-21). Selectively dampening the inflammatory response in mice has been shown to increase survival following IAV infection without impairing viral clearance (16, 17, 19-22). Thus, understanding the inflammatory cascade responsible for the immunopathology observed following IAV infection is imperative for the development of novel immunotherapeutics aimed at limiting IAV-induced disease and pathology.

Macrophages and neutrophils are recruited at excessive levels following infection with the 1918 or H5N1 influenza strains (16). More recently, it has been demonstrated

that mast cells play a pivotal role in initiating and/or amplifying the immunopathological ‘cytokine storm’ and inflammatory leukocyte recruitment in the respiratory tract during IAV infection (23-25). Mice infected with either H1N1 or H5N1 IAV demonstrated elevated levels of inflammatory cytokines and chemokines during infection. Conversely, mice lacking mast cells or treated with mast cell stabilizing agents show a reduction in the levels of these inflammatory mediators that correlates with a decrease in the recruitment of inflammatory cells to the lungs during infection (23, 24). Therefore, it is crucial that the individual and collective roles of these inflammatory cells, with each other and with the epithelial and endothelial compartments, during pathological IAV and other pathological viral infection, be more thoroughly examined.

Mast Cell Biology

Mast cells are tissue resident, granule-containing cells capable of regulating both the innate and adaptive immune response (26). Enrichment of mast cells at environmental interfaces allows these cells to be among the first to respond during pathogen invasion, along with dendritic cells and epithelial cells (27). Moreover, mast cells are typically situated near blood vessels, lymphatics, and nerve endings, enabling them to have long range effects on the host response to pathogens (27, 28). As such, mast cells are critical to immune surveillance, eliciting an immediate reaction to invading pathogens, and initiating an appropriate innate and adaptive immune response.

Phases of the Mast Cell Response

Mast cells have two distinct phases of activation; immediate degranulation, resulting in the release of pre-synthesized mediators, and delayed secretion of secondary *de novo* synthesized mediators (27, 29, 30). The delayed secretion of secondary *de novo* effector molecules produced by mast cells can be further segregated into two classes: 1) prostaglandins and eicosanoids released within minutes of activation, and 2) cytokines, chemokines, and growth factors that are released within hours of stimulation (Figure 1). Together, these mast cell outputs can increase epithelial and endothelial cell permeability and activation state, which together with chemotactic molecules, results in increased inflammatory cell recruitment to infected tissues (Figure 2).

Mast cell granules contain histamine, TNF-α, amines, β-hexosaminidase, serotonin, antimicrobial peptides, and proteases (tryptases and chymases) bound to either heparin or chondroitin sulfate through electrostatic interactions (29, 31-33). Upon stimulation, the granules are released from the cell via a calcium-dependent exocytosis process. Once expelled, the granules can either discharge the stored mediators into the immediate environment or intact granules can travel through the bloodstream and lymphatics, acting as a signaling mechanism to activate and recruit other cells to the infected tissue (34, 35). Histamine is a potent inflammatory molecule, which increases vascular permeability, induces vasodilation, and stimulates bronchial smooth muscle contraction. The inflammatory cytokine TNF-α promotes local and systemic inflammation while enhancing neutrophil recruitment to the site of infection. Granule proteases are capable of increasing vascular permeability and enhancing the recruitment of neutrophils to the site of inflammation (36-39) or can act directly to detoxify toxic

proteins (40-43). Interestingly, the local homeostatic cytokine milieu of a tissue modulates the precise granule components, allowing mast cells to adapt to their local environment to mount a tissue appropriate inflammatory response (44, 45). Following activation, mast cells are unique in that they replenish their granules, usually within weeks of activation (46, 47). This ability to regranulate allows mast cells to tailor the composition of their granules, and thus be more prepared for reinfection (Figure 2) (27).

After the immediate mast cell degranulation response, the arachidonic acid-dependent inflammatory mediators, such as leukotrienes and prostaglandins, are rapidly produced and released from mast cells due to enzymatic, rather than transcriptional, changes within the mast cell (48). These lipid mediators contribute to local vascular permeability, tissue edema, and the recruitment of neutrophils and other inflammatory cells (49-51).

Finally, *de novo* synthesized cytokines, chemokines, and growth factors are released hours following activation through transcriptional and translational up-regulation. The multitude of cytokines, chemokines, and growth factors released by mast cells include *de novo* synthesized TNF-α, IL-4, IL-5, IL-6, IL-13, IL-17, and (32, 52). These mediators activate tissue-resident cells, while recruiting additional effector leukocytes and lymphocytes to maintain the inflammatory state for a prolonged time. In conclusion, through the release of numerous chemotactic factors and vasodilators, mast cells are optimized for the rapid initiation and propagation of an acute inflammatory response through degranulation, production of bioactive lipids, and secretion of cytokines and chemokines. The resulting leukocyte and lymphocyte infiltrate can then help to maintain the inflammatory state if the infection persists (Figure 2).

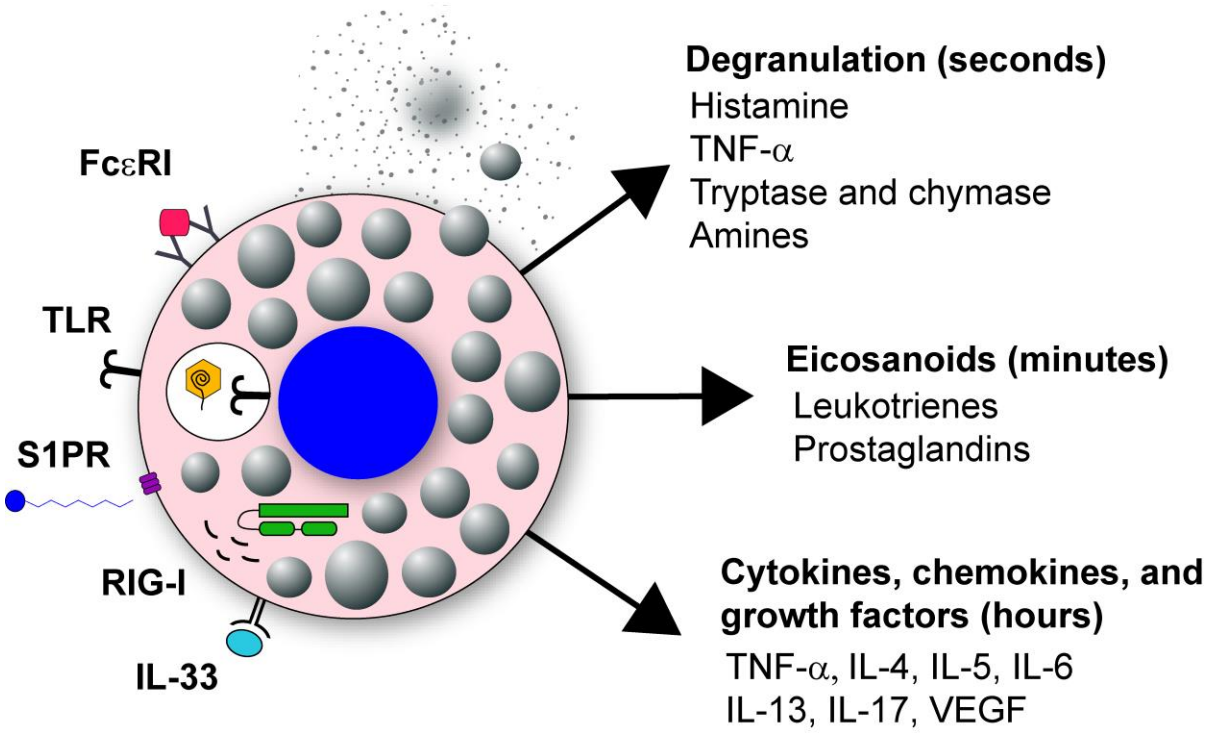

Figure 3.1**.** Mast cell activation in response to viral infection. Mast cells are classically known for their response to polyvalent cross-linking of IgE in the FcεR1 receptor, which is important in protective immunity to helminth worm infection and pathologically associated with allergic disease. However, mast cells also are important tissue sentinel cells for initiating inflammatory response to pathogens. Mast cells can recognize and respond to viruses through several different receptors. These receptors include TLR signaling, such as TLR3 detection of dsRNA, sphingosin-1-phosphate (S1P) binding to its receptor S1PR, and RIG-I recognition of uncapped vRNA. Engagement of these receptors results in mast cell activation leading to immediate degranulation, the *de novo* synthesis of eicosanoids within minutes of activation, and the *de novo* synthesis of numerous cytokines, chemokines, and growth factors within hours of activation.

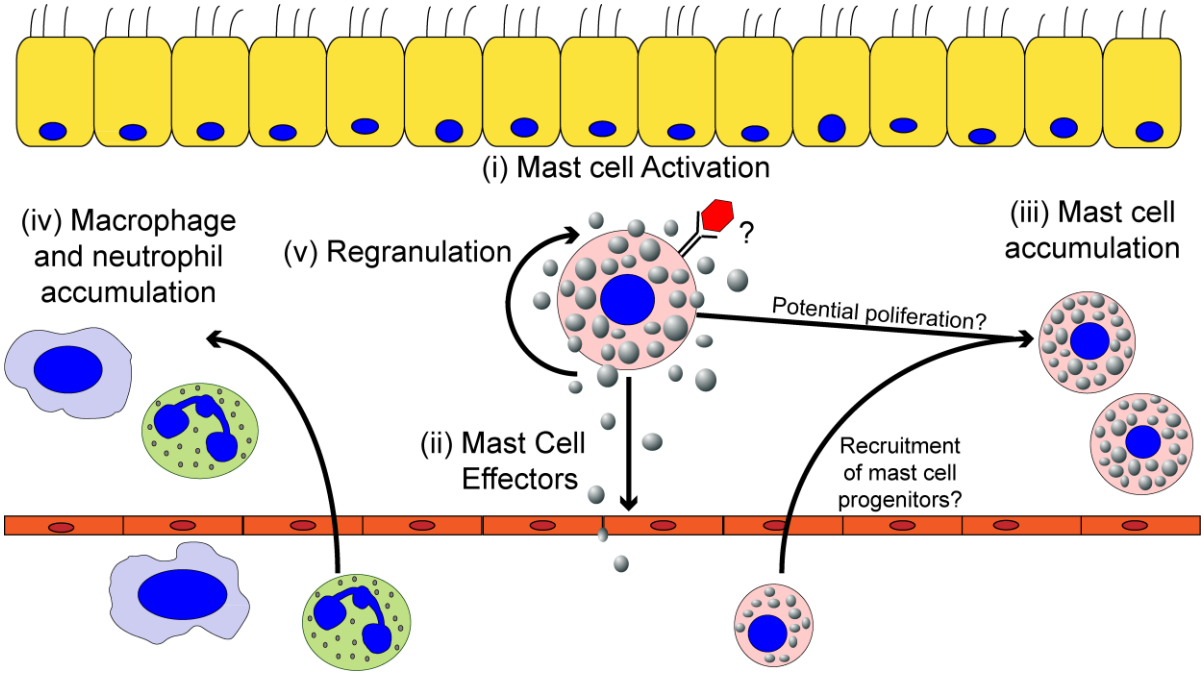

Figure 3.2. The effects of mast cell activation on the inflammatory environment induced by viruses. Within the tissues, mast cells can be activated by viruses (i) resulting in the secretion of effector molecules (ii). Mast cell-derived effector molecules act within the local tissue environment or at distal site to mediate the accumulation of mast cell progenitors (iii) and leukocytes (iv) to the site of infection. Mast cell accumulation in the infected tissues could be due to either the recruitment and differentiation of mast cell progenitors to the infected tissue and/or proliferation of the tissue-resident mast cell population. Mast cell activation can participate in limiting viral replication in the local tissue and viral dissemination, but if left unchecked can cause significant tissue damage, vascular leakage, and tissue edema. Finally, activated mast cells can survive the pathogenic insult and replenish mast cell granules to return the mast cell to a basal state to survey the tissue for future pathogenic insults

De novo Mast Cell
Recruitment during Inflammation

In addition to tissue-resident mast cells, mast cell progenitors can be recruited to sites of acute or chronic inflammation. How the recruitment of these mast cell progenitors is regulated is just now beginning to be understood. Mucosal mast cells (MMC), the

dominant type of mast cell in the lung, develop from the bone marrow as mast cell progenitors (53). In an asthma model, following aerosolized challenge with ovalbumin, mast cell progenitors are rapidly recruited into the lungs, peaking day 1 after challenge (54). In this ovalbumin-induced allergic airway inflammatory disease, multiple pathways are critical for mast cell progenitor accumulation in the lungs. Integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ regulate the migration of mast cell progenitors to the lungs through VCAM-1 interactions (55). Moreover, CXCR2 expression in a radio-resistant cell population is important in regulating mast cell progenitor recruitment to the lungs, likely through its regulation of VCAM-1 on the pulmonary endothelium (56). NKT cells are also able to induce mast cell progenitor accumulation in the lungs through an IL-9 dependent pathway (57). Finally, both prostaglandin E_2 and leukotriene B_4 (LTB_4), which can be highly produced by mast cells, have been shown to enhance chemotaxis of mast cell progenitors (58, 59). In addition to their well-elucidated role in allergic airway disease, there is strong evidence for an accumulation of mast cells in the intestinal tract during helminth infections (60). Furthermore, mast cell precursors appear to accumulate at sites of viral infection including IAV, Sendai virus, infectious bursal disease virus, and Newcastle disease infection virus (61-65). Accumulation of mast cell progenitors occurs either in a mast cell degranulation-dependent (24, 62-65) or -independent manner (61, 66). Therefore, mast cell activation can result in the local accumulation of mast cells in infected tissue, further augmenting the role these cells can play during infection (Figure 2).

Expression of Pattern-Recognition Receptors by Mast Cells for Sensing Invading Microbes

Mast cells express a large array of innate cell surface and cytosolic receptors that mediate their activation, and as such are integral cells in initiating appropriate immune responses to infectious agents. Notably, mast cells express a large array of Fc receptors including FcεRI, FcγRI, and FcγRIII (67). Mast cells are also able to respond through a wide variety of pattern-recognition receptors (PRR), including Toll-like receptors (TLR), Nod-like receptors (NLR), Retinoic-acid inducible gene 1-like receptors (RLR), and C-type lectin receptors (CLR), each of which play an essential role in innate immunity by detecting conserved molecular patterns expressed by pathogens (68-82). Mast cells can also be activated through engagement of complement receptors (28), CD48 (83, 84), and integrins (85). Lastly, mast cells can respond to pathogens indirectly through the IL-33 signaling pathway (48). Thus, mast cells are capable of responding to a broad range of pathogen-derived or pathogen-induced stimuli (Figure 1). Interestingly, mast cells do not respond uniformly to all input stimuli (86). For example, signaling through TLR4 leads to a strong pro-inflammatory cytokine response, but limited mast cell degranulation. Conversely, signaling through TLR2 induces both an inflammatory cytokine response and mast cell degranulation (87). Importantly, mast cell activation therefore is an important rheostat for the immune system, which will likely modulate to the appropriate response. However aberrant activation or prolonged activation may elicit tissue immunopathology.

Role of Mast Cells in Allergies and Asthma

Mast cells are most frequently recognized for their detrimental role during an allergic response. Following an initial exposure to antigen (Ag), activated B cells can undergo class switching, resulting in the secretion of IgE. The high-affinity IgE receptor, FcεRI, expressed on the surface of mast cells, binds to the Fc portion of IgE, sensitizing the mast cells. Upon subsequent exposures, polyvalent Ag cross-links the surface bound IgE resulting in mast cell degranulation and the production of bioactive lipids and cytokines and chemokines (67, 88, 89).

Mast cells have also been recognized for their role in asthma. Asthma is a pleomorphic disease characterized by recurrent airway restriction, shortness of breath, wheezing, and coughing. Within asthma patients, including both atopic (allergic) and non-atopic (intrinsic), the number, localization, and phenotype of mast cells is altered. Repeated activation of the pulmonary mast cells by the allergen results in mast cells, which are more likely to degranulate, compared to non-asthmatic patients (90, 91). Overall, the mast cell response contributes to the bronchial constriction, chronic inflammation, and tissue remodeling typical of asthma patients.

It is now well documented that infection with respiratory viruses, including IAV, rhinovirus, and respiratory syncytial virus (RSV), often exacerbate asthma (92-96). These upper respiratory tract infections frequently lead to hospital admission for asthma patients (97). Interestingly, asthma was the most common co-morbidity among hospitalized patients during the 2009 H1N1pdm IAV pandemic (98-101). A state of hyperresponsiveness in the asthmatic patients, as well as increased levels of inflammatory molecules (e.g. histamine, IL-6, and leukotriene) are believed to contribute to asthmatic

exacerbation from viral infection (102). Thus, it is critical we understand the interactions of mast cells with viruses in both naïve hosts and those with chronic inflammatory conditions, which alter mast cell numbers and function.

Is There a Role for Mast Cell Activation and Mediators during Pathological Viral Infections?

Numerous highly pathological viral infections cause significant disease through immune-mediated pathology to tissue and/or induction of vascular permeability. For example, during dengue virus infections there is significant vascular permeability, which is associated with severe disease and mast cell activity (51, 103). Additionally, severe respiratory virus infection can induce acute respiratory disease syndrome (ARDS), which is associated with significant epithelial-endothelial dysfunction and excessive activation of macrophages and neutrophils (104). ARDS has been observed during experimental IAV infection of animal models, as well as in people naturally infected with highly pathological IAV isolates, such as the 1918 H1N1 'Spanish' influenza strain and the recent zoonotic outbreaks of avian H5N1 and H7N9 IAV strains (105-107). An eloquent transcriptome analysis by Josset *et al*, which compared highly pathological versus seasonal IAV infections, detected a strong transcriptional signature of macrophages and neutrophils in the lungs of mice with severe IAV infection (108), which fits with prior histological observations (16). Intriguingly, Josset *et al* also saw a strong transcriptional contribution of mast cells during these severe IAV infections (108); however, these authors did not explore the role this cell population might play in the observed disease. We propose that, in addition to macrophages and neutrophils, mast cells may contribute

to the excessive inflammatory response and vascular problems observed not only during highly pathogenic IAV, but also in a range of highly pathogenic viral infections as further discussed below.

Influenza Virus

Pandemic isolates and the emerging highly pathogenic avian strains of IAV are capable of inducing a robust inflammatory response, which causes significant damage within the lungs and the ultimate restructuring of the lung architecture (1). In humans experimentally infected with IAV, detection of histamine metabolites correlates with clinical symptoms (109, 110). Moreover, emerging data in the murine model of IAV suggests a link between mast cell recruitment and activation with lung immunopathology. Following inoculation with a mouse adapted strain of the 2009 H1N1pdm IAV (A/California/04/2009) mice develop significant pathology and inflammation, recapitulating clinical observations from the 2009 pandemic in humans, while mice infected with a non-adapted strain do not (108, 111). In those mice inoculated with the mouse-adapted 2009 H1N1pdm IAV an enrichment of genes for activated macrophages, neutrophils, and mast cells were observed when compared to mice inoculated with the non-pathogenic strain (108). Moreover, this same observation was made during infection with recombinant 1918 H1N1 (108). Thus, it appears that early accumulation of activated macrophages, neutrophils, and mast cells correlates with the immunopathology associated with pandemic IAV infections.

As this prior transcriptomic study suggested (108), increased mast cell density was observed in the nasal mucosa, trachea, lung parenchyma, and mediastinal lymph

node following infection with a highly pathological H5N1 isolate (A/chicken/Henan/1/2004) (24). While these data demonstrated that mast cells are increased in the lungs of mice during highly pathological IAV infection, their role in the inflammatory response induced by IAV remained elusive. In this regard, recent data demonstrates that mast cells can play a detrimental role during IAV infection in a strain specific manner. Specifically, following infection with A/WSN/1933, B6.Cg-*Kit*$^{W\text{-}sh}$ mice, which lack mast cells (112), exhibit a reduction in weight loss, lung pathology, and pulmonary inflammation compared to wild-type mice (23). Importantly, when mast cells are reconstituted into B6.Cg-*Kit*$^{W\text{-}sh}$ mice, the weight loss and inflammatory response are restored to wild-type levels (23). In studies using a highly pathogenic H5N1 virus (A/chicken/Henan/1/2004), mice administered ketotifen, a mast cell stabilizing agent, demonstrate reduced lung inflammation and epithelial cell apoptosis than untreated mice (24). Furthermore, combination therapy with ketotifen and oseltamivir (an NA inhibitor) improves survival better than either drug alone (24). Taken together, these data show mast cells can contribute to the pathology observed during IAV infection in mice. The newly emerging zoonotic strains of highly pathogenic IAV, such as H7N2, are also presenting with high cellular infiltrate and damage within the lungs of mice suggestive of mast cell activation (25, 107). If mast cells participate in the immunopathology elicited by these emerging zoonotic IAV isolates remains to be seen.

Dengue Virus

Human infection with dengue virus can result in a wide range of pathologies. In its most severe forms, dengue virus induces dengue hemorrhagic fever and dengue shock

syndrome, both of which are characterized by increased vascular permeability. The production of cross-reactive antibodies during a primary infection can lead to more severe disease upon secondary infection with a heterologous serotype (113, 114). The urine and blood of infected patients display elevated levels of histamine (115, 116), the presence of vasoactive factors (117, 118), and increased serum levels of chymase, a mast cell specific enzyme (103). As each of these mediators are released by mast cells, numerous studies have examined the role mast cells play during dengue virus infection. Upon exposure, dengue virus induces both degranulation and cytokine production by mast cells (82, 103, 119, 120). Mast cell derived LTB_4 and granule proteases increase vascular permeability (82, 103), while the synthesis and release of TNF-α, IL-6, IFN-α, CCL2, CCL3, CCL5, and CX3CL1 recruit NK cells and T cells to the site of infection (82, 121-123). Mast cell deficient mice show a reduction in symptoms, demonstrating that mast cells play an important role in dengue virus-induced immunopathology (103). Moreover, administration of the mast cell stabilizing drugs, cromolyn and ketotifen, or the LTB_4 antagonist montelukast, results in reduced vascular leakage compared to untreated mice (103). Current data suggests that early after infection, mast cell activation by dengue virus is beneficial, as it recruits NK and T cells to promote viral clearance (82, 122, 123). However, widespread mast cell activation is detrimental, as it increases vascular leakage, leading to the more severe forms of dengue-induced disease (103). In a murine model, the presence of non-neutralizing IgG enhances mast cell degranulation during dengue infections through interactions with FcγRIII (124)). Therefore, dengue virus can activate mast cells both directly, through an as yet unidentified mechanism, or indirectly through FcγRIII.

Hantavirus

The zoonotic transmission of hantavirus to humans can result in hemorrhagic fever with renal syndrome or hantavirus cardiopulmonary syndrome, both of which are characterized by increased vascular permeability and thrombocytopenia (125). Patients with hemorrhagic fever with renal syndrome exhibit significantly elevated histamine levels, indicating a possible role for mast cells in potentiating this syndrome (125). Endothelial cells, epithelial cells, and dendritic cells are all permissive to hantavirus infection *in vitro* (125-127), and recent evidence suggests mast cells are also susceptible to this virus (125). Inoculation of *in vivo* differentiated mast cells results in productive infection and mast cell activation, though the ability of hantavirus to directly induce degranulation is not known (125). Furthermore, the ability of various strains of hantavirus to infect and replicate within mast cells directly correlates with the pathogenicity of the strains (125). Thus, mast cells may be an important factor during hantavirus-induced disease.

Sendai Virus

Sendai virus is a respiratory parainfluenza virus that is highly transmissible in both rodents and swine. In neonatal rats, Sendai virus causes viral bronchiolitis and airway hyperresponsiveness, which is associated with elevated levels of bronchiolar mast cells and eosinophils (66, 128-130). The elevated numbers of bronchiolar mast cells observed after Sendai virus infection results from both the proliferation of tissue-resident mast cells and recruitment of mast cell progenitors to the airways (61). Sendai virus can also infect human mast cells, resulting in their activation (131). While the release of β-

hexosaminidase (a major granule component) has not been detected from human mast cells, both histamine release in rats and tryptase release in pigs have been detected following Sendai virus challenge (131-133). Following Sendai virus infection, human mast cells produce type I and III interferon (131), which have been implicated in asthma exacerbations (134). Interestingly, in the rat model, animals previously infected with Sendai virus subsequently sensitized to ovalbumin one-month later display heightened allergic airway inflammatory cell reactions (66). Thus, mast cells are important contributors to the inflammatory response to parainfluenza viruses and participate in their pathological role during allergic airway disease.

Infectious Bursal Disease Virus (IBDV)

IBDV is a contagious disease with a high mortality rate, which impacts the poultry industry worldwide. IBDV infected chickens have increased inflammatory lesions, which leads to susceptibility to secondary infections (135-137). Mast cell numbers are increased at the site of infection during IBDV. Moreover, these mast cells are activated, as mast cell tryptase accumulates in the infected tissue (64). Treatment with ketotifen not only decreases mast cell numbers in infected birds, but also correlates with reduced injury during infection without altering expression of IBDV antigens (65). Thus, by reducing the release of mast cell mediators, one can decrease mast cell accumulation in the infected tissue and ultimately decrease tissue damage and increase survival during IBDV infection.

Newcastle Disease Virus (NDV)

NDV is another highly contagious poultry disease which infects the gastrointestinal tract, resulting in high mortality and economic losses (138). Similar to IBDV, mast cells are found in and around NDV lesions during infection, correlating with an increase of mast cell tryptase levels in the tissues (63). Chickens pretreated with ketotifen show a reduction in tissue damage during NDV infection (62). Thus, similar to IBDV, inhibition of mast cell mediators reduces mast cell accumulation in the infected tissue and decreases tissue damage, increasing survival following NDV infection.

Porcine Reproductive and
Respiratory Syndrome Virus (PRRSV)

PRRSV is associated with high mortality in pigs. Infection with low pathogenic PRRSV (LP-PRRSV) results in minimal histopathological changes with no mortality. In contrast, infection with a high pathogenic strain of PRRSV (HP-PRRSV) results in significant mortality associated with extensive tissue damage within the lungs (139, 140). Pigs infected with HP-PRRSV display significant respiratory distress, which is associated with pulmonary lesions characterized by inflammatory cell infiltrates, interstitial and alveolar edema, and hemorrhaging, which is not observed following LP-PPRSV infection. Infection with the HP-PRRSV results in higher virus titers and higher levels of pro-inflammatory cytokines and immune cell infiltrate, including neutrophils, mononuclear phagocytes, and mast cells. Both histamine and LTB_4 are significantly increased in the serum of HP-PRRSV infected pigs (141). Because, these mediators play an important role increasing blood vessel permeability and disease severity during

dengue virus infection (51), it is likely they contribute to the increased lung edema and hemorrhage observed during HP-PRRSV (141).

How Are Mast Cells Activated by Viruses?

Is Virus Entry and Replication in Mast Cell Required for Activation?

Both pathogenic and non-pathogenic hantavirus nucleoprotein can be detected in mast cells (125). In addition, the human mast cell lines KU812 and HMC-1 are permissive to dengue virus in the presence of human dengue virus immune sera (119). This data demonstrate that these highly pathogenic viruses can infect mast cells. RSV activates mast cells resulting in the production of cytokines and chemokines including CXCL10, CCL4, CCL5 and type I interferons (142). RSV antigen can be detected in both primary cord blood mast cells and the human mast cell lines following infection (142). However, similar to many other pathogenic viruses, mast cell infection does not result in the release of infectious progeny virions (142).

While respiratory epithelial cells are the primary target for IAV replication, IAV can infect a wide range of cells, including endothelial cells (21), macrophages (143), dendritic cells (144), and mast cells (23, 121, 145). In mast cells, IAV is able to mediate viral entry, but largely appears to undergo an abortive infection. Inoculation of murine bone marrow derived mast cells (BMDMC) with A/WSN/1933 results in *de novo* expression of the viral NS-1 protein, but does not produce any new infectious particles (23). Interestingly, treatment of murine BMDMC with another H1N1 isolate, A/PR/8/1934, does not result in detectable NS-1 expression (23). On the other hand, infection of the human mast cell line LAD and human cord blood derived mast cells with

the A/PR/8/1934 strain results in viral mRNA and protein synthesis, but does not produce *de novo* infectious particles (145). In contrast, recent data demonstrates the murine mastocytoma cell line P815 can be productively infected with A/WSN/1933 (H1N1), A/Chicken/Henan/1/2004 (H5N1), and A/Chicken/Hebai/2/2002 (H7N2), producing infectious virus over the first 24 hours of infection, as measured by qRT-PCR, hemagglutination assay, and plaque forming assay (25). These differences likely reflect the different types of mast cells used for these studies and the infectious dose of the virus. Overall, these data demonstrate that IAV, dengue virus, RSV, and hantavirus can at least bind to and enter mast cells which is likely important for mast cell activation. More studies are needed to understand the fine specificity of these viruses and specifically the different IAV isolates for distinct mast cell populations, and the cellular factors that may be present in some of these populations that limit IAV propagation.

How are Viral Particles Recognized by Mast Cells? Mast cells express a wide range of PRR which allows these cells to respond to a variety of stimuli, including bacteria, parasites, fungi, and viruses (Figure 1) (86). RIG-I is a cytosolic receptor that can detect IAV RNA and many other single stranded RNA viruses (73, 80). Once RIG-I detects vRNA, it signals through the mitochondrial adaptor MAVS resulting in an antiviral response. In mast cells, signaling through the RIG-I/MAVS pathway is important for the secondary response to IAV, but not for the immediate degranulation of mast cells (23). Virus recognition through RIG-I by mast cells is also important during dengue virus and vesicular stomatitis virus (VSV) infections (82, 121, 146). However, our studies suggest the RIG-I dependent responses in mast cells do not significantly contribute to the pulmonary

immunopathology associated with IAV infection (A.C. Graham & J.J. Obar, unpublished observation); rather mast cell degranulation appears to be the dominant mediator of immunopathology (24). In addition to RIG-I detection, TLR3 is also important for the recognition of IAV, type I reovirus, RSV, VSV, and NDV by murine BMDMC for the production of secondary mast cell mediators (79, 147, 148). Moreover, viral recognition by both Mda5 and 2'-5 oligoadenylate synthase (OAS) can participate in the initiation of the secondary response of mast cells induced by VSV (148). Thus, detection of viral nucleic acids appears to be central for production of *de novo* synthesized mast cell mediators following viral infection. Alternatively, infection can be detected indirectly by mast cells, as occurs with herpes simplex virus (HSV). Infected epithelial cells secrete IL-33, which is in turn detected by mast cells, resulting in the secretion of IL-6 and TNF-α without degranulation (149).

Mast cell degranulation not only appears to play a critical role in regulating mast cell dependent inflammation following IAV infection (23, 24), but also in a number of other viral systems (62, 65, 103). The mast cell degranulation inhibitor, ketotifen, reduces inflammation in response to H5N1 IAV infection of mice (24), and the inflammation associated with IBDV and NDV in poultry (62, 65). Additionally, mast cell stabilization using cromolyn limits dengue virus induced immunopathology (103). Together, these data strongly support a role for mast cell degranulation in the mast cell-dependent inflammatory response to highly virulent viral infections. Thus, it appears critical we understand how viruses drive mast cell degranulation to appropriately target these cells pharmacology.

How mast cells degranulate in response to viral infections remains largely unknown. Degranulation still occurs in response to A/WSN/1933 infection in RIG-I-deficient BMDMC, demonstrating that degranulation is a RIG-I-independent response (23). As degranulation occurs within 30 minutes following treatment with IAV, other PRR and/or early signaling events necessary for the virus attachment and/or entry processes are likely important in regulating mast cell degranulation. With dengue virus, mast cells degranulate occurs prior to RIG-I signaling (82). Moreover, UV-inactivated dengue virus (82) and IAV (147) retain the ability to activate mast cells suggesting this occurs early in the viral replication cycle. While FcγIII-deficient mast cells are able to degranulate in response to dengue virus, mast cells pre-treated with anti-dengue IgG demonstrate enhanced degranulated in response to all four serotypes of dengue virus compared to dengue virus alone, suggesting that antibody binding enhances degranulation in response to dengue virus (124). Although mast cell degranulation appears to be pivotal for the immunopathology associated with highly pathological IAV (24) and dengue virus infections (103), we do not understand how degranulation is initiated. To date, the only virus for which the mechanism of mast cell degranulation has been well elucidated is vaccinia virus. The activating event is fusion of the viral envelope with the mast cell plasma membrane (31). Specifically, the vaccinia virus envelope contains sphingomyelin (150) which is converted to sphingosin-1-phosphate (S1P) and signals through the S1PR2 G-coupled receptor to cause degranulation (31). Signaling through the S1PR2 has also been shown to regulate mast cell responses in general (31, 151-154). However, the role of S1P receptor signaling in other viral infections remains unknown. Further understanding the molecular signals necessary for mast cell

degranulation could lead to novel therapeutic avenues for these highly virulent viral infections.

Mast Cells as Drug Targets for Limiting Virus-Induced Immunopathology

Predicting the next pandemic IAV strain is nearly impossible, as IAV has a high mutation rate resulting in significant yearly antigenic drift and can randomly re-assort resulting in antigenic shift. Even deciding which IAV strains to produce for the yearly vaccine is difficult, as the strains must be chosen months ahead of the yearly influenza season. If these predictions are inaccurate or the seasonal IAV strains drift significantly, then the vaccine will not be highly effective resulting in a high incidence of IAV-induced disease (2). The current antiviral treatments against IAV are becoming increasingly ineffective due to the emergence of resistant strains. Therefore, alternative therapeutics avenues are needed. Targeting host-derived factors necessary for viral replication or host factors participating in the excessive pathological inflammatory response during highly pathogenic IAV are promising alternatives (2).

The literature review presented here shows the strong correlation between mast cell accumulation and degranulation at local sites of infection with the observed tissue damage and pathology, not only during highly pathological IAV infections, but many other pathogenic viral infections of humans and animals. Additional studies examining other highly pathological viruses that are known to cause ARDS and/or vascular leakage are thus warranted, which would include the emerging coronaviruses, SARS-CoV, and MERS-CoV, and hemorrhagic viruses such as Marburg and Ebola. Overall, we

hypothesize that excessive mast cell activation may be a common feature of highly pathological viral infections that cause ARDS and/or vascular leakage. This novel pathway could be pharmacologically targeted to limit the morbidity and mortality associated with these infections. Additionally, understanding how mast cells accumulate in the infected tissues, through mast cell proliferation and/or mast cell progenitor recruitment, could provide additional therapeutic targets (Figure 3).

Because mast cells and their products are known to play a dominant role in both allergic and asthmatic reactions, many drugs that stabilize and neutralize mast cells are already approved for human use (Figure 3). The mast cell stabilizing drugs, which inhibit the release of granules following mast cell activation, have proven effective at reducing vascular leakage and limit inflammatory cellular recruitment, thus increasing survival in the murine dengue virus and IAV models (24, 103, 155). Furthermore, these compounds have proven very effective at limiting lung pathology following IBDV and NDV in poultry (62, 65). Compounds are also available which block the activity specific mast cell products including TNF-α, histamine, mast cell proteases, and leukotrienes (Figure 3). Many anti-TNF-α compounds are already approved for the treatment of inflammatory arthritis. Numerous anti-histamines, including hydroxyzine, desloratadine, diphenhydramine, fexofenadine, and loratadine, are approved to treat allergy symptoms. Drugs are currently in development which target the mast cell proteases, especially the mast cell derived chymase which has been implicated in cardiovascular disease. Finally, there are two classes of leukotriene antagonists, the leukotriene-receptor antagonists (zafirlukast and montelukast) and the leukotriene synthesis inhibitors (zileuton).

In addition to stand alone treatments targeting mast cell activation and mediators, adjunct therapies utilizing both antiviral and mast cell targeting compounds might be fruitful. Earlier studies using human peripheral blood leukocytes exposed to neuraminidases or IAV at the time of IgE stimulation resulted in significantly greater histamine release (156-158). These data suggest the presence of multiple stimuli may result in an additive or synergistic effect. Therefore, mast cell targeting drugs could be used in parallel with antiviral drugs for greatest efficacy. Following infection with a highly pathogenic H5N1 IAV strain, the only cohort of mice which survived infection were those treated with both antiviral and mast cell stabilizing compounds (24). This approach may prove especially beneficial during asthmatic exacerbations following viral infection.

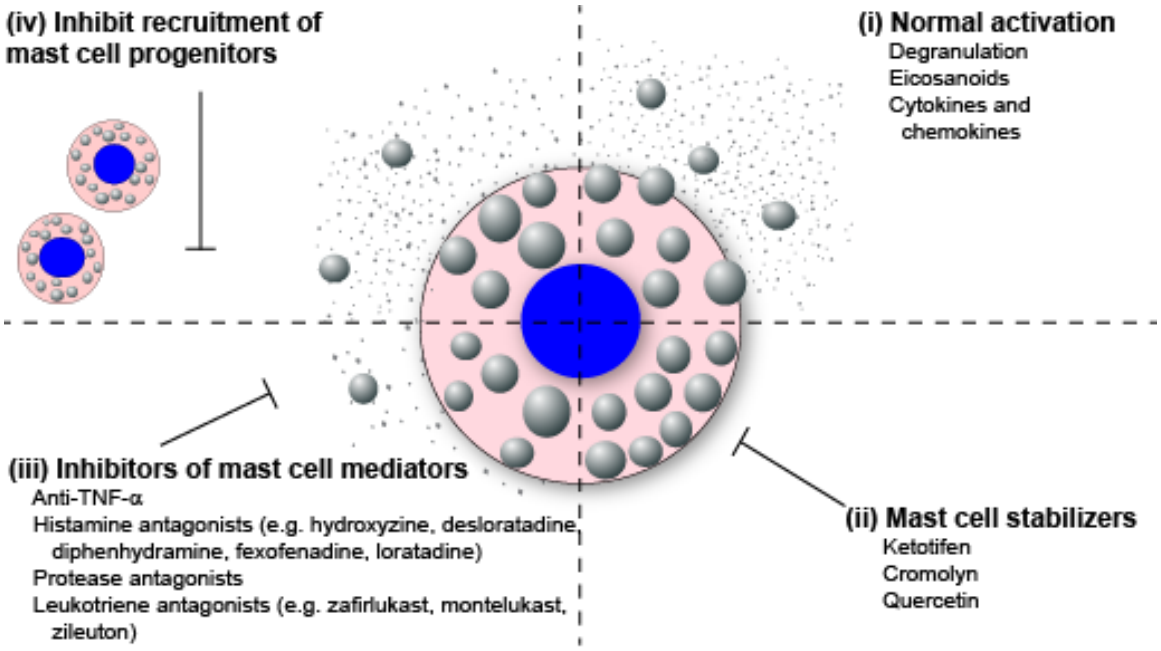

Figure 3.3. Mast cell inhibitors. Various classes of mast cell inhibitors already exist for the treatment of various conditions. (i) Uninhibited, activated mast cells will degranulate and synthesize eicosanoids, cytokines, and chemokines which are released into the surrounding tissue. (ii) The mast cell stabilizing drugs (e.g. ketotifen, cromolyn, and quercetin) block the release of mast cell granules following activation. (iii) Second broad class of mast cell inhibitors target the activity of specific mast cell mediators. These includes anti-TNF-α compounds, anti-histamines (e.g. hydroxyzine, desloratadine, diphenhydramine, fexofenadine, loratadine), protease antagonists, and leukotriene antagonists (e.g. montelukast, zafirlukast, zileuton). (iv) A potential third class of mast cell inhibitors could target the recruitment of mast cells to inflamed tissue following infection.

Concluding Remarks

Mast cells are important players in pathogen defense. Their location at environmental barriers allows them to quickly respond to invading pathogens. In parasitic and bacterial infections, mast cells are essential in preventing the spread of infection (26-28). While in certain viral infections mast cells can be protective (31, 122, 123, 149), in

highly pathogenic viral infections, such as IAV or systemic dengue infections, the data demonstrate that mast cells are more detrimental than beneficial (23, 24, 103). If the role of mast cells during IAV infections, and other highly pathogenic viral infections, can be elucidated, these cells may serve as a lucrative target for new therapeutics. Activation and release of mediators from mast cells in response to these viruses correlates with severity of disease in mice. Application of existing allergy medications that target mast cells may decrease the severity of IAV infections, limiting the morbidity and mortality associated with future pandemics.

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CHAPTER FOUR

INFLAMMATORY RESPONSE OF MAST CELLS DURING INFLUENZA A VIRUS INFECTION IS MEDIATED BY ACTIVE INFECTION AND RIG-I SIGNALING

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INFLAMMATORY RESPONSE OF MAST CELLS DURING INFLUENZA A VIRUS INFECTION IS MEDIATED BY ACTIVE INFECTION AND RIG-I SIGNALING

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Abstract

Influenza A virus (IAV) is a major respiratory pathogen of both humans and animals. The lung is protected from pathogens by alveolar epithelial cells, tissue resident alveolar macrophages, dendritic cells, and mast cells. The role of alveolar epithelial cells, endothelial cells, and alveolar macrophages during IAV infection has been previously studied. Here we address the role of mast cells during IAV infection. Respiratory infection with A/WSN/33 causes significant disease and immunopathology in C57BL/6 mice, but not B6.Cg-*Kit*$^{W\text{-}sh}$ mice that lack mast cells. During *in vitro* co-culture, A/WSN/33 caused mast cells to release histamine, secrete cytokines and chemokines, and produce leukotrienes. Moreover, when mast cells were infected with IAV, the virus did not replicate within mast cells. Importantly, human H1N1, H3N2, and influenza B virus isolates could also activate mast cells *in vitro*. Mast cell production of cytokines and chemokines occurs in a RIG-I/MAVS-dependent mechanism; in contrast, histamine production occurred through a RIG-I/MAVS-independent mechanism. Our data highlight that following IAV infection the response of mast cells is controlled by multiple

receptors. In conclusion, we have identified a unique inflammatory cascade activated during IAV infection that could potentially be targeted to limit morbidity following IAV infection.

Introduction

Influenza A virus (IAV) is one of the most common respiratory infections in humans. IAV can cause a range of disease courses from asymptomatic or symptomatic seasonal outbreaks to severe forms of respiratory infection including acute respiratory distress and acute lung injury, which are observed during pandemic outbreaks. Typically seasonal IAV infections cause limited morbidity and mortality associated with specific patient populations. However, severe IAV infection such as with the avian H5N1 isolates and 1918 Spanish flu isolate causes pathological changes to the lung architecture (1). Recent evidence indicates that highly pathogenic strains of IAV lead to an uncontrolled inflammatory response characterized by excessive lung infiltration of macrophages and neutrophils and a dramatic ‘cytokine storm’, which participates in causing excessive lung damage (2-5). Dampening the ‘cytokine storm’ response can significantly enhance the survival of mice during IAV infection (6). Thus it is imperative that we understand the mechanisms of the early events triggered by IAV infection, which result in inflammatory cell infiltration, and initiation of the ‘cytokine storm’.

The initial lines of defense in the respiratory tract include alveolar epithelial cells, endothelial cells, tissue resident alveolar macrophages, dendritic cells, and mast cells. However, the role of mast cells during respiratory infections is an understudied area (7).

Mast cells are tissue sentinel cells of hematopoietic origins found in most vasculature tissue, but are enriched tissues that are at environmental interfaces such as skin, gastrointestinal tract, urinary tract, and lungs (8). This localization is significant because mast cells are poised to play an important role in the early immunosurveillance for pathogens. Mast cells express numerous molecules which can recognize pathogens including CD48, FcR, complement receptors, TLR, Retinoic acid inducible gene I (RIG-I)-like receptors (RLR), Nod-like receptors (NLR), and C-type lectin receptors (CLR) (9). After activation the mast cell response can be separated into two distinct phases: 1) immediate degranulation and secretion of stored mediators and 2) delayed secretion of *de novo* synthesized mediators. The immediate response is characterized by release of histamine, serotonin, tryptases, chymases, and TNF-α, while the delayed response includes secretion of leukotrienes, prostaglandins, cytokines, chemokines, and growth factors (10). Interestingly, mast cells do not respond uniformly to all stimuli. Stimulation of TLR4 by LPS causes mast cells to facilitate a strong inflammatory cytokine response, but not degranulation; in contrast, TLR2 activation results in both inflammatory cytokine release and degranulation by mast cells (11). Thus, the mast cell response is extremely adaptable, which enables them to have dramatic effects on the composition and regulation of subsequent inflammatory responses.

It is well documented that mast cells play a crucial role in immunity against certain parasitic and bacterial infections (reviewed in (8, 9, 12)). More recently, the role of mast cells during viral infections has been explored. *In vitro,* mast cells have been shown to be capable of responding to vesicular stomatitis virus, Sendai virus, Hantavirus, dengue virus, and reovirus (13-17). However, there is a limited understanding about the

in vivo relevance of mast cells during viral infections. In a peritonitis model of Newcastle disease virus infection, mast cells were shown to be important in inflammatory cell infiltration in a TLR3-dependent manner (18). During cutaneous dengue virus infection mast cells have been shown to play an important role in immunosurveillance through RIG-I and Mda5-dependent recognition of the virus (19, 20). In humans, dengue shock syndrome has recently been associated with elevated serum levels of mast cell-derived VEGF and proteases (21). Additionally, mast cells have been shown to play a protective role during skin vaccinia virus infection (22). However, the *in vivo* relevance of mast cells during respiratory virus infections remains understudied. IAV has been shown to enhance IgE-mediated histamine release from basophilic leukocytes, but IAV alone caused minimal histamine release (23). Moreover, IAV infections can sensitize mice leading to flu-specific cutaneous anaphylaxis (24). Together these data demonstrate that IAV infection can have effects on mast cells, but whether mast cells are important in the inflammatory response to respiratory IAV infection remains unresolved.

Here we specifically demonstrate that mast cells play an important role in the pathological response during A/WSN/33 infection of mice. Importantly, mast cell activation was also observed with human influenza virus isolates from the H1N1 IAV, H3N2 IAV, and influenza B virus families. The ability of IAV isolates to activate mast cells correlated with their ability to infect those cells *in vitro*. Interestingly, upon infection cytokine and chemokine production by mast cells were entirely dependent on RIG-I signaling, while mast cell degranulation occurred even in the absence of RIG-I signaling. Thus, we demonstrate that mast cells can play a central role in the inflammatory pathology induced by IAV infection.

Materials and Methods

Viral Strains

Allantoic fluid containing the A/Puerto Rico/8/34 virus was purchased from Charles River. Allantoic fluid containing the A/WSN/33 virus was originally obtained from Dr. David Topham (University of Rochester) and was subsequently grown in embryonated chicken eggs. Human influenza virus isolates used in Figure 4.5 were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH.

Mouse Strains and Infectious Protocol

B6.Cg-*kit*$^{W\text{-}sh}$ mice were original purchased from Jackson Laboratories and subsequently bred in house. C57BL/6J mice were bred in house. Specific knock-out bone marrow was kindly provided by multiple investigators: RIG-I by Dr. Michael Gale (University of Washington), MAVS by Dr. Mathias Schnell (Thomas Jefferson University), MYPS/STING by Dr. John Cambier (National Jewish Health), CARD9 by Dr. Tobias Hohl (Fred Hutchinson Cancer Center), and STAT6 by Dr. Daniel Campbell (Benoyra Institute).

Mice were intranasally infected with 1500 plaque forming units (PFU) of A/PR/8/34 or A/WSN/33 under 2,2,2-tribromoethanol (Avertin) anesthesia. At the indicated times after IAV infection, mice were given a lethal overdose of pentobarbital. Broncheoalveolar lavage fluid (BALF) was collected by washing the lungs with 2 ml of PBS containing 50 mM EDTA. Lungs were saved for viral titers and stored at -80°C.

BALF was spun down, cells were analyzed by cytospins, and BALF supernatant was analyzed using a lactate dehydrogenase kit (Promega) and BCA assay (Thermo Scientific). For weight loss studies, mice were infected as previously stated and weighed daily. All animal protocols were approved by the Montana State University Institutional Animal Care and Use Committee.

IAV Plaque Assay

IAV viral titers in the lungs were quantified using a standard plaque assay. Briefly, lungs were homogenized in 2 ml of medium using a dounce homogenizer. Next, 10-fold serial dilutions of the lung homogenates were plated in duplicate on MDCK cells (ATCC) in a 6-well plate (dilutions 10^{-1}–10^{-6}). Virus was left to adhere to the cells for 1 h at 35°C, tipping every 10 minutes. Cells were overlayed with 2 ml of 0.8% SeaKem® agarose (Lonza) in DMEM containing 0.5 μg TPCK trypsin (Worthington) and 0.2% bovine serum albumin (Millipore). Plates were incubated at 35°C for 3 days after which plates were fixed overnight by adding 3 ml of 1:1 methanol:acetone. After fixation, monolayers were stained with 0.1% crystal violet.

Histological Analysis of Lungs

Lungs were inflated with 10% buffered formalin phosphate, placed in the same solution for at least 24 hours, then paraffin embedded. 5μm sections were then stained with hematoxylin and eosin (H&E) to assess lung inflammatory cell infiltration. H&E stained lungs were observed on an upright 80i eclipse microscope (Nikon) and images were captured with a DS Ri1 color camera (Nikon).

Growth of Bone Marrow
Cultured Mast Cells (BMCMC)
and Generation of Mast Cell Knock-in Mice

BMCMC were grown as previously described (25). Briefly, femurs from 4-8 week old mice were removed and bone marrow cells were collected by centrifugation for 30 seconds at 5000 rpm. Cells were resuspended in RPMI supplemented with 1% non-essential amino acids, 1mM sodium pyruvate, 1% HGPG, 10 mM HEPES, 10% fetal bovine serum, and 0.1% 2-mercaptoethanol. For the first three weeks, the cells were incubated with 10 ng/mL recombinant murine IL-3 (Peprotech). In subsequent weeks, 25 ng/mL recombinant murine stem cell factor (Peprotech) was added along with IL-3. After five weeks, the purity of the population was >90% mast cells as determined by flow cytometry analysis using anti-FcεR1α (BioLegend) and anti-CD117 (BioLegend). To generate BMCMC 'knock-in' mice, we followed the methods previously used by others (26-28). Briefly, BMCMC were generated as described above. Mice 3-4 weeks of age were reconstituted with $5x10^6$ BMCMC via intravenous injection and rested for ~8-10 weeks before use.

In vitro Mast Cell Activation Assay

For activation assays, $2.5x10^5$ BMCMC were plated per well in a 96 well U-bottom plate. Virus was added at an MOI of ~1 and the final volume was brought to 100 ml and incubated for 4-6 hours. As positive controls BMCMC were stimulated with LPS (5 mg/ml; List Biologicals Laboratory) for cytokine/chemokine release or the calcium ionophore A23187 (40 nM; Fisher Scientific) for leukotriene synthesis and mast cells

degranulation. Additionally, naive allantoic fluid could not activate the BMCMC similar to medium alone (Supplemental Figure 4.1).

Luminex Assay for
Cytokine and Chemokine Secretion

In vitro BMCMC activation assay supernatants from 4-6 h post-treatment and BALF from the indicated time points were analyzed for cytokine and chemokines according to the manufacturer's instructions. Custom Milliplex® plates were used for cytokine and chemokine analyses (Millipore), while murine IFNα and IFNβ were measured using Procarta® 2-plex assay (Affymetrix). Plates were read using a BioPlex® 200 (Bio-Rad).

Histamine and Leukotriene B_4
Enzyme Immunoassays (EIA)

Histamine and leukotriene B_4 EIA assays were conducted following the manufacturer's instructions (Cayman Chemical). Briefly, the samples were incubated with an acetylcholinesterase linked histamine or leukotriene B_4 tracer for 24 hours or overnight, respectively. Plates were washed and Ellman's Reagent was added to detect the tracer labeled histamine or leukotriene B_4. Obtained results are inversely proportional to free histamine or leukotriene B_4 present in the well.

Intracellular Staining for Viral Proteins

To measure virus infectivity of mast cells, we stained BMCMC looking for intracellular expression of the NS-1 protein. Briefly, $2.5x10^5$ BMCMC were plated per well in a 96 well U-bottom plate. Virus was added at MOI ~1 and the final volume was

brought to 100 ml and incubated for 4-6 hours. The cells were then fixed with 100 □l BD Cytofix/Cytoperm (BD Biosciences) for 30 minutes. Cells were then stained with a mouse monoclonal antibody against the IAV NS1 protein (NS1-1A7; BEI Resources) for 30 minutes in BD Perm-wash buffer. Subsequently, the cells were washed with BD Perm-wash buffer and then stained with PE-labeled goat anti-mouse IgG F(ab') fragments (Jackson ImmunoResearch Laboratories). Cell were then washed with BD Perm-wash buffer and resuspended in FACS Buffer. Samples were collected using a FACS Calibur and analyzed via FlowJo.

Statistical Analysis

Statistical significance was determined by either a Mann-Whitney U-test or one-way ANOVA using Prism 5 (Graphpad Software). Significance was set as $p < 0.05$.

Results

Mast Cells are Critical for Inducing the Pulmonary and Systemic Inflammatory Disease Induced by A/WSN/33 Infection of Mice

Mast cells have been found to be crucial participants in the immune responses to parasitic and bacterial infections (8, 12). Recently, the role of mast cells during respiratory infection with bacterial pathogens has begun to be explored (29-31), but the role of mast cells during respiratory infection with viral pathogens remains understudied. IAV has been reported to enhance IgE-mediated histamine secretion from basophilic leukocytes, but those studies failed to observe any direct activation of mast cells by IAV alone (23). Furthermore, genetic analysis of lungs from mice infected with a model strain

of the 2009 IAV pandemic virus demonstrated an enrichment of genes associated with mast cells (32). Thus, we looked to address the role mast cells might play during IAV infections. To assess the role of mast cells during respiratory infection with IAV, 10-12 week old B6.Cg-*Kit*$^{W\text{-}sh}$ mice, which are known to have a mast cell deficiency (26), and C57BL/6 mice were infected with 1500 PFU of A/WSN/33. Body weight of individual animals was longitudinally monitored for 2 weeks. When infected with A/WSN/33, B6.Cg-*Kit*$^{W\text{-}sh}$ mice did not lose substantial amounts of their initial body weight, while C57BL/6 mice displayed significant weight loss (Figure 4.1a). Weight loss during A/WSN/33 infection correlated with the amount of vascular leakage and tissue damage observed in the lungs of A/WSN/33 infected mice as measured by total protein (Figure 4.1b) or lactate dehydrogenase (data not shown) levels present in the BALF. Moreover, B6.Cg-*Kit*$^{W\text{-}sh}$ mice infected with A/WSN/33 had reduced numbers of inflammatory cells infiltrating into the BALF 7 days after infection (Figure 4.1c). The reduced BALF cell numbers in the B6.Cg-*Kit*$^{W\text{-}sh}$ mice were observed in all cell populations (macrophages, neutrophils, and lymphocytes). Reduced inflammatory cells infiltrating the BALF was not due to significantly decreased cell numbers found in the BALF of naïve B6.Cg-*Kit*$^{W\text{-}sh}$ mice (C57BL/6 = $1.00\text{x}10^5 \pm 0.48\text{x}10^5$ versus B6.Cg-*Kit*$^{W\text{-}sh}$ = $0.77\text{x}10^5 \pm 0.54\text{x}10^5$; $p=0.29$) or BALF composition which was >90% alveolar macrophages in both naïve C57BL/6 and B6.Cg-*Kit*$^{W\text{-}sh}$ mice (data not shown). Moreover, leukocyte numbers are largely normal in B6.Cg-*Kit*$^{W\text{-}sh}$ mice, if anything those mice display neutrophilia (33). Histologically analysis of lungs from A/WSN/33 infected C57BL/6 mice display robust interstitial inflammatory infiltrates on both day 7 and 10 post-infection; additionally, by day 10 airway infiltration was seen by histology in the infected C57BL/6 mice (Figure

4.1e). In contrast, B6.Cg-*Kit*$^{W\text{-}sh}$ mice displayed minimal interstitial inflammatory infiltrates at either time-point. These data track with the amount of IAV-induced immunopathology observed as measured by protein in the BALF (Figure 4.1b). The reduction in IAV-induced immunopathology in B6.Cg-*Kit*$^{W\text{-}sh}$ mice was not the result of decreased viral growth in the respiratory tract because viral titers were similar after A/WSN/33 infection in C57BL/6 and B6.Cg-*Kit*$^{W\text{-}sh}$ mice and both mouse strains cleared the IAV infection by 10 dpi (Figure 4.1d).

It appears that mice devoid of mast cells are highly resistant to IAV-induced inflammatory disease (Figure 4.1). However, B6.Cg-*Kit*$^{W\text{-}sh}$ mice have other abnormalities in addition to the mast cell deficiency (26, 33). To ensure the defect in the B6.Cg-*Kit*$^{W\text{-}sh}$ mice was within the mast cell compartment we made mast cell knock-in mice as previously described (26-28). Selective reconstitution of the mast cell compartment of B6.Cg-*Kit*$^{W\text{-}sh}$ mice with $5x10^6$ C57BL/6-derived bone marrow cultured mast cells (BMCMC) 8-10 weeks prior to IAV infection was able to complement the reduced weight loss and inflammatory cell recruitment observed in B6.Cg-*Kit*$^{W\text{-}sh}$ mice (Figure 4.2a & b). Thus, our data demonstrate that during respiratory infection with IAV, mast cells are crucial participants in the pathological innate immune response.

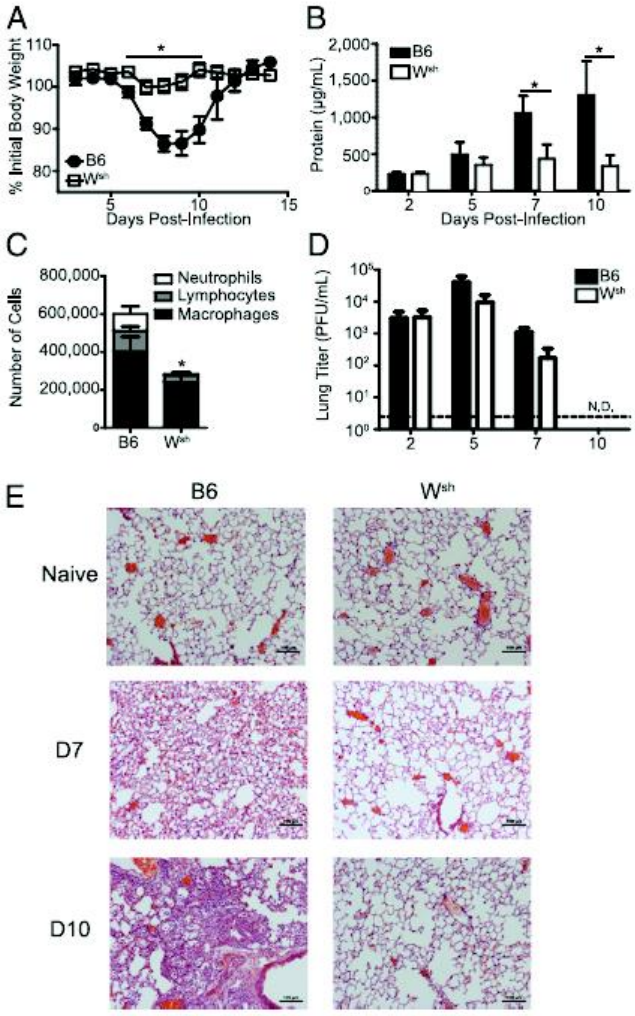

Figure 4.1. B6.Cg-*Kit*$^{W\text{-}sh}$ is less sensitive to A/WSN/33. Age-matched C57BL/6 (B6) or B6.Cg-*Kit*$^{W\text{-}sh}$ (W^{sh}) mice were infected nasally with 1500 PFU of A/WSN/33. (A) Body weights were measured daily. The data is normalized to each mouse's starting body weight. (B) Damage to the lung was assessed by measuring the total protein levels in the BALF at the indicated time points. (C) Total leukocyte recruitment to the BALF was measured day 7 post-infection. (D) Lung viral titers were determined at indicated time points post-infection by plaque assay on MDCK cells. Dashed line represents the limit of detection of the plaque assay (2 PFU/mL). (E) Formalin fixed lungs were paraffin embedded and then sectioned and stained with H&E for analysis by microscopy. Representative lung sections from naïve and A/WSN/33-infected (7 of 10 d) C57BL/6 and W^{sh} mice are shown. Scale bars, 100 μm. Data are representative of two to four independent experiments consisting of four to eight mice/group. *$p<0.05$, Mann-Whitney U-test. N.D., None detected.

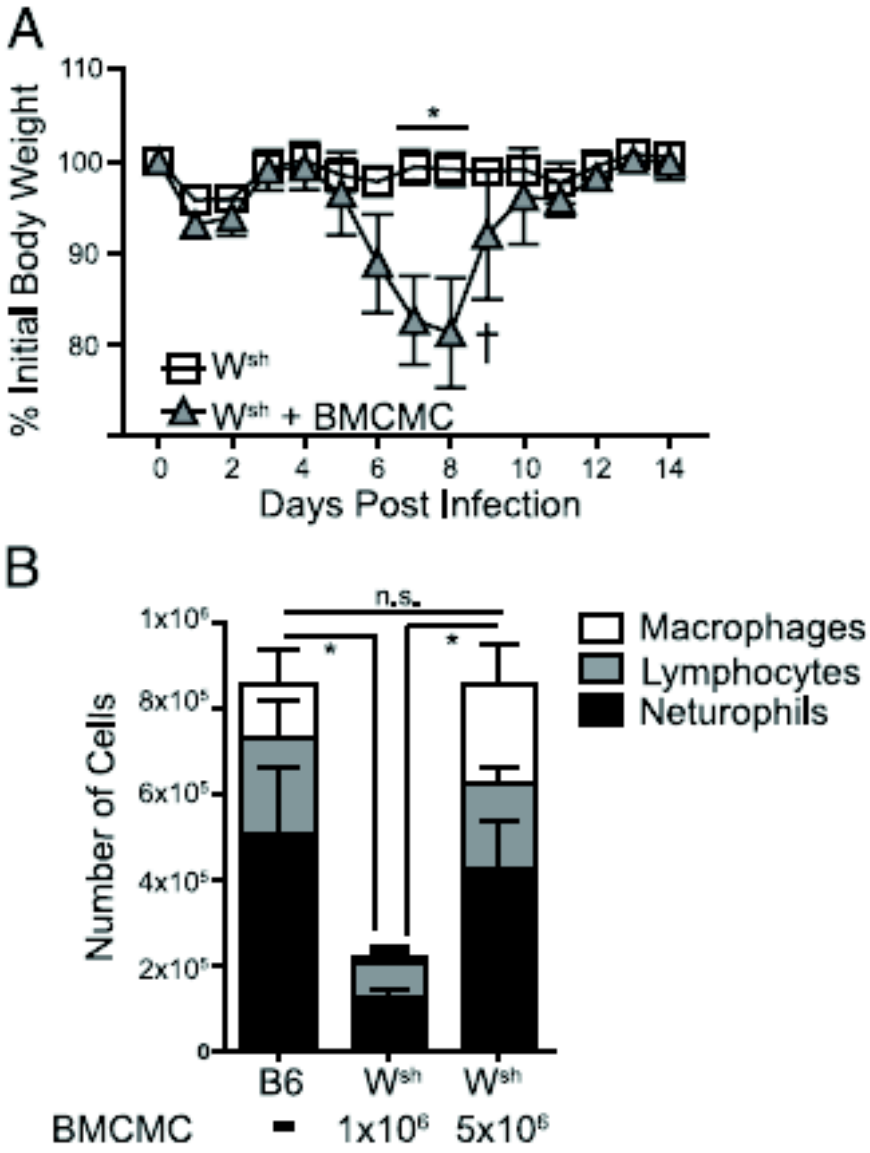

Figure 4.2. B6.Cg-*Kit*$^{W\text{-}sh}$ mice reconstituted with BMCMC were susceptible to WSN. (A) Age-matchedC57BL/6 (B6), B6.Cg-*Kit*$^{W\text{-}sh}$ (W^{sh}), or B6.Cg-*Kit*$^{W\text{-}sh}$ mice reconstituted with $3x10^6$ BMCMC 10 wk prior were infected nasally with 1500 PFU of A/WSN/33 (WSN). Body weights were measured daily. The data is normalized to each mouse's starting body weight. (B) Age-matched C57BL/6 mice and B6.Cg-*Kit*$^{W\text{-}sh}$ mice, reconstituted with either $1x10^6$ or $5x10^6$ BMCMC, were infected nasally 10 wk later with 1500 PFU of A/WSN/33, and total lymphocyte, neutrophil, and macrophage counts in the BALF was measured 7 d post-infection. Data are representative of two independent experiments consisting of four to eight mice/group. Statistically significant differences were determined using a Mann-Whitney U-test (A) or one-way ANOVA (B). † = mouse had to be euthanized. *$p<0.05$.

Altered Inflammatory Milieu in B6.Cg-*Kit*$^{W\text{-}sh}$ Mice during A/WSN/33 Infection

Because our data demonstrated a dramatic reduction in the number of inflammatory cells infiltrating into the BALF of B6.Cg-*Kit*$^{W\text{-}sh}$ mice (Figures 4.1 & 2), we next wanted to assess the inflammatory cytokine and chemokine production pattern in C57BL/6 and B6.Cg-*Kit*$^{W\text{-}sh}$ mice after A/WSN/33 infection. To do this, C57BL/6 and B6.Cg-*Kit*$^{W\text{-}sh}$ mice were infected with 1500 PFU of A/WSN/33. BALF was collected from individual mice 7 days after A/WSN/33 infection and analyzed by multiplex bead-based assays. We found that there was a marked alteration of the inflammatory milieu found in the BALF of B6.Cg-*Kit*$^{W\text{-}sh}$ mice after A/WSN/33 infection as compared with C57BL/6 mice. Specifically, there was a reduction in TNF-α, CCL2, CCL3, CCL4, CXCL2, and CXCL10 at this time point in the B6.Cg-*Kit*$^{W\text{-}sh}$ mice (Figure 4.3). Moreover, there was a trend toward reduced levels of IL-6 and IFNγ (Figure 4.3). However, not all cytokine expression was lost in B6.Cg-*Kit*$^{W\text{-}sh}$ mice because there was equivalent expression of CCL5, CCL12, IL-5, IL-16, LIF, G-CSF, and CX_3CL1 (Figure 4.3). All cytokine and chemokines were below the limit of detection in naïve BALF from C57BL/6 mice, except IL-16 (36.9 ± 5.9 pg/ml) and CX_3CL1 (140.6 ± 79.3 pg/ml). Thus, our data suggest that mast cells can be important for initiating the inflammatory milieu found during IAV infection.

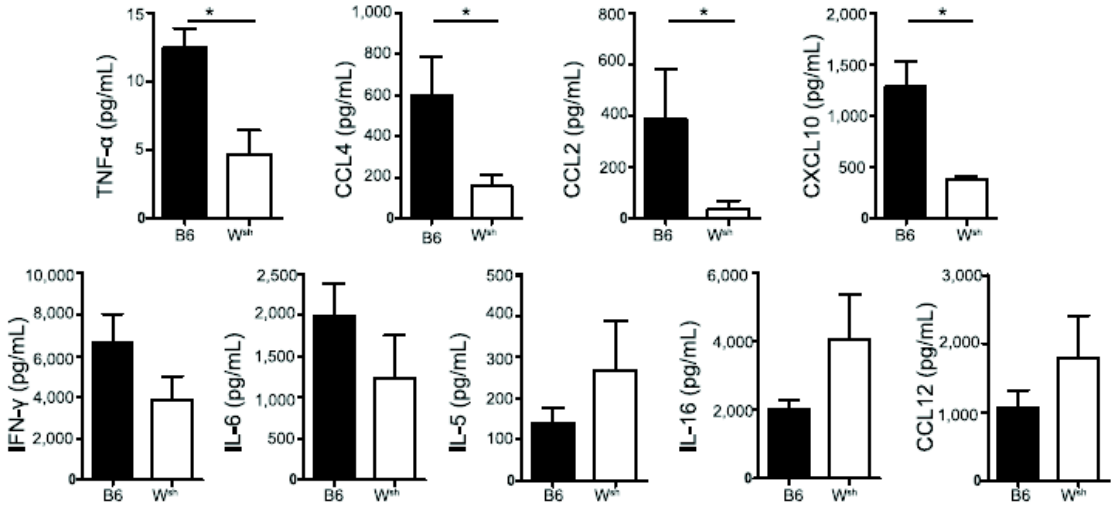

Figure 4.3. B6.Cg-*Kit*$^{W\text{-}sh}$ mice had lower levels of cytokines in the lung during A/WSN/33 infection. Age-matched C57BL/6 (B6) or B6.Cg-*Kit*$^{W\text{-}sh}$ (W^{sh}) mice were infected nasally with 1500 PFU of A/WSN/33 (WSN). Cytokine and chemokine levels in the BALF were assessed 7 d post-infection using Milliplex™ multiplex assays. Data are representative of two independent experiments with four to six mice/group. *$p<0.05$, Mann-Whitney U-test.

In vitro Activation of Mast Cells
in Response A/WSN/33 Treatment

Next we wanted to assess whether IAV had the potential to directly activate mast cells *in vitro*. To do this, $2.5x10^5$ BMCMC were treated with medium or A/WSN/33 for 6 hours or at the indicated times. BMCMC supernatants were then analyzed for mast cell mediators, which included histamine, leukotriene B_4 (LTB_4), and cytokines. Medium treated BMCMC showed minimal expression of all three mediators (Figure 4.4a-c). Additionally, uninfected allantoic fluid did not cause mast cell mediator release greater than medium alone (Supplemental Figure 4.1). A/WSN/33 treatment of BMCMC resulted in histamine release, CCL2 secretion, and LTB_4 production (Figure 4.4a-c). Specifically, we found that mast cells released histamine in two waves: the first peaking ~30 minutes

after A/WSN/33 treatment and the second beginning ~4 hours after stimulation (Figure 4.4a). Additionally, 6 hours after A/WSN/33 inoculation mast cells produced substantial quantities of CCL2 (Figure 4.4b) and LTB_4 (Figure 4.4c). In a broader analysis of the cytokines and chemokines secreted by BMCMCs after A/WSN/33 activation, we found that mast cells produced CCL2, CCL3, CCL4, CCL5, CXCL2, CXCL9, CXCL10, IL-6, and TNF-α, but not CXCL1, IFN-α, IFN-β, IFN-γ, IL-1□, IL-5, IL-9, IL-15, and VEGF in response to A/WSN/33 (Supplemental Table 4.1). Furthermore, cytokine and chemokine expression began ~2 hours after IAV treatment (Supplemental 4.10). Thus, at least *in vitro*, IAV-induced mast cell activation appears to occur through direct recognition of the virus.

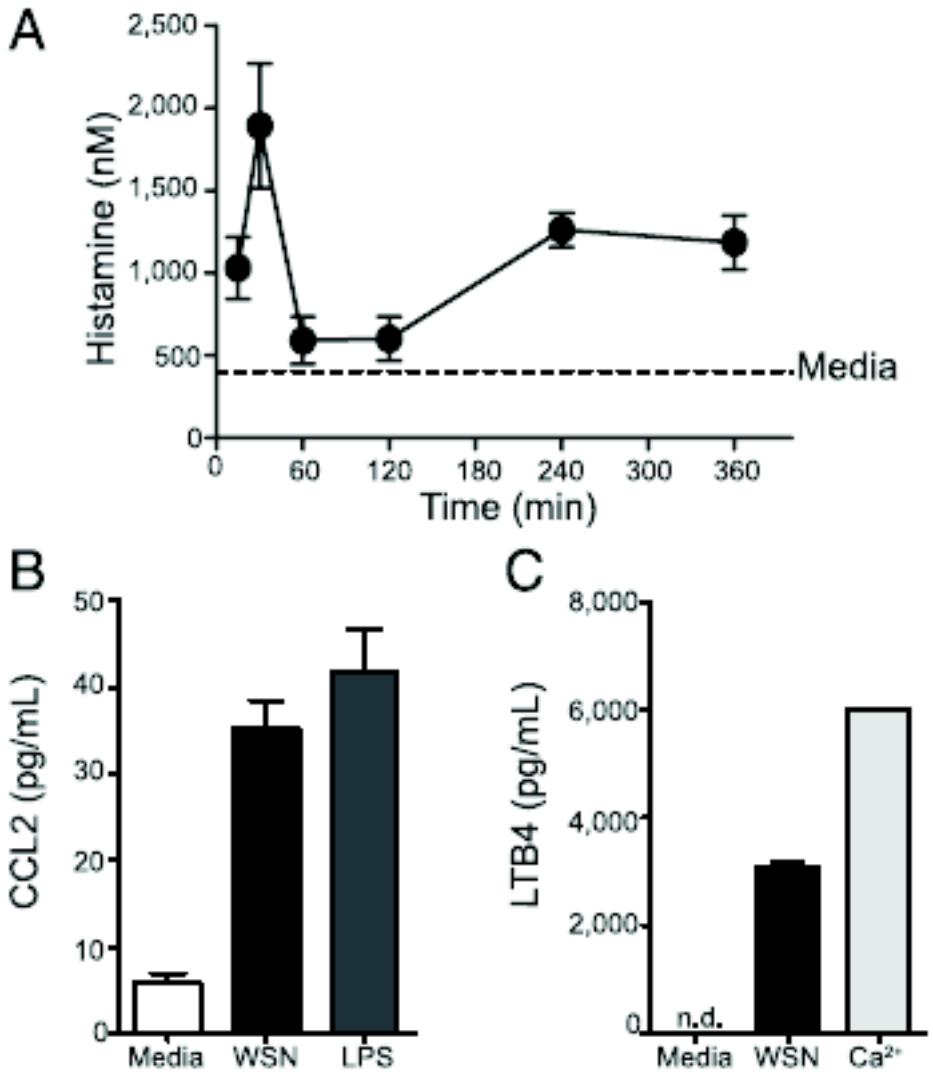

Figure 4.4. *In vitro* BMCMC activation with A/WSN/33 results in mast cell activation. BMCMCs were derived by culturing total bone marrow with IL-3 for 5 wk and supplemented with stem cell factor for the last 2 wk. A total of $2.5x10^5$ $FcR\varepsilon1^+$ and $CD117^+$ mast cells were treated with media, A/WSN/33 (WSN), or a positive control of calcium ionophore (Ca^{2+}; 40nM) or LPS (5μg/mL). (A) Histamine levels were measured by EIA at the indicate time points. As a control histamine levels from unstimulated BMCMC were assayed after 6 h of culture. Cytokine/chemokine (B) and LTB_4 (C) secretion were measured 6 h after stimulation by either Milliplex™ multiplex analysis or EIA, respectively. Each virus was added at an MOI of 1. Data are representative of two to four independent experiments. N.d.. = none detected.

Mast Cells can be Activated by
Human Influenza Virus Isolates

Since A/WSN/33 is a highly mouse-adapted strain of IAV, we next wanted to assess whether human-derived isolates which had not been passed through mice could activate mast cells *in vitro*. To do this, $2.5x10^5$ BMCMC were treated with 50 □l of each influenza virus isolate for 6 hours. BMCMC supernatants were then analyzed for the cytokine and chemokine levels. In agreement with our earlier findings, A/WSN/33 treatment of BMCMC resulted in secretion of IL-6, CCL2, and TNF-α (Figure 4.5, black bar). Interestingly, another mouse adapted IAV strain, A/Puerto Rico/8/34 resulted in minimal activation of mast cells (Figure 4.5), which corresponds with our *in vivo* observation that B6.Cg-*Kit*$^{W\text{-}sh}$ mice are as susceptible as C57BL/6 to A/PR/8/34 infection (Figure 5.9). Human-derived IAV isolations of both H1N1 (Figure 4.5, light gray bars) and H3N2 (Figure 4.5, dark gray bars) families were both able to activate murine BMCMC to produce cytokines and chemokines to varying levels. Furthermore, the influenza B virus isolate B/Florida/4/2006 could also activate murine BMCMC *in vitro* to produce these cytokines and chemokines (Figure 4.5, white bar). Our data support the notion that mast cell could play an important role in the inflammatory response to a range of influenza virus isolates.

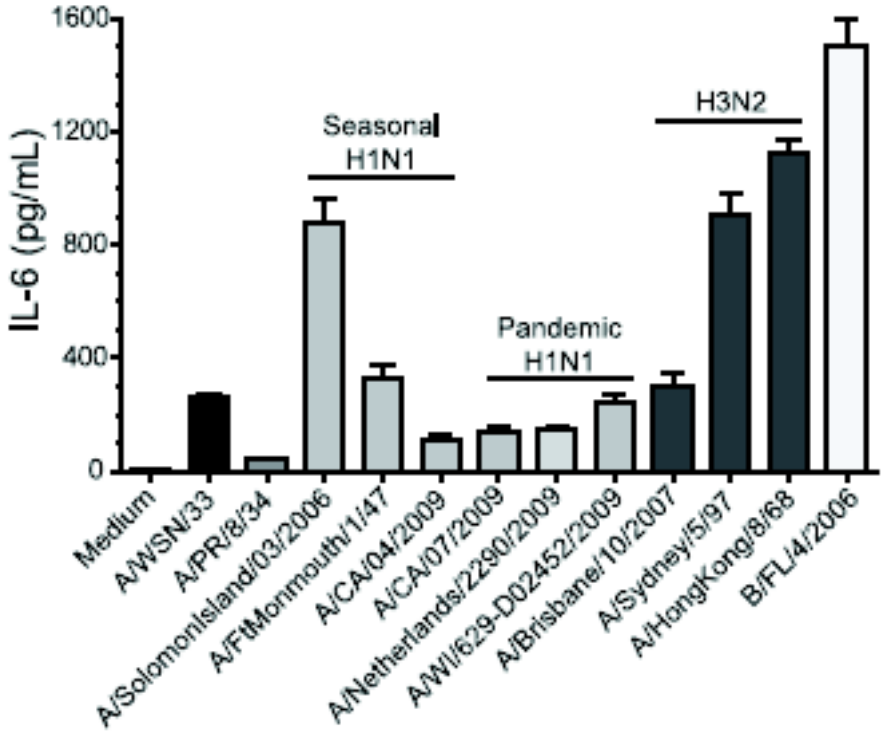

Figure 4.5. Human influenza virus isolates can activate mast cells. BMCMC were derived by culturing total bone marrow with IL-3 for 5 wk and supplementing with stem cell factor for the last 2 wk. A total of $2.5x10^5$ FcRε1$^+$ and CD117$^+$ mast cells were treated with media or 50μL of each virus for 6 h at which time supernatants were collected and analyzed for cytokine and chemokine analysis with Milliplex™ multiplex analysis. Expression of IL-6 is shown for each virus: Mouse adapted strains of IAV H1N1: A/WSN/33 and A/PR/8/34 (EID_{50}/ml log_{10}=8.8); Human derived isolates of IAV H1N1 which are either seasonal or pandemic viruses: A/Solomon Island/03/2006 (EID_{50}/ml log_{10}=7.2), A/FtMonmouth/1/47 (EID_{50}/ml log_{10}=7.4), A/CA/04/2009 (EID_{50}/ml log_{10}=6.9), A/CA/07/2009 (EID_{50}/ml log_{10}=8.4), A/Netherlands/2290/2009 (EID_{50}/ml log_{10}=7.4), and A/WI/629-D02452/2009 (EID_{50}/ml log_{10}=7.2); Human derived isolates of IAV H3N2: A/Brisbane/10/2007 (EID_{50}/ml log_{10}=8.4), A/Sydney/5/97 (EID_{50}/ml log_{10}=7.9), and A/Hong Kong/8/68 (EID_{50}/ml log_{10}=8.4); and Human derived isolates of influenza B virus B/FL/4/2006 (EID_{50}/ml log_{10}=8.4). Similar results were observed with CCL2 and CCL4. Data are representative of two independent experiments. All IAV isolates, with the exception of A/PR/8/34, induced an IL-6 response that was statistically significantly different than the medium control, as determined by a one-way ANOVA ($p<0.05$).

Mast Cells are Infected by A/WSN/33

We next wanted to ask whether mast cells were capable of being infected with IAV. To do this, $2.5x10^5$ BMCMC were treated with medium, A/PR/8/34, or A/WSN/33 for 6 hours. Cells were then fixed, permeabilized, and stained for intracellular NS1 of IAV. NS1 is a non-structural protein which is not found in the IAV virion and therefore require *de novo* synthesis of viral proteins to detect protein. Uninfected BMCMC displayed minimal anti-NS1 staining over our secondary antibody alone (Figure 4.6, black histogram). Interestingly, A/WSN/33 treated BMCMC expressed intracellular NS1 (Figure 4.6, red histogram); while A/PR/8/34 treated BMCMC did not (Figure 4.6, blue histogram). Thus, the A/WSN/33 strain of IAV is able to directly infect mast cells, which correlates with the virus' ability to activate mast cells, but could not propagate IAV.

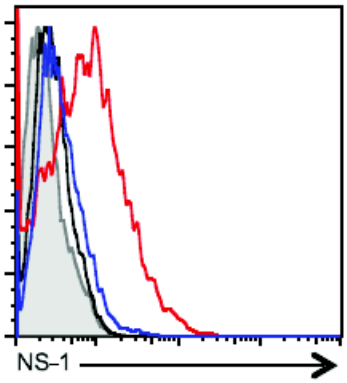

Figure 4.6. Mast cells are infected by A/WSN/33. BMCMC were derived by culturing total bone marrow with IL-3 for 5 wk and supplementing with stem cell factor for the last 2 wk. A total of $2.5x10^5$ $FcR\varepsilon1^+$ and $CD117^+$ mast cells were treated with medium, A/WSN/33, or A/PR/8/34. Each virus was added at an MOI of 1. BMCMC were harvested after 5 hr and fixed, permeabilized, and stained for intracellular NS-1 using mAb NS1-1A7 for 30 min. BMCMC were then stained with PE-labeled anti-mouse IgG F(ab') fragments. Representative histograms are shown for two independent experiments. Shaded histogram = PE-labeled anti-mouse IgG F(ab') fragments alone; Black histogram = media treated BMCMC; Blue histogram = A/PR/8/34 infected BMCMC; Red histogram = A/WSN/33 infected BMCMC.

RIG-I Detection of IAV is Involved
in Mast Cell Cytokine, Chemokine, and
Leukotriene Production, but Not Degranulation

Numerous pattern recognition receptors have been shown to play a role in sensing IAV infection. These include the TLR3, TLR7, NLRP3, and RIG-I receptors (34-43). Because BMCMC were productively infected with A/WSN/33 we hypothesized that detection of cytosolic genomic RNA might be crucial for mast cell activation. The major cytosolic receptor for IAV RNA is RIG-I, which subsequently docks on its adaptor MAVS (also known as IPS-1, VISA, and Cardif) to initiate IRF3- and NF□B-dependent signaling (44-47). We first decided to examine the role of RIG-I in the activation of mast cells by A/WSN/33. To do this $2.5x10^5$ BMCMC from either C57BL/6 or RIG-$I^{-/-}$ mice were treated with medium, A/WSN/33, or LPS for 6 hours. In agreement with our previous findings, A/WSN/33 treatment of C57BL/6-derived BMCMCs resulted in significant production of histamine, IL-6, and LTB_4 (Figure 4.7a-c). In contrast, RIG-$I^{-/-}$-derived BMCMCs had significantly blunted IL-6 and LTB_4 responses after A/WSN/33 treatment (Figure 4.7a & b). However, RIG-$I^{-/-}$-derived BMCMCs produced equivalent levels of histamine when compared with A/WSN/33 treated C57BL/6-derived BMCMCs (Figure 4.7c). Similarly, when BMCMCs from $MAVS^{-/-}$ mice were used cytokine and chemokine production was significantly reduced (Supplemental Figure 4.11). Importantly, RIG-$I^{-/-}$ BMCMC responded similarly to C57BL/6 BMCMC to an irrelevant stimulus, such as LPS or A23187. Our data demonstrate that RIG-I/MAVS signaling is crucial for *de novo* generated mediators, but not the immediate degranulation response of mast cells during IAV treatment.

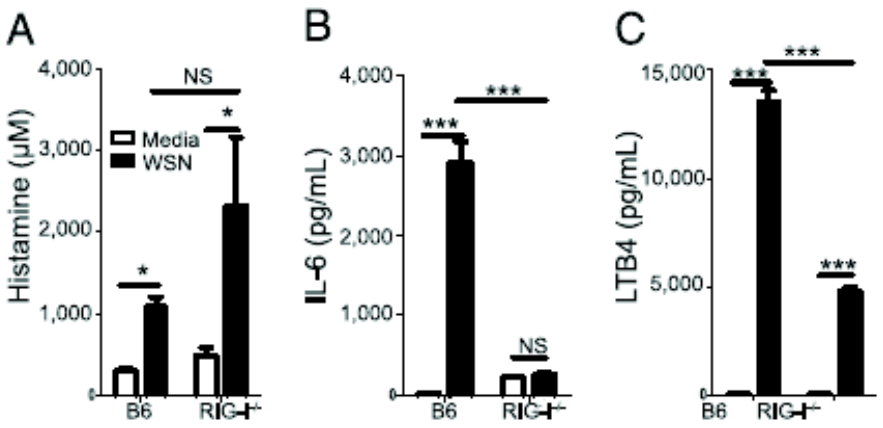

Figure 4.7. Mast cell activation by A/WSN/33 is dependent on RIG-I. BMCMC were generated by culturing total bone marrow from either C57BL/6 (B6) or RIG-I$^{-/-}$ mice with IL-3 for 5 wk and supplementing with stem cell factor for the last 2 wk. A total of 2.5x10^{5} FcRε1^{+} and CD117^{+} mast cells were treated with media or A/WSN/33 (WSN). Virus was added at an MOI of 1. Six hours after inoculation histamine (A), IL-6 (B), and LTB4 (C) levels were measured by Milliplex™ multiplex analysis or EIA. Similar data were observed for CCL2 and CCL4 expression. Data are representative of two to four independent experiments. *p<0.05, ***p<0.001, one-way ANOVA.

Cytokine and Chemokine
Expression by Mast Cells in
Response to A/WSN/33 Treatment
is Partially STING and STAT6 Dependent

Our knowledge about the signaling networks downstream of RIG-I recognition of ssRNA has expanded to include STING, CARD9, and STAT6. RIG-I/MAVS interactions result in the activation of IRF3 and NF-□B (44-47). Recently, CARD9 and MALT10 were shown to be necessary for activation of NF-□B, but not IRF3 in response to synthetic RIG-I ligands or VSV infection (48). STING is an ER resident protein that has been shown to be important in the RIG-I dependent anti-viral response to VSV (49). Additionally, others have found that STAT6 is recruited to the STING-MAVS-RIGI

complex during Sendai virus or VSV infection to mediate CCL2 and CCL20 expression, but is dispensable for IL-6 production (50). Since RIG-I and MAVS were critical in the cytokine/chemokine response of BMCMC, we next wanted to examine the role of CARD9, STING, and STAT6 in the response of BMCMCs to A/WSN/33 infection. To do this, $2.5x10^5$ BMCMC from C57BL/6, $CARD9^{-/-}$, $STING^{-/-}$, or $STAT6^{-/-}$ mice were treated with medium, A/WSN/33, or LPS for 6 hours. In agreement with our previous findings, A/WSN/33 treatment of C57BL/6-derived BMCMCs resulted in cytokine and chemokine secretion in all experiments (Figure 8a-c). The response of $CARD9^{-/-}$-derived BMCMCs was similar to C57BL/6-derived BMCMCs after A/WSN/33 infection (Figure 4.8a). However, $STING^{-/-}$- and $STAT6^{-/-}$-derived BMCMCs had a ~50% reduction in their production of IL-6, CCL2, and CCL4 (Figure 4.8b & 8c). Importantly, $CARD9^{-/-}$, $STING^{-/-}$ and $STAT6^{-/-}$ BMCMC responded similarly to C57BL/6 BMCMC to an irrelevant stimulus, such as LPS. Thus, while RIG-I and MAVS are absolutely essential for the cytokine response of BMCMCs in response to IAV infection (Figure 4.7), CARD9 is completely dispensable, while STING and STAT6 play partial roles in the inflammatory cytokine and chemokine response to IAV infection (Figure 4.8).

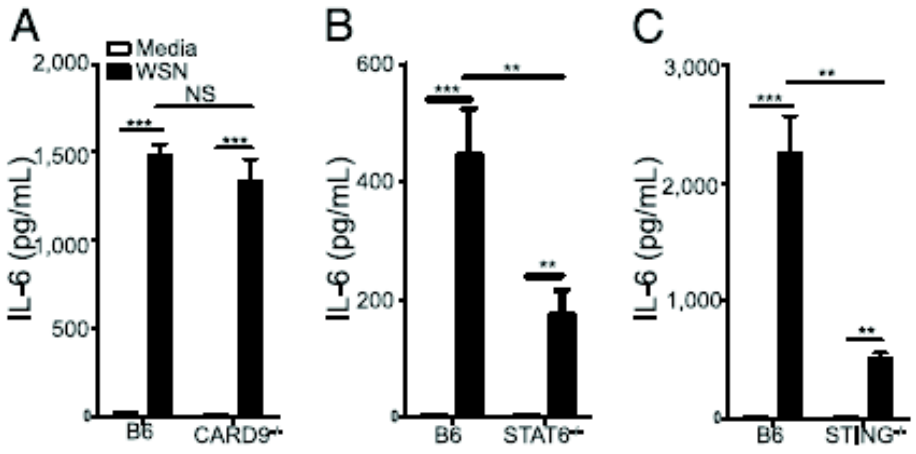

Figure 4.8. Mast cell activation is partially dependent on STING and STAT6. BMCMC were generated by culturing total bone marrow from either C57BL/6 (B6) or CARD9$^{-/-}$ mice (A), C57BL/6 or STAT6$^{-/-}$ mice (B), or C57BL/6 or STING$^{-/-}$ mice (C) with IL-3 for 5 wk and supplemented with stem cell factor for the last 2 wk. A total of $2.5x10^5$ FcRε1$^+$ and CD117$^+$ mast cells were treated with media or A/WSN/33 (WSN). Virus was added at an MOI of 1. Six hours after inoculation, IL-6 levels were measured by Milliplex™ multiplex analysis. Similar data was observed for CCL2 and CCL4 expression. Data are representative of two to four independent experiments **$p<0.01$, ***$p<0.001$, one-way ANOVA.

Discussion

Mast cells are tissue resident hematopoietic cells. Since infectious agents enter the host through environmentally exposed barriers, such as the skin, gastrointestinal tract, and respiratory tract, mast cells are poised to be one of the first cell types to respond to invading pathogens. Furthermore, mast cells express a wide array of pattern-recognition receptors that endow them with the ability to respond to a broad range of stimuli, such as infections and pathogenic conditions (51). To date it is well established that mast cells play crucial immune surveillance roles during bacterial and parasitic infections (8, 12). In contrast, the role of mast cells in the immune surveillance of viral infections has received

less attention. In the current study, we examined the role of mast cells in sensing IAV infection and initiating the subsequent inflammatory response.

A primary rationale for our work stems from the recent work by Teijaro *et al* that demonstrated that blunting the 'cytokine storm' significantly improves the health of animals infected with either the mouse-adapted IAV strain A/WSN/33 or the 2009 H1N1pdm strain A/Wisconsin/WSLH34939/2009 (6). Thus, we reasoned if mast cells are critical mediators of the inflammatory response to IAV, a further understanding of this interaction may lead to new therapeutic option(s) to improve IAV patient outcomes.

In further support of our hypothesis regarding mast cells and IAV, recent genetic analysis of the lungs from mice infected with a model strain of the 2009 IAV pandemic virus demonstrated an enrichment of genes associated with mast cells (32). However, whether respiratory mast cells play a role during IAV infection remains elusive. Here our data demonstrate that mast cells are critical participants in local lung inflammation and systemic IAV-induced disease during A/WSN/33 infection of mice. *In vivo* we found that mast cells contributed to the establishment of the inflammatory milieu and lung damage during A/WSN/33 infection. This appears to not be due to dramatic differences in the lung viral burden in the absence of mast cells; rather, in the absence of mast cells (B6.Cg-$Kit^{W\text{-}sh}$), mice had decreased inflammatory cell infiltration into the BALF and levels of inflammatory cytokines and chemokines. Importantly, this reduction in inflammatory cells and mediators in the absence of mast cells correlated with reduced lung damage and/or vascular leakage as measured by protein levels in the BALF.

Our results fit with a recent report that the H5N1 isolate A/chicken/Henan/1/2004 was capable of activating the mastocytoma cell line P815 and inducing a significant

increase in mast cell numbers within the respiratory tract over the first 5 days of IAV infection in mice (52). We extend these findings to show that IAV can activate primary mast cells (BMCMC) and importantly that H1N1, H3N2, and influenza B viruses could also activate mast cells. Additionally, Hu *et al* demonstrated that ketotifen, an H1 receptor inhibitor (53), could limit epithelial cell death *in vivo* (52). However, ketotifen can also inhibit cytokine and chemokine expression by macrophages after LPS stimulation (54) and the H1 receptor has a broad expression pattern including airway epithelial cells (55). These facts complicate the interpretation by Hu *et al* that mast cells are the crucial cell inhibited by ketotifen (52). In our study we specifically demonstrate that mice lacking mast cells do not develop as severe an IAV-induced inflammatory response and lung damage, which could be complemented by reconstitution of those mice with mast cells. How mast cells are mediating their response *in vivo* is the focus of our future studies. *In vitro* Hu *et al* demonstrated mast cells produced high levels of IFNγ which caused significant epithelial cell death (52). However, we failed to detect any IFNγ expression by BMCMC after IAV treatment. The major differences between our two studies are the source of mast cells (primary BMCMC versus P815 cell line) and viral isolates studied (H1N1 versus H5N1 isolates), which could likely contribute to this difference. However, both our study and Hu et al. (52) suggest that human IAV isolates can activate mast cells. Interestingly, IAV infection of humans results in elevated urinary histamine levels, whose major source *in vivo* are mast cells and basophils (56), which peaked ~2 days after infection and its expression correlated with virus-induced illness (57). Thus, mast cells appear to be an attractive target for a host-targeted therapeutic to limit the pathological response induced by IAV infection.

Interestingly, similar to our results with IAV, mast cells have been shown to be a key role during cutaneous viral infections. Cutaneous mast cells were recently shown to play a protective role during skin vaccinia virus infection (22). Additionally, mast cells have been shown to be crucial for the *in vivo* immune surveillance of dengue virus after a foot-pad inoculation (19). Mast cells can also regulate endothelial cell function during dengue virus infection *in vitro* (58). Importantly, dengue shock syndrome in humans displays elevated levels of mast cell-derived tryptases and chymases (21). Thus, mast cells are likely crucial participants in the inflammatory response during dengue virus infection through the skin. These data, together with ours, demonstrate that mast cells may play a crucial role in the immune surveillance of viral infections in general.

A major question then is how mast cells detect viral infections and whether mast cells can serve as a suitable environment for virus replication. Again, there appears to be numerous correlations between IAV and dengue virus. We found that mast cells were directly infected with IAV *in vitro* as demonstrated by *de novo* NS1 expression, but BMCMC could not propagate A/WSN/33 *in vitro*. This fits with Befus and colleagues recent finding that IAV did not replicate in human mast cells (59). Similarly, the mast cells could be infected with dengue virus (19, 20); however, in contrast to our results, dengue virus could replicate within mast cells (19). BMCMC could be infected with vaccinia virus at higher multiplicities of infection, but whether vaccinia virus could replicate within mast cells *in vitro* was not explored (22).

Interestingly, it appears each phase of the mast cell response, immediate degranulation and *de novo* mediators synthesis, is regulated by a distinct innate immune sensing receptor and signaling network during the course of IAV infection. Expression of

de novo synthesized cytokines and chemokines following IAV inoculation required RIG-I and MAVS. Similar to our results with IAV, chemokine expression by mast cells in response to dengue virus infection was dependent on sensing by both the cytosolic RNA sensors, RIG-I and Mda5 (19, 20). Moreover, IL-6, CXCL10, and type I interferon expression by BMCMC in response to VSV treatment was dependent on RIG-I and Mda5 (60). Upon interacting with RNA, RIG-I and Mda5 undergo a conformational change allowing it bind with the scaffolding protein MAVS. Originally, MAVS was described as a mitochondrial localized protein (44), but recently MAVS has been found to also localize to the peroxisome (61). Interestingly, the downstream signaling events of mitochondrial or peroxisome localized RIG-I/MAVS complexes differs (61). Specifically, peroxisome localized RIG-I/MAVS results in the activation of early interferon-regulated proteins in the absence IFNα□β expression (61). In contrast, mitochondrial localized RIG-I/MAVS results in the production of IFN□□□ with delayed kinetics (61). RIG-I appears to interact with MAVS at the mitochondria-associated endoplasmic reticulum membrane (MAM) (62). STING is an endoplasmic reticulum protein which is important for the scaffolding protein for signaling of receptors detecting cytosolic nucleic acids (49). STING-RIG-I interactions were recently demonstrated to be important for the anti-viral response to Japanese encephalitis virus (JEV) (63). Moreover, STING recruits Stat6-dependent anti-viral immunity through the production of CCL2, CCL20 and CCL26, but not IL-6 and IFNα□β (50). Our data are consistent with a more central role of peroxisome-mediated RIG-I/MAVS mediated signaling during IAV infection of BMCMC for two reasons: 1) we observed nominal expression of IFN α□β, CXCL9, and CXCL10 and 2) we observed only a partial STING- and Stat6-dependency

for the inflammatory response of BMCMC. However, formal testing of whether peroxisome or mitochondrial MAVS is more important during IAV infection of mast cells remains to be explored. Additionally, the PB1-F2 protein of IAV was shown to interfere with MAVS-mediated signaling through disruption of the mitochondrial membrane potential (64-66). Potentially, PB1-F2's expression in mast cells could strongly impair mitochondria localized MAVS, thus making peroxisome localized MAVS the dominant signaling pathway in mast cells. Thus, it will be intriguing to test the anti-viral cellular response of BMCMC in the absence of these IAV proteins.

In addition to viral detection mechanisms, how mast cells degranulate in response to IAV, VSV, or dengue virus infection remains an open question. Histamine production from BMCMC occurred rapidly after IAV inoculation and appears to occur prior to endosomal escape of the virus (Figure 5.3). Furthermore, histamine production by BMCMC after IAV infection was normal in the absence of RIG-I. Analogously, BMCMC degranulation in response to VSV treatment occurred normally following RIG-I or Mda5 knock-down by siRNA (60). Other pattern-recognition receptors detecting early stages of the IAV infectious cycle could be important, such as TLR3 or TLR7 (34-36). However, triggering of synthetic polyI:C could not induce the degranulation of human mast cells (67). Alternatively, STING was recently shown to 'recognize' viral membrane fusion (68), but it is role in mast cell degranulation has not be explored. The one viral infection we have at least a partial understanding of how the virus is inducing mast cell degranulation is vaccinia virus infection. During vaccinia virus infection BMCMC degranulation occurred in a membrane fusion-dependent and S1PR2-dependent manner (22). S1P signaling is important inducing the cytokine storm during IAV infection (6) and

in vitro can regulate IAV growth (69); however, whether S1P signaling plays a role in regulating mast cell function during IAV infection remains unknown.

In conclusion, we demonstrate that mast cells can be infected by IAV resulting in their activation. Importantly, mast cells were critical participants in inducing IAV-induced cytokine storm and systemic disease during A/WSN/33 infection of mice. Our results suggest that the mast cell response to IAV infection is regulated by multiple receptors controlling either degranulation or *de novo* mediator synthesis. Importantly, based on our findings it is important to consider the role mast cells are playing in regulating the excessive inflammatory response during infections when trying to control such adverse events. It remains to be answered whether degranulation, *de novo* mediator synthesis, or both are required for mast cells to enhance the inflammatory response and pathology during IAV infection. Understanding these events is imperative for developing a host-targeted therapeutic which is aim at curtailing mast cell function to limit IAV-induced morbidity and mortality.

Acknowledgements

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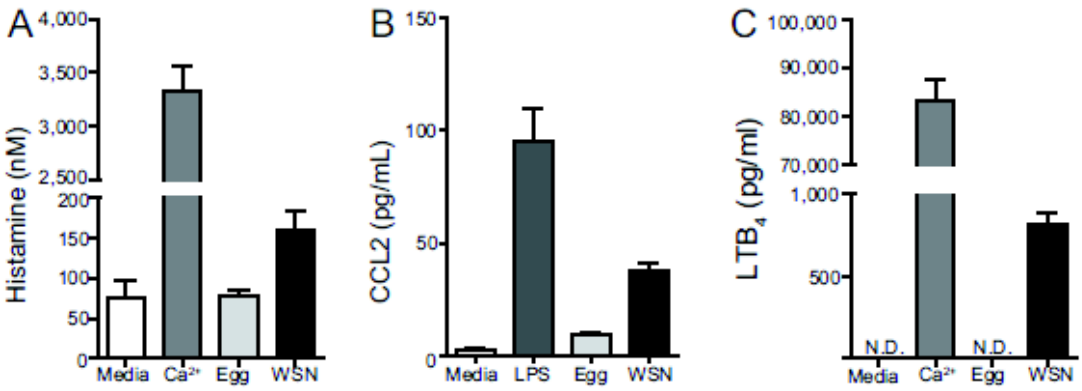
(NIH/NIAID), Dr. Allen Harmsen (Montana State University), and Dr. Robert Cramer (Geisel School of Medicine at Dartmouth College) provided many helpful suggestions about this project.

Footnotes

[1]Work in this study was supported by NIH grants P20 RR-020185 (JJO), P20 GM-103500 (JJO), and K22 AI-091647 (JJO), the Montana State University Agricultural Experimental Station, and equipment grant from the M.J. Murdock Charitable Trust.

[2]Abbreviations: IAV = influenza A virus; RIG-I = Retinoic acid inducible gene I; RLR = RIG-I like receptors; NLR = Nod-like receptors; CLR = C-type lectin-like receptors; BALF = broncheoalveolar fluid; BMCMC = bone marrow cultured mast cells; LTB_4 = leukotriene B_4



Supplemental Figure 4.9. Naive allantoic fluid does not cause BMCMC activation. BMCMC were derived by culturing total bone marrow with IL-3 for 5 weeks, supplementing with stem cell factor for the last 2 weeks. $2.5x10^5$ $FcRr1^+$ and $CD117^+$ BMCMC were treated with media, naive allantoic fluid, A/WSN/33 (WSN), or a positive control of calcium ionophore (Ca^{2+}) at 40nM or LPS at 5mg/mL. (A) Histamine levels were measured by an EIA 30 minutes after stimulation. (B-C) Cytokine/chemokine (B) and LTB_4 (C) secretion were measured 6 hours after stimulation by either Milliplex^{TM} multiplex analysis or an EIA, respectively. A/WSN/33 was added at an MOI of 1. Data are representative of 2 independent experiments. N.D. = none detected.

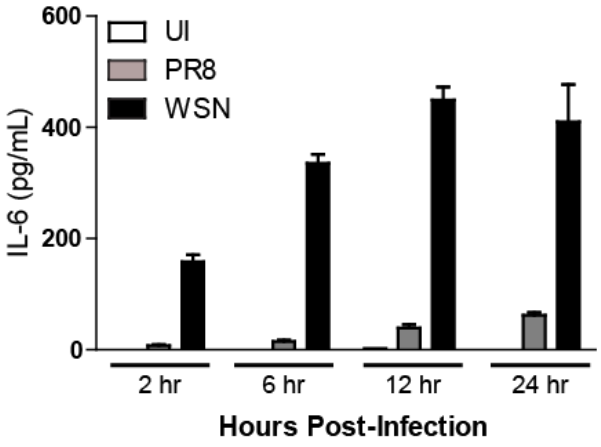
Supplemental Table 1. Cytokine and Chemokine Produced by BMCMC after A/WSN/33 treament.[1]

	A/WSN/33 (pg/ml)[2]
IL-1β	< LOD[3]
IL-5	< LOD
IL-6	742.8 ± 92.8
IL-9	< LOD
IL-15	< LOD
IFNα	< LOD
IFNβ	< LOD
IFNγ	< LOD
TNFα	12.7 ± 3.2
VEGF	< LOD
CCL2 (MCP-1)	89.1 ± 5.8
CCL3 (MIP-1α)	169.6 ± 81.2
CCL4 (MIP-1β)	420.2 ± 36.8
CCL5 (RANTES)	71.3 ± 38.8
CXCL1 (KC)	< LOD
CXCL2 (MIP-2)	17.4 ± 1.4
CXCL9 (MIG)	4.9 ± 2.7
CXCL10 (IP-10)	11.9 ± 2.3

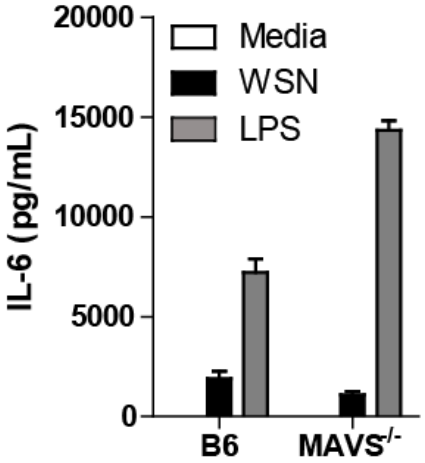
[1]To measure the ability of IAV to induce each cytokine or chemokine, $2.5x10^5$ BMCMC mast cells were treated with A/WSN/33 at an MOI+1 for 6 h. At which time cytokine or chemokine levels in the supernatants were measured by Luminex™ multiplex assays.

[2]Uninfected BMCMC had cytokine levels below the limit of assays

[3]LOD = Limit of Detection; IL-1β = 2.0 pg/ml, IL-5 = 0.5 pg/ml, IL-9 = 6.0 pg/ml, IL-15 = 6.5 pg/ml, IFNα = 10pg/ml, IFNβ = 10 pg/ml, IFNγ = 0.9 pg/ml, VEGF = 0.3 pg/ml, CXCL1 (KC) = 1.4 pg/ml



Supplemental Figure 4.10 Cytokine and chemokine expression began ~2 hours after A/WSN/33 treatment. BMCMCs were derived by culturing total bone marrow with IL-3 for 5 wk and supplemented with stem cell factor for the last 2 wk. A total of $2.5x10^5$ $FcR\varepsilon1^+$ and $CD117^+$ mast cells were treated with media, A/WSN/33 (WSN), or A/PR/8/34 (PR8). Cytokine/chemokine secretion was measured 2, 6, 12, and 24 h after stimulation by either Milliplex™ multiplex analysis. Each virus was added at an MOI of 1. Data are representative of two to four independent experiments.



Supplemental Figure 4.11 Mast cell activation is partially dependent on MAVS. BMCMC were generated by culturing total bone marrow from either C57BL/6 (B6) or $MAVS^{-/-}$ mice with IL-3 for 5 wk and supplemented with stem cell factor for the last 2 wk. A total of $2.5x10^5$ $FcR\varepsilon1^+$ and $CD117^+$ mast cells were treated with media or A/WSN/33 (WSN). Virus was added at an MOI of 1. Six hours after inoculation, IL-6 levels were measured by Milliplex™ multiplex analysis. Similar data was observed for CCL2 and CCL4 expression.

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CHAPTER FIVE

DIFFERENTIAL ROLE OF INFLUENZA A VIRUS BINDING PREFERENCE IN MAST CELL ACTIVATION

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CHAPTER FIVE

DIFFERENTIAL ROLE OF INFLUENZA A VIRUS BINDING PREFERENCE IN MAST CELL ACTIVATION

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Abstract

Influenza A virus (IAV) is a seasonal respiratory pathogen that typically causes a mild respiratory disease, although in some years infection can result in severe lung pathology, leading to acute respiratory distress syndrome. Recent global analysis of lungs from mice infected with highly pathological IAV strains, such as the 1918 H1N1 strain, demonstrated an enrichment of a mast cell genetic signature in the lungs compared to non-pathological IAV strains (1). This is supported by histological analyses exhibiting increased mast cell number in the upper and lower respiratory tracts and nasal mucosa (2). However, the role of mast cells during severe pulmonary viral infections has been under studied. We have recently shown that A/WSN/33 causes significant immunopathology in C57Bl/6 mice and that this pathology was mast cell-dependent. A/WSN/33 is able to directly activate mast cells *in vitro* to produce histamine, leukotrienes, inflammatory cytokines, and anti-viral chemokines. Interestingly, mast cell cytokine production occurs in a RIG-I/MAVS-dependent fashion, but reconstitution of mast cell-deficient mice with RIG-$I^{-/-}$ BMCMC generated lung pathology similar to wild-type mast cells. Thus, it appears that mast cell degranulation rather than production of

cytokines drives lung pathology observed following A/WSN/33 infection. We demonstrate that mast cell degranulation occurs early in the viral entry cycle through a RIG-I-independent mechanism. Using recombinant A/WSN/33 strains, we found an association between the A/WSN/33 hemagglutinin and neuraminidase proteins in the activation of mast cell degranulation and A/WSN/33-induced disease. Thus, we have identified a mechanism that could potentially be therapeutically targeted to limit morbidity following infection with influenza virus.

Introduction

Influenza A virus (IAV) is a common seasonal respiratory pathogen that has the potential to cause global pandemics, inducing severe lung injury that can culminate in acute respiratory distress syndrome. Seasonal IAV causes limited morbidity and mortality associated with only certain susceptible populations. However, severe IAV can affect the entire human population, as seen during the 1918 Spanish influenza pandemic and more recently with the 2009 pandemic H1N1 IAV (H1N1pdm). Strains that cause pandemics are impossible to predict; thus, understanding the pathological mechanisms of these pandemic viruses, including the immune response to the virus, are important to limit IAV-associated illnesses.

Highly pathogenic strains of IAV can cause changes in lung architecture due to an uncontrolled immune response, characterized by an uncontrolled influx of neutrophils, macrophages, and a ‘cytokine storm’ (3-9). Limiting this cellular influx and ‘cytokine storm’ can increase the survival of mice infected with these pathogenic strains of IAV (9, 10). However, lack of an immune response during IAV infection is also detrimental, as

depletion of neutrophils in mice led to decreased survival during infection (11-13). Therefore, understanding the balance of the immune response induced by IAV is important to prevent damage and control infection.

The respiratory tract contains alveolar epithelial cells, endothelial cells, tissue resident macrophages, dendritic cells, and mast cells that make up the initial lines of defense against invading respiratory pathogens. Mast cells are hematopoietic cells enriched in tissues exposed to the environment, including the skin, gastrointestinal, and respiratory tracts, allowing them to be among the sentinel responders to pathogens (15-20). There are two distinct phases of mast cell activation: 1) immediate degranulation of pre-synthesized mediators including histamine, serotonin, tryptases, and stored TNF-α, and 2) delayed secretion of *de novo* synthesized mediators consisting of leukotrienes, prostaglandins, growth factors, cytokines, and chemokines (21). The activation of mast cells by pathogens will dramatically influence the downstream immune response to mediate an appropriate response to aid in the clearance of the invading pathogen.

The role that mast cells play during bacterial and parasitic infections has been well characterized, but their function during viral infections is only recently becoming known (16, 19, 22). Mast cells have been shown to respond to a variety of viruses, including vesicular stomatitis virus (VSV), Sendai virus, hantavirus, dengue virus, Newcastle disease virus, and reovirus (15, 23-33). Activation of mast cells can lead to both defensive anti-pathogen and tissue damaging immune responses. The role that mast cells play during IAV infections is only beginning to be unraveled. Experimental infection of humans with IAV resulted in detectable levels of histamine in their blood, which correlated with the onset of disease symptoms (34, 35). Also, a pulmonary

transcriptome analysis of mice infected with a pathological model strain of the 2009 H1N1 IAV pandemic virus revealed an enrichment of mast cell associated transcripts in the lung during infection but the functional role of mast cells in the model was not explored (1). We have recently shown that mice lacking mast cells are not as susceptible to A/WSN/33 IAV-induced immunopathology, suggesting a detrimental role of mast cells during influenza infection (36). Moreover, mice treated with mast cell stabilizing compounds during H5N1 IAV infection fared better than their untreated counterparts (2). Therefore, it appears that mast cells can be activated during severe IAV infection and influence the immunopathological response to the certain IAV isolates. Conversely, detection of dengue virus by mast cells is important in viral control early after infection, but can be disadvantageous as dengue virus infection persists (15, 37, 38). Thus, mast cells appear to have central roles in modulating the innate inflammatory response to viruses, which can be disadvantageous to infected hosts.

Although, we have previously shown mast cells to be infected by A/WSN/33 (36), how this affects activation is not understood. An important step in the viral replication cycle is the binding of viral hemagglutinin (HA) to sialic acid receptors located on the cell surface. Viral HA is a trimeric protein located on the viral envelope (39). During attachment, the viral HA binds to sialic acid linkages on the surface of cells, initiating entry of the virus into the cellular endosome (39-43). IAV HA preferably binds to α2,3- or α2,6-sialic acids linkages containing glycoproteins and glycolipids, and different IAV strains bind to either sialic acid linkage with distinct preferences (39-43). Generally, human IAV binds to α2,6-sialic acid linkages, while avian IAV preferably binds to α2,3-linkages (40, 42, 43).

Once attachment has occurred, the virus is able to induce endocytosis into the cell. Endocytosis of IAV occurs either through clathrin-mediated endocytosis (CME) or non-CME, which includes mechanisms like macropinocytosis (44). CME is the most well characterized mechanism of IAV uptake and involves the formation of clathrin-coated pits after the virus binds to the cell. Once the pit is formed, it is 'pinched off' by dynamin to form clathrin-coated vesicles, which then fuse with other endocytic vesicles or endosomes (44-46). However, in certain conditions IAV is still able to enter cells when CME is inhibited, suggesting IAV may utilize other mechanisms for entry (44-46). Macropinocytosis of IAV occurs independent of dynamin and can be induced by serum (44). Once pinocytosed, the vacuole containing the virus will then fuse to the endolysosomal system. A drop in the pH of the virus containing endosome initiates release of the viral genome into the cytoplasm. The viral genome then translocates to the nucleus, where replication takes place. Viral mRNA is translated in the cytoplasm and protein assembles at the host cell membrane for packaging and budding. For efficient viral spread, neuraminidase (NA) cleaves sialic acids, releasing the newly formed virion from the cell (47).

We have previously shown that mast cells are important in the pathological immune response to the A/WSN/33 isolate of IAV. A/WSN/33 induced secondary mediators through RIG-I-dependent signaling, and active replication of the viral genome is important for this secondary response from mast cells (36). While the mechanism driving A/WSN/33-induced degranulation from mast cells is currently unknown. In this study, we show that the binding of viral HA is important for mast cell activation. Additionally, we have found that NA also plays a role in mast cell degranulation. We

demonstrate that A/WSN/33, which preferably binds to α2,6-linked sialic acids activates mast cells and leads to increased lung damage during infection. We have found that another H1N1 strain, A/PR/8/34, preferably binds to α2,3-linked sialic acids, does not activate mast cells. Most importantly, a recombinant A/WSN/33 virus that switches the sialic acid binding preference from α2,6-linked sialic acids to α2,3-linked sialic acids was also unable to drive of mast cell degranulation. Interestingly, *in vivo* infection with these viruses leads to differential downstream responses. A/PR/8/34 result in higher neutrophil recruitment, as opposed to A/WSN/33, which induces lung damage due cells more of a monocytic nature. Thus, we show that preferential sialic acid binding of IAV HA to sialic acids located on mast cells can influence the downstream immune response during IAV infection.

Materials and Methods

Viral Strains

A/WSN/33 virus in allantoic fluid was originally obtained from Dr. David Topham (University of Rochester) and A/Puerto Rico/8/34 (A/PR/8/34) was originally purchased from Charles River. A/WSN/33 NA130 was originally obtained from Dr. Peter Palese (Mount Sinai School of Medicine). The viruses were then subsequently grown in embryonic chicken eggs. WSN D225G HA plasmid was a gift from Dr. Leo Poon (University of Hong Kong) and all other WSN plasmids were gifts from Dr. Robert Webster (St. Jude). Recombinant viruses were created by using a plasmid-based reverse genetics system (48). Briefly, $4x10^5$ 293T cells were transfected with the 8 bidirectional purified IAV plasmids using *Trans*IT®-293 Transfection Reagent (Mirus Bio LLC;

Madison, WI) per the manufacturer's protocol. Roughly, 0.8μg of each of the 8 plasmids were combined with serum-free DMEM and allowed to incubate at RT for 45 minutes with 2 μL/μg transfection reagent. This mixture was then added drop-wise to the cultured cells and moved to a 37°C 5% CO_2 incubator for 72 hours. The transfected cells and supernatant were then transferred to a confluent flask of MDCK cells in low-serum media (DMEM high glucose, 1.4% BSA. 0.14% sodium bicarbonate) for 72 hours to amplify the recombinant viruses. Virus-containing supernatants were cleared by centrifugation for ten minutes at 400 x *g*. Supernatant was aliquoted and stored at -80°C until they were grown in embryonic chicken eggs and used in BMCMC activations.

Mouse Strains and Infectious Protocol

B6.Cg-*kit*$^{W\text{-}sh}$ mice were original purchased from Jackson Laboratories and successively bred in house. C57Bl/6J mice were bred in house. Mice were intra-nasally infected with 1000 plaque forming units (PFU) of A/WSN/33, A/PR/8/34, A/WSN/33 NA130, or A/WSN/33 D225G, as indicated in the text, under anesthesia with 2,2,2-tribromoethanol (Avertin). At the indicated times after IAV infection, mice were given a lethal overdose of pentobarbital. Broncheoalveolar lavage fluid (BALF) was collected by washing the lungs with 2 ml of PBS containing 50 mM EDTA. BALF was centrifuged, cells were analyzed by differential cytospins, and BALF supernatant was analyzed using luminex assay for cytokine or chemokine analysis (Millipore). For weight loss studies, mice were infected as previously stated, weighed daily, and normalized to starting weight. All animal protocols were approved by the Montana State University Institutional Animal Care and Use Committee.

UV Virus Inactivation

To UV inactivate A/WSN/33, egg-grown virus was placed in a 6cm petri dish in a thin layer. This was placed on ice ~10cm from a 254nm UV lamp for 60 minutes. As a control, A/WSN/33 was placed in a petri dish and kept on ice without being exposed to UV light. The virus was aliquoted and stored at -80°C until use. The virus was titered using IAV plaque assay to confirm inactivation of the virus. UV inactivated virus had decreased PFU compared to control A/WSN/33. To further confirm UV inactivation, cytokine and chemokine production from infected mast cells was assessed, because their production requires the production of newly synthesized vRNP (36). Mast cells were treated with equivalent numbers of infectious particles of A/WSN/33 for these assays based on the starting PFU values.

IAV Plaque Assay

IAV viral titers in the lungs were quantified using standard plaque assay. Briefly, MDCK cells (ATCC) were grown to confluence. On the day of titering, MDCK cells were serum starved for 30 minutes before virus was added in 10-fold serial dilutions. Virus was left to adhere for one hour at 35°C, tipping every ten minutes. Cells were overlaid with 1.6% agarose (SeaKem®) in DMEM with 0.5µg/mL TPCK-treated trypsin (Worthington Biochemical Corporation). Once the agarose had set, the plates were set in a 35°C 5% CO_2 incubator for 3-4 days. To fix the plaques, 3mL of a 1:1 methanol:acetone solution were added to the wells overnight. Plaques were stained with 0.1% crystal violet.

Growth of Bone Marrow
Cultured Mast Cells (BMCMC)
and Generation of Mast Cell Knock-in Mice

BMCMC were grown as previously described (49). Briefly, femurs from 4-8 week old mice were removed and bone marrow cells were collected by centrifugation for 30 seconds at 5000 rpm. Cells were resuspended in RPMI supplemented with 1% non-essential amino acids, 1mM sodium pyruvate, 1% HGPG, 10 mM HEPES, 10% fetal bovine serum, and 0.1% 2-mercaptoethanol. The cells were supplemented with 10 ng/mL recombinant murine IL-3 (Peprotech). After three weeks, 25 ng/mL recombinant murine stem cell factor (Peprotech) was added to the media along with IL-3. After five weeks, the purity of the population was >95% mast cells as determined by flow cytometry analysis using anti-FcεR1α (BioLegend) and anti-CD117 (BioLegend). To generate BMCMC 'knock-in' mice, we followed the methods previously used by others (50-52). Briefly, BMCMC were generated as described above. B6.Cg-*kit*$^{W\text{-}sh}$ at 3-4 weeks of age were reconstituted with $5x10^6$ C57Bl/6 or RIG-I$^{-/-}$ BMCMC via intravenous injection and rested for ~8-10 weeks before use.

In vitro Mast Cell Activation Assay

For all activation assays, 5-6 week cultures of BMCMC were washed and $2.5x10^5$ BMCMC were plated per well in a 96-well U-bottom plate. Virus was added at an MOI of ~0.4 in a final volume of 100μL for up to one hour for degranulation studies and 4-6 hours for secondary mediator activation, as indicated in the text. As positive controls, BMCMC were stimulated with the calcium ionophore A23187 (40 nM; Fisher Scientific),

for leukotriene synthesis and mast cells degranulation or LPS (5 mg/ml; List Biological Laboratory) for cytokine/chemokine release.

The above BMCMC activation procedure was also used to study the viral entry pathways necessary for IAV-induced mast cells responses. Each compound was diluted into media or PBS before being added to the cells. Compounds used in these experiments were bafilomycin A (200nM; Calbiochem), cytochalasin D (20μM; Sigma-Aldrich), Dynasore hydrate (120μM; Sigma-Aldrich), and 5-(N-Ethyl-N-isopropyl) amiloride (EIPA; 120μM; Sigma-Aldrich). BMCMC were pre-incubated with for one hour with each compound. The cells were then washed and stimulated with IAV in the presence or absence of the compound throughout the activation assay.

Detection Assays for Cytokine and Chemokine Secretion

In vitro BMCMC activation assay supernatants from 4-6 h post-treatment were analyzed for cytokine and chemokines with Custom Milliplex® plates (Millipore). IL-6 levels were also determined by ELISA (Biolegend). Murine IFN-α were measured using Procarta® 2-plex assay (Affymetrix). Plates were read using a BioPlex® 200 (Bio-Rad) or a SpectraMax Paradigm plate reader (Molecular Devices).

Histamine Enzyme Immunoassays (EIA)

Histamine assays were conducted following the manufacturer's instructions (Cayman Chemical). Briefly, the samples were incubated with an acetylcholinesterase linked histamine tracer for 16-24 hours. Plates were washed and Ellman's Reagent was

added to detect the tracer labeled histamine. Obtained results are inversely proportional to free histamine present in the well.

Annexin V Expression
for Mast Cell Degranulation

To measure degranulation of mast cells, cell surface staining with annexin V was done as previously described (53). Briefly, BMCMC were activated as described above. After activation, BMCMC were washed with cold PBS. Cells were stained with 5μL/well annexin V (BioLegend) in 100μL annexin V binding buffer (10mM HEPES, 140mM NaCl, 2.5nM $CaCl_2$, pH 7.4) for 30 minutes at room temperature. Immediately after incubation, cells were diluted with an additional 100μL annexin V binding buffer for a final volume of 200 μL . Samples were collected on a LSRII (BD Biosciences) and data was analyzed via FlowJo software (Tree Star).

Intracellular Staining for Viral Proteins

To measure virus infectivity of mast cells, we stained BMCMC for intracellular expression of the NS-1 protein. A total of $2.5x10^5$ BMCMC were plated per well in a 96-well U-bottom plate. Virus was added at MOI ~0.4 in a final volume of 100 μL and incubated for 4-6 hours. The cells were then fixed with 50μL BD Cytofix/Cytoperm (BD Biosciences) for 30 minutes at room temperature. Cells were then stained with a mouse monoclonal antibody against the IAV NS1 protein (NS1-1A7; BEI Resources) for 30 minutes in BD Perm-wash buffer (BD Biosciences). Subsequently, the cells were washed with BD Perm-wash buffer and then stained with PE-labeled goat anti-mouse IgG F(ab') fragments (Jackson ImmunoResearch Laboratories). Cells were then washed with BD

Perm-wash buffer and resuspended in FACS Buffer. Samples were collected using a LSRII (BD Biosciences) and data was analyzed via FlowJo software (Tree Star).

Statistical Analysis

Statistical significance was determined by either a Mann-Whitney U-test or one-way ANOVA using Prism 5 (Graphpad Software). Significance was set as $p < 0.05$.

Results

Secondary Mast Cell Mediators are not Responsible for Mast Cell-Induced Disease with A/WSN/33

Mast cells have been well characterized in bacterial and parasitic infections, but their role during viral infections has only recently begun to be explored (16, 54-56). In dengue virus infections, activation of mast cells leads to increased severity of disease (24). Moreover, mast cells appear to play a detrimental role during IAV infections as well (2, 36). In IAV infections we have previously shown that secondary mediator production in response to A/WSN/33 is RIG-I-dependent (36), but the role of this pathway during *in vivo* infection was not explored. Using RIGI-I$^{-/-}$ BMCMC, we can determine if expression of these secondary mediators that are dependent on RIG-I are playing a role in mast cell-induced disease during IAV infections. To define the role these *de novo* mediators play during A/WSN/33 infection, we utilized mast cell deficient B6.Cg-*Kit*$^{W\text{-}sh}$ mice that had been reconstituted with RIG-I$^{-/-}$ BMCMC, and therefore these mice will have mast cells that are not able to produce cytokines, chemokines, and leukotrienes. Selective reconstitution of the mast cell compartment of B6.Cg-*Kit*$^{W\text{-}sh}$ mice used $5x10^6$ C57Bl/6-

derived BMCMC or RIG-I$^{-/-}$ BMCMC 10 weeks occur prior to infection. These mice, along with C57Bl/6 and non-reconstituted B6.Cg-*Kit*$^{W\text{-}sh}$ mice were inoculated with 1000 PFU A/WSN/33 and weights were measured daily to determine susceptibility to the virus. As previously published, wild-type C57Bl/6 mice were sensitive to the virus as they lost weight during infection, while mice lacking mast cells did not (Figure 5.1). B6.Cg-*Kit*$^{W\text{-}sh}$ reconstituted with C57Bl/6 BMCMC complemented the reduced weight loss, as body weights were similar to what was seen in wild-type mice (Figure 5.1). Interestingly, B6.Cg-*Kit*$^{W\text{-}sh}$ reconstituted with RIG-I$^{-/-}$ BMCMC also lost weight during infection to a similar degree as those reconstituted with C57Bl/6 BMCMC (Figure 5.1). This indicates that the RIG-I-dependent response within mast cells, and mast cell secondary mediators, are not playing a role in the pathological inflammatory response, but instead suggests the degranulation mediators are the important factors in the pathological inflammatory response.

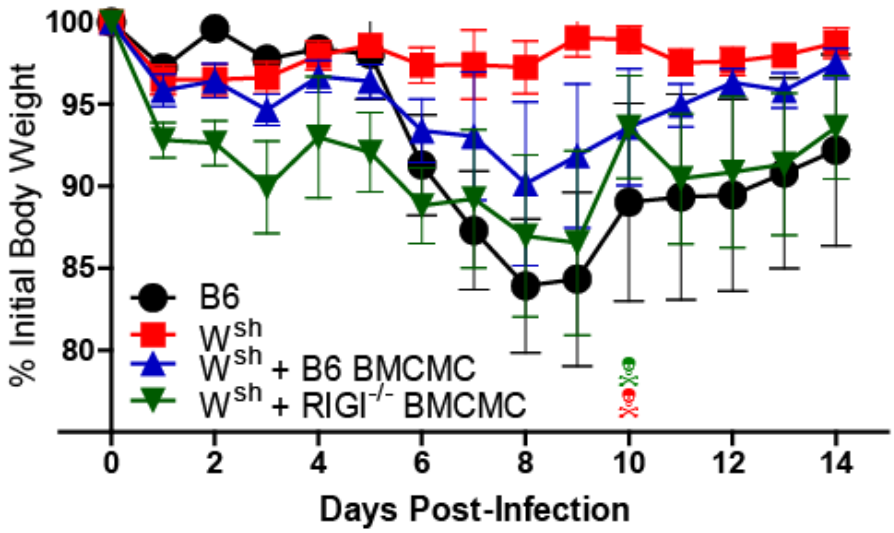

Figure 5.1 RIG-I in the mast cell compartment is not important for A/WSN/33 induced disease *in vivo*. Age matched C57Bl/6 (B6), B6.Cg-*kit*$^{W\text{-}sh}$ (W^{sh}), or B6.Cg-*kit*$^{W\text{-}sh}$ mice reconstituted with 5x10^{6} of either C57Bl/6 or RIG-I deficient BMCMC 10 weeks prior were infected with 1000 PFU of A/WSN/33. Body weights were measured daily. Each mouse weight is normalized to the starting weight at day 0. ☠ = Mouse was euthanized. Five mice per group.

BMCMC Degranulation Occurs
Early during IAV Replication Cycle

Mast cells have been shown to respond and activate *in vitro* to a variety of viruses, including VSV, Sendai virus, Hantavirus, dengue virus, and reovirus (25, 28, 30, 57, 58). We have also previously shown that A/WSN/33 (H1N1) infects and activates mast cells to degranulate and produce *de novo* mediators including cytokines, chemokines, and leukotrienes (36). While production of secondary mediators occurred in a RIG-I/MAVS-dependent fashion, degranulation occurred through a RIG-I/MAVS-independent mechanism (36). As it appears degranulation mediators are crucial for

A/WSN/33-induced disease in mice, we next wanted to explore how mast cell degranulation was occurring in response to A/WSN/33.

Mast cell degranulation typically occurs immediately upon stimulation. We can detect histamine within 30 minutes after stimulation with A/WSN/33 (36). We wanted to determine how mast cells were degranulating in response to A/WSN/33, so we tested annexin V binding as a possible marker for degranulation, which would enable us to have greater throughput in our *in vitro* BMCMC activation assay. Annexin V does not bind to resting mast cells, but does bind to the membranes of BMCMC that have degranulated (53). We wanted to determine if annexin V could be used as a marker for A/WSN/33–induced degranulation in our studies. For these studies, $2.5x10^5$ BMCMC were plated and cultured with media, A/WSN/33, or the calcium ionophore A23187. BMCMC treated with media alone showed no annexin V binding (Figure 5.2A, filled black graph), while BMCMC treated with A/WSN/33 showed higher annexin V binding (Figure 5.2A, filled red graph). The calcium ionophore is a positive control that is known to induce BMCMC degranulation and had the highest amount of annexin V binding (Figure 5.2A, filled gray graph). Importantly, annexin V binding correlated with histamine production (Figure 5.2B). Thus, we conclude that annexin V can be used as a measurement for BMCMC degranulation in response to IAV.

We next wanted to identify when in the viral replication cycle mast cell degranulation was occurring. To confirm if virus replication and the production of new viral proteins is important for BMCMC degranulation, we took A/WSN/33 and inactivated the virus by exposure to UV light for 60 minutes before adding to BMCMC. The UV-inactivated virus will still be able to bind to sialic acids and enter the cell, but

the viral genome will not be able to successfully replicate. UV light treatment successfully inactivated A/WSN/33 virus because the virus was unable to form viral plaques equivalent to wild-type IAV. UV-inactivated virus was added to $2.5x10^5$ BMCMC such that equal particles of A/WSN/33 were utilized. Activation was then allowed to occur for 30 minutes and annexin V binding was used to assess BMCMC degranulation. UV-inactivated A/WSN/33 had similar annexin V binding as did our original A/WSN/33 virus (Figure 5.3A). As we have previously shown, cytokine and chemokine production from BMCMC requires *de novo* vRNP production (36). Therefore, to confirm the UV-treated virus was indeed fully inactivated, we also measured cytokine and chemokine production from BMCMC cultured for 5 hours with the UV-inactivated A/WSN/33. UV-inactivated A/WSN/33 had no detectable production of IL-6, confirming that our UV-inactivated virus was not actively infecting the BMCMC (Figure 5.3B).

As histamine is detected rapidly from BMCMC cultured with A/WSN/33, we next wanted to extend our UV-inactivated virus results using pharmacological inhibitors that impede earlier events in the IAV replication cycle. Bafilomycin A (BafA) is a vATPase inhibitor that blocks acidification of the endosome, thus preventing the vRNP from escape into the cytoplasm. BMCMC were pre-treated for one hour with media alone or 200nM BafA. After one hour, cells were stimulated with media or A/WSN/33 with or without BafA for 30 minutes to determine if the cells were able to degranulate. Even in the presence of BafA, A/WSN/33 was still able to induce BMCMC degranulation as detected by annexin V binding (Figure 5.4A), suggesting that A/WSN/33 activated BMCMC to degranulate prior to IAV genome translocation from the endosome into the cytoplasm. Using BafA on calcium ionophore treated cells did not affect BMCMC

degranulation in response to calcium (Figure 5.4A). To verify that BafA was inhibiting IAV escape from the endosome, we looked for production of *de novo* synthesized mediators from BMCMC in cells that had been treated with BafA. To do this, $2.5x10^5$ BMCMC were pre-treated for one hour with media or 200nM BafA followed by infection with A/WSN/33 with or without BafA for 5 hours. BMCMC supernatants were then evaluated for cytokines and chemokines. We found that when BMCMC were treated with BafA, there was a significant decrease in the amount of secondary mediator production (Figure 5.4B), confirming that BafA was inhibiting the viral replication cycle. Thus, our data show that that BMCMC degranulation in response to A/WSN/33 occurs prior to viral escape from the endosome during the earliest steps of the viral replication cycle.

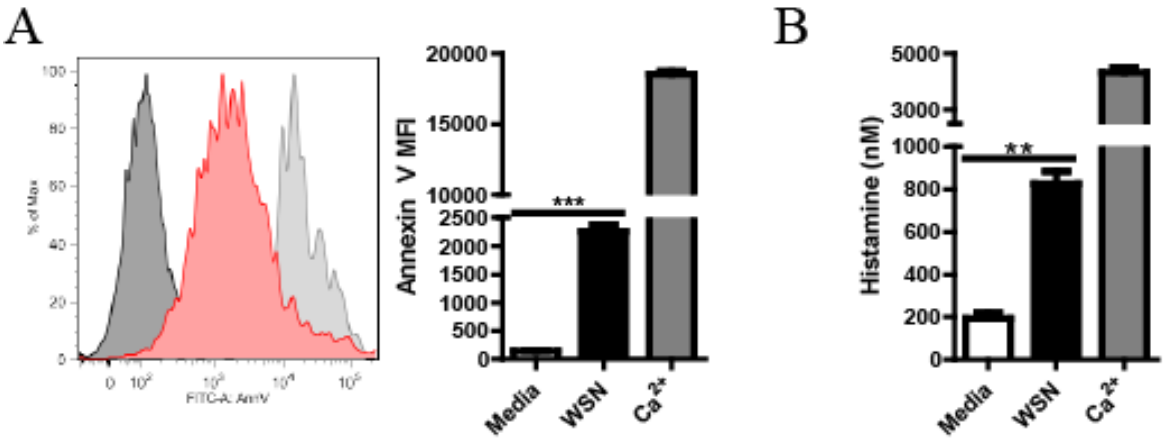

Figure 5.2 Annexin V as a measurement for degranulation. $2.5x10^6$ FcεR1$^+$ and CD117$^+$ BMCMC were cultured with media, A/WSN/33 (WSN), or calcium ionophore (Ca^{2+}) for 30 minutes. Virus as added at a MOI of 0.4. BMCMC were stained with annexin V FITC for 30 minutes (A). Supernatant was collected and histamine levels were measured by EIA (B). Black shaded graph = Media; Red shaded graph = WSN; Gray shaded graph = Ca^{2+}. Data is representative of two to four independent experiments with three replicates per condition. Significance was determined by one-way ANOVA with Tukey's post-test. $^{**}p<0.05$.

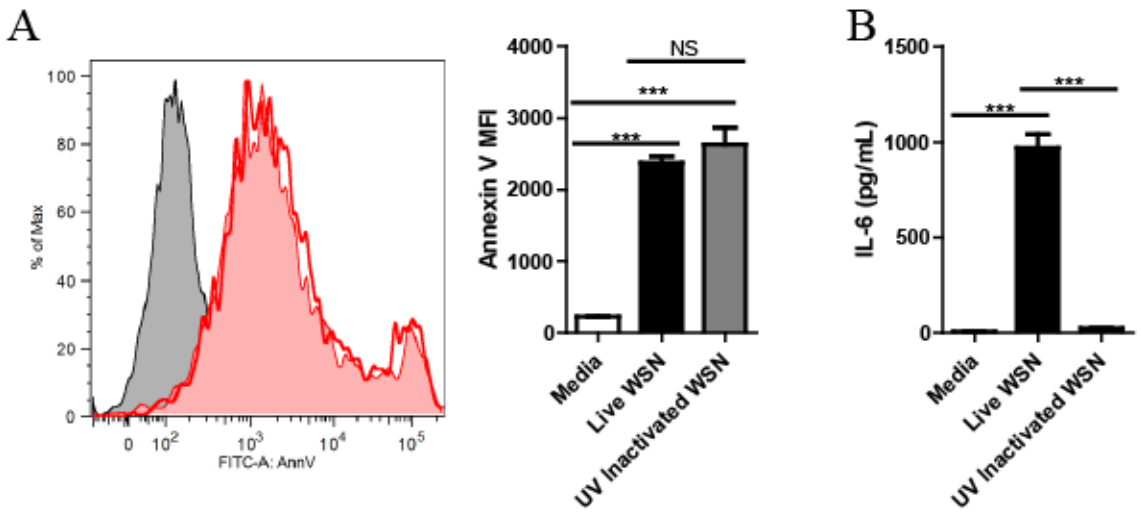

Figure 5.3 UV inactivated A/WSN/33 causes BMCMC degranulation. A/WSN/3 was UV inactivated on ice for one hour and confirmed to have lower titers with plaque assays. A total of $2.5x10^6$ FcεR1$^+$ and CD117$^+$ BMCMC were cultured with A/WSN/33 or UV inactivated A/WSN/33 for 30 minutes or five hours. Degranulation was determined annexin V binding on BMCMC after 30 minutes. Black shaded graph = Media treated BMCMC; Red shaded graph = WSN treated BMCMC; Red graph = UV inactivated WSN treated BMCMC. (A). IL-6 was measured by ELISA on five hour BMCMC supernatants (B). Virus was added at a MOI of 0.4. Data is representative of two to four independent experiments with three replicates per condition. Significance was determined by one-way ANOVA with Tukey's post-test. $^{**}p<0.05$.

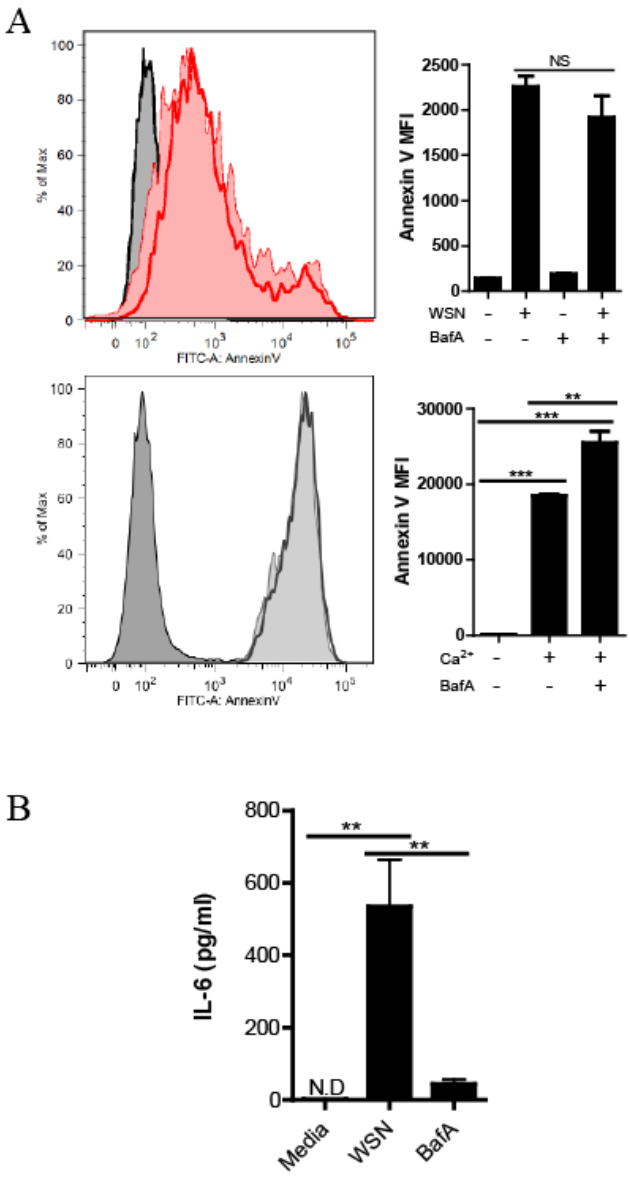

Figure 5.4 Degranulation from BMCMC takes place early during viral replication cycle. A total of $2.5x10^6$ FcεR1$^+$ and CD117$^+$ BMCMC were pretreated with or without 200nM Bafilomycin A (BafA) for one hour. Cells were then incubated with media or A/WSN/33 (WSN) with or without 200nM of BafA for 30 minutes to measure degranulation or five hours to determine infection. As a control, BafA treated cells were treated with calcium ionophore. Degranulation was determined by annexin V binding on BMCMC. Black shaded graph = media treated BMCMC; Red shaded graph = WSN treated BMCMC; Red graph = BafA treated BMCMC then treated with WSN (A). Five hour supernatants were collected and cytokine/chemokine production was with Milliplex multiplex analysis (B). Virus was added at a MOI of 0.4. Data are representative of two independent experiments with three replicates per condition. Significance was determined by one-way ANOVA with Tukey's post-test. $^{**}p<0.05$. $^{***}p<0.005$

BMCMC Degranulation is not
Triggered by Entry of A/WSN/33 into the Cell

Before acidification of the endosome and release of the vRNP into the cytoplasm, the virus must be endocytosed into the cell. IAV can enter cells through CME or non-CME (such as macropinocytosis) mechanisms. We first treated BMCMC with the actin inhibitor cytochalasin D, as both mechanisms of endocytosis require actin rearrangement. BMCMC were pre-treated for one hour with media or 20uM of cytochalasin D. Cells were then stimulated with media or A/WSN/33 with or without the same concentration of cytochalasin D for 30 minute to determine if the cells were able to degranulate, as measured by annexin V. In the presence of cytochalasin D, there was a slight decrease in annexin V binding (Figure 5.5A). However, there was no change in degranulation to calcium when the cells were treated with the inhibitor (Figure 5.5A). This suggests that actin rearrangement is partially required for BMCMC degranulation in response to A/WSN/33.

Dynamin is a key protein of CME and can be inhibited in cells by treatment with dynasore. Alternatively, macropinocytosis has been identified as a secondary means of viral entry for IAV and can be inhibited by Na^+/H^+ antiporters inhibitor EIPA (44, 59). Therefore, to determine if and which means of endocytosis is necessary to induce mast cell degranulation, $2.5x10^5$ BMCMC were treated with 120uM of either dynasore, EIPA, or both prior to and during BMCMC activation with A/WSN/33. As serum triggers macropinocytosis of IAV (44), inhibition of endocytosis was done in PBS. Similar to previous results, BMCMC degranulated in response to A/WSN/33 (Figure 5.5B, filled red graph). Interestingly, BMCMC treated with dynasore, or EIPA prior to stimulation

with A/WSN/33 had minimal alterations in annexin V binding (Figure 5.5B). BMCMC treated with both inhibitors had less annexin V MFI that untreated BMCMC, but it was significantly higher than untreated BMCMC. As a control, BMCMC were treated with dynasore, EIPA, or both and then stimulated with a calcium ionophore. Again, no change in degranulation was observed (Figure 5.5B).

To confirm that we were inhibiting IAV entry, we looked at cytokine production from BMCMC treated with the compounds for 5 hours after one hour pretreatment. As expected, there was lower production of IL-6 from BMCMC pre-treated with dynasore, EIPA, or both prior to A/WSN/33 stimulation when compared to A/WSN/33 treatment alone, indicating that we successfully inhibited IAV entry and blocked the viral replication cycle (Figure 5.5C). These data suggests that while actin rearrangement appears to be partially required for BMCMC degranulation in response to A/WSN/33, degranulation of BMCMC is not dependent on either CME or non-CME IAV entry mechanisms. Thus, it is likely cell surface recognition of IAV is sufficient to induce mast cells degranulation.

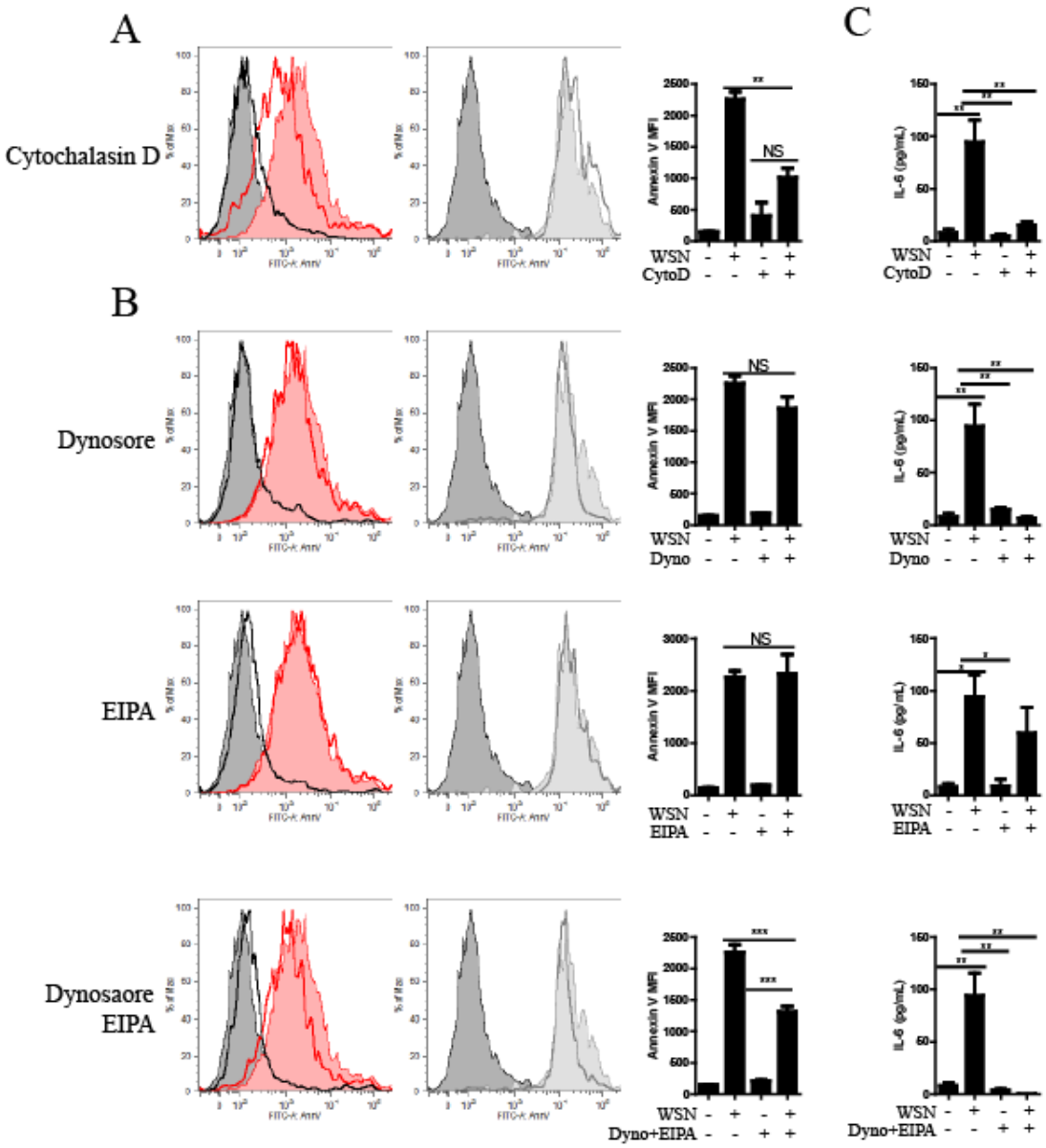

Figure 5.5. Inhibiting A/WSN/33 endocytosis do not alter BMCMC activation. A total of $2.5x10^6$ FcεR1$^+$ and CD117$^+$ BMCMC were pretreated with 20μM of cytochalasin D (A), 120μM of Dynasore, 120μM of EIPA, or 120μM each of Dynasore and EIPA (B) for one hour. Cells were then incubated with A/WSN/33 for 30 minutes or five hours with or without the compounds. Degranulation was determined by annexin V binding on BMCMC after 30 minutes. BMCMC supernatants were measured with IL-6 by ELISA (C). Virus was added at a MOI of 0.4. Black shaded graph = Media; Red shaded graph = WSN; Gray shaded graph = Ca^{2+}; Black graph = media treated with compound; Red graph = WSN treated with compound; Light gray graph = Ca^{2+} treated with compound. Data is representative of two to four independent experiments with three replicates per condition. Significance was determined by one-way ANOVA with Tukey's post-test. $^{**}p<0.05$, $^{***}p<0.005$.

Sialic Acid Binding is Important for BMCMC Degranulation

The first step in the viral replication cycle is the binding of viral HA to sialic acids located on the surface of host cells. IAV preferably binds to α2,3- or α2,6- sialic acid terminal linkages on glycoproteins (42, 43). In humans, both sialic acid linkages are expressed, with α2,3-linkages more highly expressed in the lower respiratory and α2,6-linkages primarily expressed in the upper respiratory tract (60). A/WSN/33 has been shown to preferably bind to α2,6-linked sialic acids (61). Another commonly used H1N1 IAV strain used in the laboratory is A/PR/8/34, which has been shown to primarily bind to α2,3-linkages (13).

We have previously shown that A/PR/8/34 does not infect or activate BMCMC to produce secondary mediators (36). We next wanted to establish if IAV with different sialic acid binding preferences would still be able to cause mast cell degranulation. To determine if A/PR/8/34 was directly activating mast cells, $2.5x10^5$ BMCMC were cultured with media alone, A/WSN/33, or A/PR/8/34 for 30 minutes and subsequently BMCMC were stained with annexin V to determine the extent of degranulation (Figure 5.6). A/WSN/33 induced marked annexin V staining, which was in contrast to A/PR/8/34 that induced little annexin V binding, which was comparable to media only BMCMC (Figure 5.6). It was already published that A/PR/8/34 does not induce expression of cytokines or chemokines from BMCMC (36). Taken together, these data suggests that A/PR/8/34 is not inducing BMCMC activation. The lack of secondary mediators also indicates that A/PR/8/34 is unable to infect BMCMC. In correlation, A/PR/8/34 had NS-1 expression comparable to uninfected controls, confirming that A/PR/8/34 is not able to

infect BMCMC, while A/WSN/33 is able to do so (36). Collectively this data confirms that A/PR/8/34 is not able to infect or activate BMCMC to degranulate or produce secondary mediators *in vitro*.

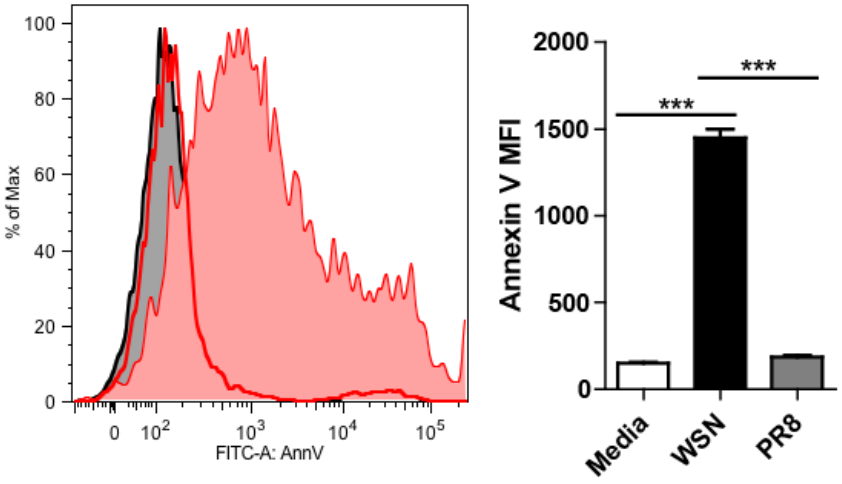

Figure 5.6 Mast cells activate in response to A/WSN/33 but not A/PR/8/34. A total of $2.5x10^6$ FcεR1$^+$ and CD117$^+$ BMCMC were treated with media, A/WSN/33 or A/PR/8/34 for 30 minutes. Degranulation was determined by annexin V binding on BMCMC after 30 minutes. Black shaded graph = media treated BMCMC; Red graph = A/WSN/33 treated BMCMC; Red graph = A/PR/8/34 treated BMCMC. Each virus was added at a MOI of 0.4. Data is representative of two to four independent experiments with three replicates per condition. Significance was determined by one-way ANOVA with Tukey's post-test. ***p<0.005.

A/WSN/33 Binding Specificity is Important for BMCMC Activation

A/WSN/33 binds to α2,6-sialic acid, whereas A/PR/8/34 binds to α2,3-sialic acids (41, 61); this sialic binding preference difference correlates with these strains ability to activate BMCMC, but other difference in the viral isolates could also account for the

observed difference in mast cell activation. To specifically test the importance of HA binding to specific sialic acid linkages within one IAV strain, we obtained a A/WSN/33 virus that contains a single amino acid change at position 225 in the viral HA that switches the sialic acid binding preference of this virus from α2,6- (WT) to an α2,3-linkages (noted as A/WSN/33 D225G) (40). To determine whether the sialic acid binding specificity of a virus isolate is important for BMCMC activation, $2.5x10^5$ BMCMC were incubated with media alone, A/WSN/33, or A/WSN/33 D225G for 30 minutes to look at degranulation or five hours to determine if the BMCMC infected and release secondary mediators. After the 30 minute activation, BMCMC were stained for annexin V and binding was observed by FACS. Similar to A/PR/8/34, A/WSN/33 D225G did not induce an upregulation of annexin V on the cell surface of BMCMC, demonstrating that the A/WSN/33 D225G virus was not causing BMCMC to degranulate (Figure 5.7A). Furthermore, virus stimulated BMCMC supernatants were analyzed for cytokines and chemokines after a five hour incubation. While A/WSN/33 induced the production of cytokines and chemokines from BMCMC after five hours, treatment of BMCMC with the A/WSN/33 D225G strain produced significantly lower amounts of these secondary mediators (Figure 5.7B). Overall, changing the binding preference of A/WSN/33 from α2,6-sialic acid linkages to α2,3-linked sialic acids altered the virus' ability to drive BMCMC degranulation, thus demonstrating that IAV binding specifically to α2,6-linked sialic acid is important for driving BMCMC activation.

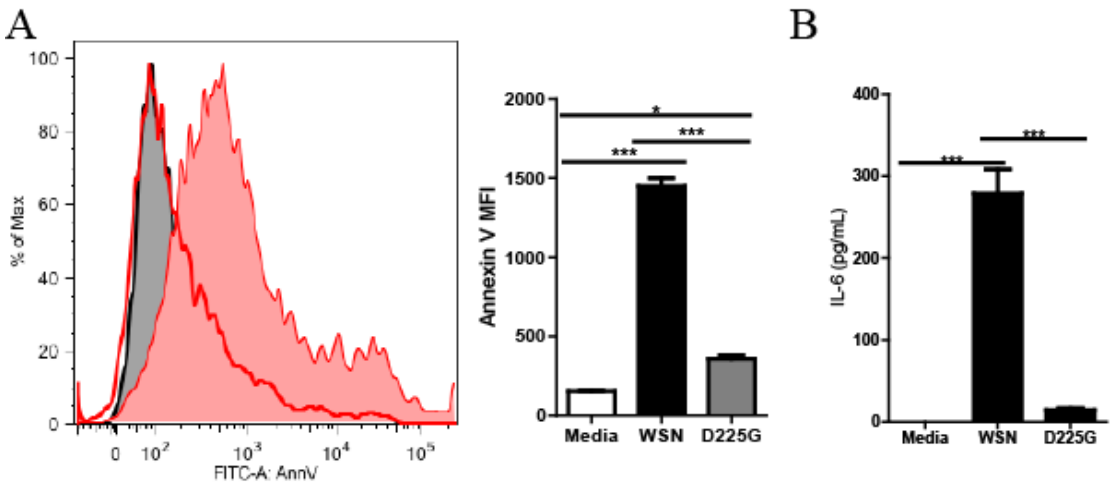

Figure 5.7 A/WSN/33 D225G that binds to α2,3-linked sialic acids is not able to activate or infect BMCMC *in vitro*. A/WSN/33 D225G (D225G) contains a single amino acid change in the HA at position 225 that changes the binding preference to α2,3-linked sialic acids. A total of $2.5x10^6$ FcεR1$^+$ and CD117$^+$ BMCMC were treated with media (black shaded graph), A/WSN/33 (red shaded graph), or A/WSN/33 D225G (red graph) for 30 minutes or five hours. Degranulation was determined annexin V binding on BMCMC after 30 minutes (A). Five hour supernatants were collected and cytokine/chemokine production was measured with Milliplex multiplex analysis (B). Virus was added at a MOI of 0.4. Data are representative of two to a million independent experiments with three replicates per condition. Significance was determined by one-way ANOVA with Tukey's post-test. ** $p<0.05$. *** $p<0.005$.

A/WSN/33 NA
Contributes to BMCMC Degranulation

In addition to the viral HA protein, the viral NA protein is also located on the viral envelope and can also interact with sialic acids. NA proteins cleaves terminal sialic acid linkage from the adjacent glycoprotein or glycolipid to which it is attached allowing the release of new virions from an infected cell (62). This is important for maturation and spread of IAV. Interestingly, treatment of mast cells with purified neuraminidases can enhance mast cell response to classic stimuli (63-67). A/WSN/33 NA130 contains an extra glycosylation site on the NA protein and cannot effectively cleave sialic acids (62).

To determine whether the IAV NA is contributing to the induction of BMCMC degranulation, $2.5x10^5$ BMCMC were incubated with media alone, A/WSN/33, or A/WSN/33 NA130 for 30 minutes to examine the degranulation response or five hours to determine infectivity and secondary mediator synthesis by BMCMC. Cells incubated for 30 minutes were stained with annexin V to assess mast cell degranulation. Interestingly, if the A/WSN/33 NA130 virus is not able induce BMCMC degranulation as strongly as wild-type A/WSN/33 (Figure 5.8). Supernatant from BMCMC cultured for 5 hours were analyzed for cytokine and chemokine production to determine if A/WSN/33 NA130 was able to successfully infect BMCMC. A/WSN/33 NA130 induced significantly less IL-6 than wild-type A/WSN/33 (Figure 5.8B), indicating that A/WSN/33 NA130 is not able to successfully infect BMCMC. Thus, alteration of the A/WSN/33 NA protein results in a virus that is not able to induce mast cell degranulation, signifying the importance of this protein in BMCMC activation.

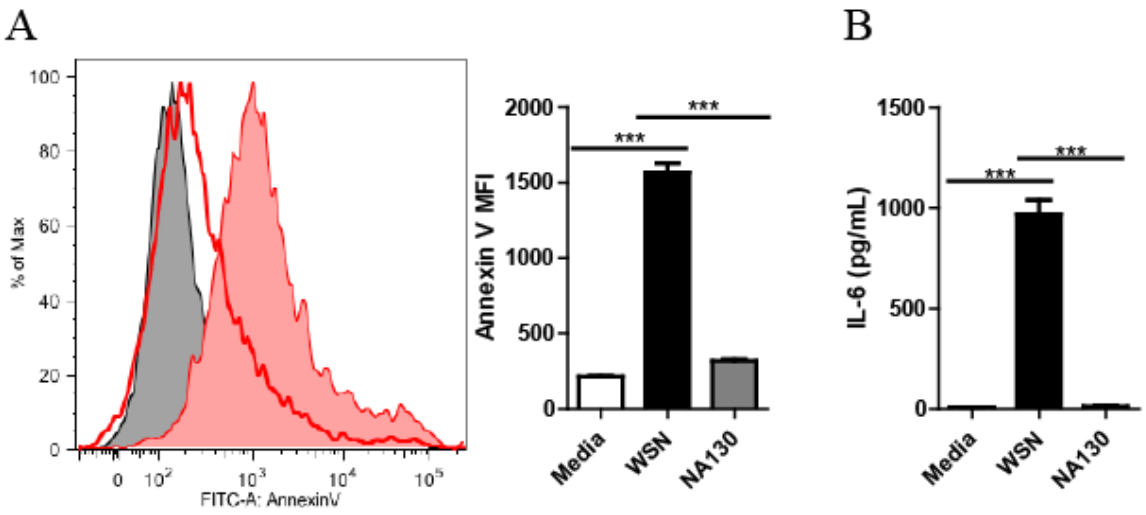

Figure 5.8 A/WSN/33 NA130 does not activate or infect BMCMC *in vitro*. A/WSN/33 NA130 (NA130) contains an additional glycosylation site at position 130 on its NA, preventing NA from effectively cleaving sialic acids. A total of $2.5x10^6$ FcεR1$^+$ and CD117$^+$ BMCMC were treated with media (black shaded graph), A/WSN/33 (red shaded graph), or A/WSN/33 NA130 (red graph) for 30 minutes or five hours. Degranulation was determined annexin V binding on BMCMC after 30 minutes (A). Five hour supernatants were collected and cytokine/chemokine production was with Milliplex multiplex analysis (B). Virus was added at a MOI of 0.4. Data are representative of two independent experiments with three replicates per condition. Significance was determined by one-way ANOVA with Tukey's post-test, ***p<0.005.

Sialic Acid Binding Preference Leads to Differential Immune Response *in vivo*

Because we found that BMCMC degranulation *in vitro* correlated with α2,6-sialic acid binding preference, we wanted to determine if viruses that had α2,3- sialic acid binding preferences elicited mast cell-dependent or –independent inflammatory response in mice. C57Bl/6 or mast cell deficient B6.Cg-*Kit*$^{W\text{-}sh}$ mice were infected intranasally with 1000 PFU of A/PR/8/34, A/WSN/33 NA130, or A/WSN/33 D225G and weights were measured daily to determine susceptibility to IAV infections. C57Bl/6 mice lost weight with infection to all viruses (Figure 5.9A). Interestingly, mice deficient in mast

cells, B6.Cg-*Kit*$^{W\text{-}sh}$ mice, were still susceptible to A/WSN/33 NA130, A/PR/8/34, and A/WSN/33 D225G-induced disease, as mice infected with these viruses still lost weight following infection (Figure 5.9A). This is in contrast to B6.Cg-*Kit*$^{W\text{-}sh}$ infected with A/WSN/33, which did not lose body weight during infection (Figure 5.1, Figure 5.9A). Therefore, IAV-induced disease during A/WSN/33 infection is mast cell-dependent, while the disease observed during A/WSN/33 NA130, A/PR/8/34, and A/WSN/33 D225G infections are mast cell-independent.

To further explore the differences that caused IAV induced disease in A/PR/8/34 and A/WSN/33 D225G infections to be mast cell-independent, C57Bl/6 mice were infected with 1000 PFU of AWSN/33, A/PR/8/34, or A/WSN/33 D225G. After five days, bronchial alveolar lavage fluid (BALF) was collected from these mice and the inflammatory cellular infiltrate was assessed. All infected mice had increased total cells in the BALF compared to naïve mice (Figure 5.9B). Interestingly, mice infected with A/PR/8/34 or A/WSN/33 D225G had significantly greater neutrophil recruitment than compared to A/WSN/33 infected mice (Figure 5.9B). Thus, it appears that the sialic acid binding preference can impact the downstream inflammatory cellular response in the lungs, as α2,3-sialic acid binding viruses induced greater neutrophilic responses.

As we saw differences in the inflammatory cellular recruitment into the lungs of mice infected with α2,3- versus α2,6-preferenced viruses, we next examined at the cytokine and chemokine expression profile within of the lungs. C57Bl/6 mice were infected with either A/WSN/33 or A/PR/8/34 and BALF was collected and analyzed for cytokine and chemokine expression using a Multiplex bead-based assay. While both IAV strains induced strong inflammatory response following infection, as demonstrated by an

increase in TNF-α and IL-6 levels over naïve mice, A/WSN/33 tended to drive higher levels of IL-10 and CCL2, while A/PR/8/34 induced increased production of CCL4 and CXCL2 (Figure 5.10). This correlates with inflammatory cellular recruitment profile, because CCL4 and CXCL2 are known neutrophil chemokines and thus correlated with the increased influx of neutrophils into the lungs of A/PR/8/34 infected mice (Figure 5.9B).

IFN-I signaling has been shown to play a role in cellular recruitment during IAV infections (68). Increases in IFN receptor (IFNAR) signaling, can result in increased recruitment of monocytic cells during IAV infections. Conversely, $IFNAR^{-/-}$ mice display a more neutrophilic inflammatory response in response to IAV infection. To examine if there is a difference in IFN-I produced between these viruses, C57Bl/6 mice were infected with A/WSN/33, A/PR/8/34, or A/WSN/33 D225G and IFN-α levels were measured in the BALF at day 5 post-infection. Interestingly, we see that A/WSN/33 infections induce higher amounts of IFN-α compared to A/PR/8/34 and A/WSN/33 D335G (Figure 5.10). Therefore, A/PR/8/34, which preferably binds to α2,3-sialic acid linkages, induces less IFN-I that correlates with the increased production of neutrophil recruiting chemokines during and higher numbers of neutrophils recruited into the lungs, while A/WSN/33 produces higher IFN-I, which may lead to an increased monocytic cellular response during infection.

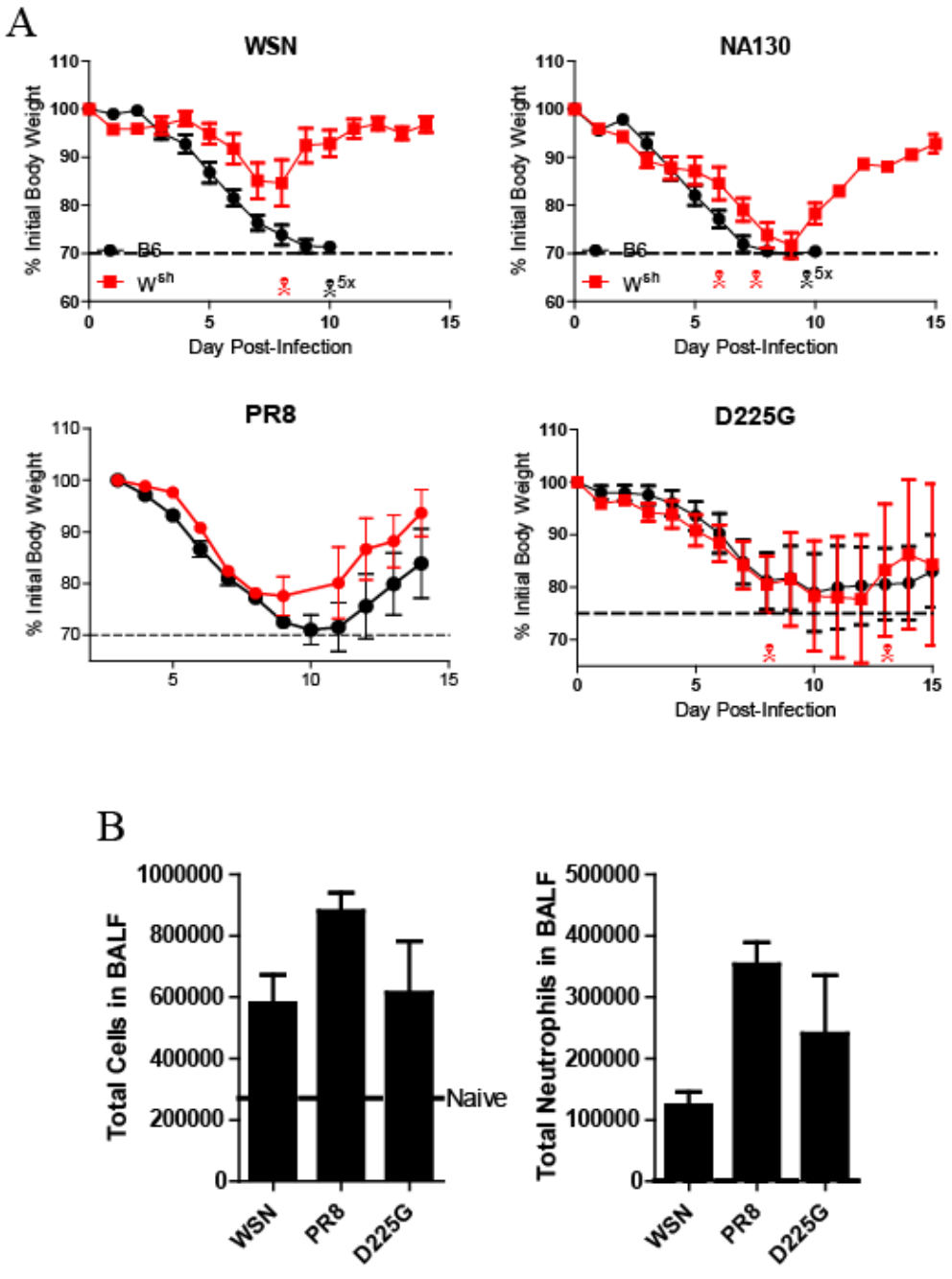

Figure 5.9 Mast cell deficient mice are susceptible to α2,3-sialic acid binding viruses. Age matched C57Bl/6 (B6) and mast cell deficient B6.Cg-*kit*$^{W\text{-}sh}$ mice were infected with nasally with 1000pfu of either A/PR/8/34 or A/WSN/33 D225G. Body weights were measured daily and normalized to each mouse's starting weight (A). Total leukocyte recruitment (left panel) and neutrophil (right panel) recruitment in the BALF at day five post-infection (B). Data is representative of two to four independent experiments. ☠ = Mouse was euthanized.

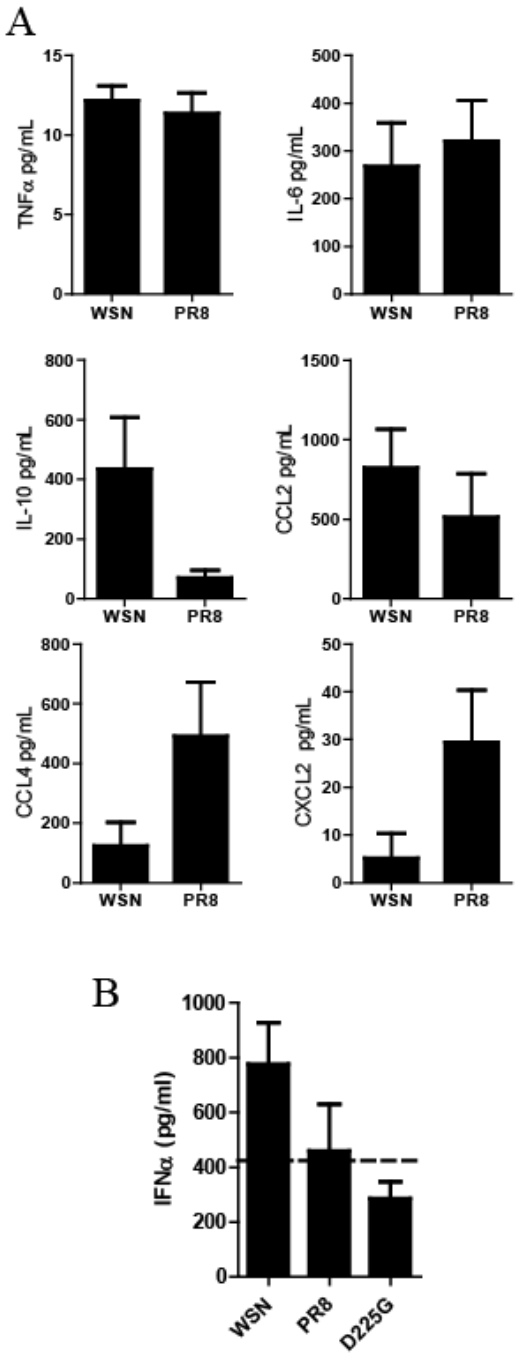

Figure 5.10 Inflammatory environment is not the same in A/WSN/33 and A/PR/8/34 infections. Age matched C57Bl/6 (B6) mice were infected with nasally with 1000pfu of either A/WSN/33 (WSN), A/PR/8/34 (PR8) (A). Inflammatory cytokines were measured in the BALF at day 5 with Milliplex© multiplex analysis. IFN-α was measured in the BALF of A/WSN/33, A/PR/8/34, or A/WSN/33 D335G mice infected for five days by Murine IFN-α were measured using Procarta® 2-plex assay.

Discussion

Mast cells are located in tissues exposed to the environment, which is an ideal location that allows them to quickly recognize and respond to invading pathogens (22, 69). The role that mast cells play during viral infections is only now beginning to be elucidated. Once mast cells encounter a pathogen, they can degranulate, releasing pre-formed mediators, while also producing *de novo* mediator's hours after their activation (21, 22, 70). We have previously shown that mast cells become activated in response during A/WSN/33 infection, resulting in the production of secondary mediators in a RIG-I/MAVS-dependent fashion (36). However, mast cells also degranulate in response to A/WSN/33 stimulation, although the cellular mechanism behind how mast cells degranulate in response to IAV remains elusive. In this current study, we showed that mast cell degranulation in response to IAV is dependent on the viral HA sialic acid binding preference and NA activity of the virus.

Although mast cells produce a variety of cytokines and chemokines in response to A/WSN/33, it appears that degranulation of mast cells during IAV infection is important for mast cell-induced disease *in vivo*. RIG-I/MAVS signaling is only required for secondary mediator production from BMCMC, while mast cell degranulation occurs independent of this signaling pathway (36). Using RIG-I-deficient BMCMC allowed us to determine whether mast cell degranulation or secondary mediator production were important for the mast cell-induced disease observed during A/WSN/33 infection of mice. When B6.Cg-*Kit*$^{W\text{-}sh}$ mice were reconstituted with RIG-I$^{-/-}$ BMCMC, mice were susceptible to A/WSN/33-induced disease similar to B6.Cg-*Kit*$^{W\text{-}sh}$ reconstituted with

C57Bl/6 BMCMC. This result was quite surprising, as the inflammatory cellular infiltrate and lung damage observed during IAV pandemics has been thought to be due to the 'cytokine storm', and we hypothesized that mast cells were contributing to the influx of cytokines and chemokines during infection (4, 5, 9). However, mast cell production of cytokines and chemokines appears not to be involved during this process. This does not exclude the 'cytokine storm' in driving the lung damage during IAV infection, rather it suggests that mast cell degranulation mediators, including histamine, serotonin, and proteases, likely are partaking in this process, either directly or indirectly by recruiting other inflammatory cells, which secrete high levels of cytokines. Therefore, understanding how mast cells degranulate in response to A/WSN/33 infection is critical in understanding how these cells are aiding in the damage observed in the lung during infection.

Mast cell degranulation has been implicated in the high degree of tissue pathology observed in some other virus infection models. We and others have previously shown that inflammatory cellular recruitment during IAV infection is dependent on mast cells (2, 36). Mast cell deficient B6.Cg-*Kit*$^{W\text{-}sh}$ mice displayed decreased cellular recruitment and decreased inflammatory cellular infiltrates during A/WSN/33 infection (36). In another study, mice infected with H5N1 A/Chicken/Henan/1/2004 and treated with mast cell degranulation inhibitor ketotifen, along with an IAV NA inhibitor, had reduced inflammation in response to H5N1 IAV infection in mice (2). Dengue virus is another highly pathogenic virus that is spread through mosquito bites (25, 71). Dengue-induced pathology was significantly decreased in mast cell deficient mice or when wild-type mice were treated with cromolyn (24). Furthermore, with highly pathological chicken viruses

IBDV and NDV, there is a strong correlation with mast cell recruitment to inflammatory lesions and the disease severity (72-74). Moreover, treatment of chickens with ketotifen reduced tissue injury during IBDV and NDV infections (72-74). Thus, reducing mast cell degranulation during these high pathogenic viruses appears to significantly decrease the overall inflammation and has the potential to increase survival during infection with these highly pathogenic viruses. Therefore, we sought to further understand the regulation of mast cell degranulation in response to viral infections, in particular A/WSN/33 stimulation.

How mast cells degranulate in response to viruses is not well understood. Dengue virus can directly activate mast cells *in vitro* to release both chymase and secondary mediators (24). Mast cell mediators have been detected in both humans and mice infected with dengue virus (24, 75-78). Similar to our observations with IAV, dengue virus can infect mast cells; however, degranulation occurs independently of infection and RIG-I-mediated detection mechanisms (15, 24). Moreover, UV-inactivated dengue virus was also able to cause mast cells to degranulate (15). Therefore, it is thought that mast cell degranulation in response to dengue virus is dependent on an unknown cell surface receptor (24).

Vaccinia virus also has the ability to cause mast cell degranulation and does so in response to the fusion of the viral envelope with the cell membrane (79). Sphingomyelin is present in the vaccinia virus envelope, which can be converted to sphingosin-1-phosphate (S1P) by sphingosine kinase resulting in signaling through the S1PR2 G-coupled receptor to induce mast cell degranulation (79). Interestingly, mast cells stimulated by IgE also utilize signaling through the S1P receptors to mediate optimal

mast cell responses (79-85). The use of S1P receptor signaling during other viral infections to drive mast cell degranulation is unknown. It is important to note that vaccinia virus envelope fusion is the first step of its viral replication cycle, reinforcing our findings that mast cell degranulation occurs at the earliest step of the virus replication cycle.

IAV initiates its viral replication cycle by the binding of the viral HA to sialic acids located on the cell surface. While IAV can bind to both α2,3- and α2,6-sialic acid linkages (40, 41), our data support that mast cell degranulation in response to IAV is dependent on viruses that preferentially bind to α2,6-linked sialic acids. Literature has shown that A/WSN/33 preferably binds to α2,6-linkages, while A/PR/8/34 primarily binds to α2,3-linked sialic acids (41, 61). To confirm that the sialic acid binding preference of A/WSN/33 plays a critical determinant role in mast cell degranulation by IAV, we obtained a A/WSN/33 mutant that had a single amino acid change (D225G) in its HA that changed its sialic acid binding preference from α2,6- to α2,3 linkages (61). Interestingly, this D225G isolate of A/WSN/33 behaved more similar to A/PR/8/34 than A/WSN/33, as it did not cause mast cell activation (Figure 5.7). Why mast cells do not degranulate in response to α2,3-linked sialic acid binding viruses is an interesting question. Mast cells express sialic acids on their surface (86), but whether they primarily express α2,3- and α2,6-sialic acid linkages remains unknown. Thus, the type of sialic acid linkages expressed by the mast cells could explain why one virus is able to activate mast cells to degranulate, while others do not.

It should be noted that the HA is not the only viral protein located on the viral envelope that has sialic acid binding properties. Neuraminidase (NA) is important for

cleaving sialic acid linkages and releasing new virions from infected cells. The role that NA activity plays in mast cell activation by IAV has not yet been clarified. One hypothesis is that viral HA is able to attach the virus to mast cells, and it is the cleavage of these sialic acids by NA that is causing mast cells to degranulation. Using a mutation of A/WSN/33, where an extra glycosylation site on the NA inhibits cleavage of sialic acids, we found no degranulation from mast cells (Figure 5.8), suggesting NA does play a role in mast cell activation. Basophils are granular cells that have the potential to produce histamine in response to IgE stimulation or bacteria. In studies with human basophils, histamine release in response to IgE cross-linking was synergistically enhanced by IAV (63, 65, 66). Interestingly, inhibiting the NA activity in these studies reduced the IAV-mediated enhancement of histamine production when either IgE cross-linking or *Staphylococcus aureus* was used as a stimulus (63-65). Moreover, adding *Vibrio cholera* NA also mimicked the IAV-mediated enhancement of histamine production (63, 65). These data, along with our data showing that A/WSN/33 NA130 is unable to drive mast cell degranulation, support the enzymatic function of NA in driving mast cell activation

We began our studies by trying to understand when in the viral replication cycle mast cell degranulation was occurring. Histamine can be detected rapidly after BMCMC are cultured with A/WSN/33. Using pharmacological inhibitors that target early steps of the viral replication cycle, we found that the signals that drive mast cell degranulation most likely occur at cell surface. As BMCMC still degranulated when IAV was unable to escape from the endosome (Figure 5.4), thus we examined the earlier processes of which the virus entry pathway was necessary for mat cell degranulation. IAV enters cells through the separate but redundant pathways of CME or non-CME mechanisms.

Although CME is highly characterized in IAV uptake, the other mechanisms are less understood (44). However, macropinocytosis of IAV into cells is beginning to be characterized (44). In certain systems, using a dynamin inhibitor the CME pathway can be inhibited, but interestingly, IAV can still be detected within cells (44). It has been demonstrated that macropinocytosis of IAV is an alternative entry pathway for the virus (44). Interestingly, we found that even when both these endocytosis mechanisms were inhibited during A/WSN/33 stimulation of mast cells, BMCMC were still able to degranulate. Therefore, endocytosis of the virus likely does not play a critical role in driving mast cell degranulation. As mast cell degranulation, but not cytokine production, still takes place in the absence of endocytosis, this suggests that degranulation is happening earlier in the viral replication cycle, likely at the cell membrane. This has lead us to hypothesize that earlier events in the viral replication cycle, for example, the IAV HA binding to host sialic acids, is critical for inducing degranulation.

Intriguingly, if actin rearrangement is inhibited by cytochalasin D, there was a slight decrease in BMCMC degranulation response. There are a couple of explanations for why this occurs. First, the process of degranulation itself requires actin, and therefore inhibiting actin polymerization will inhibit degranulation. Signaling through FcεR1 induces the formation of microtubules, and inhibition of the microtubule network decreases degranulation (87-90). However, if this were the case, then there would be a decrease of degranulation when BMCMC were treated with cytochalasin D and stimulated by calcium ionophore A23187, which was not observed (Figure 5.5A). An alternative explanation is that IAV is entering the cell through an unknown route of endocytosis, which also uses actin polymerization. While CME of IAV has been well

characterized, other mechanisms of virus entry have been less so. Finally, cell surface signaling may require actin rearrangement. During IAV budding from MDCKs, the cytoskeleton is used to localize lipid rafts containing the newly formed IAV surface proteins in preparation for budding. Using pharmacologically inhibitors to prevent actin rearrangement, lipid rafts failed to cluster, leading to decreased budding of virions (91, 92). Although the mast cell signals required for degranulation in response to IAV remains elusive, one hypothesis is the use of receptor clustering in lipid rafts is essential. Preventing lipid raft fluidity in the membrane would lead to lack of signaling, and therefore could explain why we see a decreasing in degranulation from mast cells with IAV.

Lastly, we were curious to determine whether there was a difference in the damage response between viruses that activate mast cells versus those that do not. Interestingly, in viruses that have a binding preference for α2,3- linked sialic acids, we found that the inflammatory response occurred completely independent of mast cells (Figure 5.9). Viral preference to α2,3-sialic acid linkages resulted in a substantial increase of neutrophil recruitment to the lungs (Figure 5.8). When looking specifically at the cytokine and chemokine signature within the BALF of A/WSN/33 and A/PR/8/34, we saw that while both viruses produced a strong inflammatory response as marked by the production of IL-6 and TNF-α, there was an increase in neutrophil recruiting chemokines in the A/PR/8/34 infected mice. This may explain why we saw a greater influx of neutrophils into the lungs of these mice. However, why one virus would induce a different inflammatory leukocyte response is intriguing. Type I interferons (IFN-I) are crucial in the anti-viral response, and it has been shown that signaling through the IFN-I

receptor, IFNAR, during lethal IAV infection leads to higher recruitment of $Ly6C^{hi}$ monocytes (68). However, when there is no IFN-I signaling, in $IFNAR^{-/-}$ mice, there was higher recruitment of neutrophils during infection (68). We have found that during A/WSN/33 infections, there is higher IFN-α production compared to A/PR/8/34 and A/WSN/33 D225G infections (Figure 5.10). It would be interesting to determine whether mast cells are involved in regulating the balance of IFN-I production following IAV infection.

Highly pathogenic viruses have been shown to infect and replicate in the pulmonary epithelium, and this leads to release of chemokines and induce inflammatory cell recruitment to the lungs, including an influx of neutrophils and macrophages (7). Differences we observe in cellular recruitment between our viruses could be due to the initial cells that each virus is interacting. Our *in vitro* data demonstrates that A/WSN/33 activates mast cells, and this interaction is also important *in vivo*, as mice lacking mast cells do not have the same pathological effects of infection compared to wild-type mice (36). We also see increased IFN-α production in the lungs during A/WSN/33 infection, and a major producer of IFN-I are dendritic cells, particularly plasmacytoid dendritic cells (93). Perhaps A/WSN/33 infects and interacts with mast cells and dendritic cells, producing higher amounts of IFN-I and leading to an increased monocytic cellular influx. Conversely, our data reveals that lungs of A/PR/8/34 and A/WSN/33 D225G mice had increased neutrophil recruitment compared to A/WSN/33 lungs (Figure 5.9). Infection of the epithelium with these viruses may elicit a neutrophilic chemokine response, explaining the increase in neutrophils during infection. Interestingly, one study has shown that it is the viral glycoproteins located on the cell surface that dictate neutrophil

recruitment. Although the study was using viruses based on their pathology in mice to study neutrophil recruitment, each of these viruses contain internal proteins for the A/PR/8/34 viral strain. BJx109 is non-pathogenic and neutrophils play little role during infection, while low pathogenicity HKx31 and high pathogenic A/PR/8/34 recruits neutrophils to the lungs. It would be interesting to investigate the sialic acid binding properties between these viruses. Understanding what cells these strains of IAV target would help us understand the downstream inflammatory response we observe during infections.

Neutrophils and macrophages have long been shown to be required for adequate clearing of IAV infections (7, 10-12). However, uncontrolled infiltration during IAV infection can cause significant damage to the lungs (68). One major cause for the higher mortality during the 1918 Spanish IAV pandemic is thought to be due to an excessive inflammatory response evoked by the virus (94). However, both of these cells are required for survival of mice during infection, as lack of neutrophil recruitment during IAV infection results in higher titers and decreased survival of mice (11, 12). Yet excessive neutrophil influx leads to increased damage during lethal IAV infections (7, 10). Partially depleting neutrophils during infection increases mouse survival (10). Thus, understanding how to temper a robust immune response could help reduce the more deadly outcomes during pandemic IAV infections.

In conclusion, we have shown that mast cell degranulation plays a role in mast cell-induced disease observed during A/WSN/33 infections. The cellular signaling mechanism behind mast cell degranulation to IAV remains unknown, but we have now demonstrated that viral envelope protein interactions with sialic acids on the surface of

mast cells are important in inducing the mast cell degranulation response. Degranulation of mast cells in response to IAV was dependent on the sialic acid binding preference of the virus, with IAV isolates that preferably bind to α2,6-sialic acid linkages inducing greater mast cell degranulation. Finally, we also demonstrate that IAV with different sialic acid binding preferences induce differential inflammatory environments, which affects inflammatory cell recruitment to the respiratory tract during infection. Understanding how viruses activate mast cells to induce mast cell-associated damage during infection is an important tool for being prepared for future IAV pandemics. Importantly, human isolates of IAV are capable of activating mast cells (36) and histamine release can be observed in human's infection with IAV (34, 35). If we can predict which emerging strains are going to activate mast cells, we as a society can be better prepared for the next pandemic.

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CHAPTER SIX

DISCUSSION AND FUTURE DIRECTIONS

Discussion

Influenza A virus (IAV) is a common respiratory virus that causes seasonal infections with only mild morbidity and mortality, however during pandemic years or during zoonotic spillover events, the observed morbidity and mortality rates can significantly increase. Pandemic IAV and highly pathogenic avian IAV isolates cause increased damage in the lung during infection. Because of the increasing resistance to anti-viral drugs and the fact that vaccines for IAV take over 6 months to produce (1), alternative therapies are required to treat the emergence of novel IAV strains, such as targeting the inflammatory pathways that drive the IAV-induced lung damage (2, 3). Genetic analysis of murine lungs infected with mouse-adapted 2009 H1N1pdm or recombinant 1918 IAV strain showed enriched mast cell transcripts in the lungs during IAV infections (4), although their role during IAV infection was not elucidated. We hypothesized that mast cells are actively infected with IAV, resulting in their activation and thus contributing to the amplification of the inflammatory response and lung pathology induced by IAV infection. We addressed this hypothesis with two specific aims: 1) Identify the receptors mast cells utilize to initiate IAV-induced inflammatory disease, and 2) Determine the key characteristics of the hemagglutinin protein necessary for mast cell activation.

Mast cells are tissue resident granular cells that are enriched in tissues exposed to the environment. This allows them to rapidly respond to invading pathogens, including viruses such as IAV. Moreover, mast cells tend to be located around blood vessels, lymphatics, and nerve endings, allowing their mediators to have long-range effects for the host response (5, 6). Once activated, mast cells display two distinct phases to their responses: 1) immediate degranulation of pre-synthesized mediators, and 2) delayed secretion of secondary *de novo* synthesized mediators (5, 7, 8). These mediators can drive increased vascular permeability around the site of infection and enhance recruitment of additional leukocytes and lymphocytes to resolve the infection (9-12). In our studies, we used bone marrow cultured mast cells (BMCMC) to help identify the specific interactions that occur between mast cells and IAV. While work is starting to be done to understand the role of mast cells during other viral infections (5, 13-17), the specific function of mast cells during IAV had previously not been elucidated.

To address our hypothesis and understand the role that mast cells are playing during IAV infections, we sought to understand if and how mast cells were activated by the virus, and if viral proteins, specifically HA and NA, were playing a role in driving the mast cell response. In order to understand if mast cells were participating in the inflammatory response induced by IAV infections, we utilized a mast cell deficient mouse model and observed their susceptibility to IAV infection. Interestingly, mice deficient in mast cells displayed decreased disease severity during IAV infection compared to wild-type mice, thus indicating a pathological role of mast cells during IAV infection. To fully understand how mast cells were interacting with IAV, we used a series of *in vitro* BMCMC assays and found that mast cells are directly activated by IAV to

produce both histamine and *de novo* mediators such as cytokines, chemokines, and leukotrienes. IAV HA binds to sialic acids on surface of cells to initiate viral infection, but different strains of IAV bind to different sialic acid linkages with certain specificity to initiate their entry. Interestingly, when IAV strains that preferably bind to α2,6-linked sialic acids, they drove mast cell activation and infection, and NA plays a role in this activation. Conversely, IAV strains that primarily bind to α2,3-sialic acid linkages were unable to infect or activate BMCMC. Viruses that successfully entered cells were detected by the cytosolic PRR RIG-I, and this detection was important for mast cell production of secondary mediators in response to the virus. However, production of these cytokine, chemokine, or leukotriene mediators from mast cells was not important to induce IAV-induced disease *in vivo*, suggesting that mast cell degranulation mediators are the driving force behind the pathological disease observed. Moreover, IAV strains that preferably bind to α2,3-linked sialic acids had a different inflammatory response than viruses with an α2,6-sialic acid preference. Thus, we have 1) identified that mast cell activation requires sialic acid binding and RIG-I detection for production of mediators, and 2) elucidated a viral characteristic of HA and found a role of NA that is important for degranulation and the subsequent IAV driven inflammatory response. Overall, we have found that not only are mast cells infected and activated by IAV, but also participate in the pathological lung damage observed during infection in a virus strain specific manner.

IAV can be recognized by a wide variety of PRR, including toll-like receptors (TLR), Nod-like receptors (NLR), C-type lectins receptors (CLR), and retinoic acid-inducible gene-I like receptor (RLR) (18-29), and recognition of IAV by these receptors is important for initiating an appropriate anti-viral response. All of these receptors have

been shown to be expressed on mast cells (30-33), but their roles with mast cells during IAV infection were unknown. While IAV can be recognized by TLR-3, -4, and -7 (22, 27, 29), when the TLR adapter proteins MyD88 and/or TRIF were knocked out in BMCMC, degranulation and secondary production of mediators still occurred (Appendix A). Similarly, when BMCMC lacked prominent signaling adapters for CLR (FcR, DAP12) or NLR (NLRP3, ASC) degranulation and cytokine/chemokine production still took place (Appendix A). Only when RIG-I was absent was there a decrease observed in BMCMC activation, but this was only observed for secondary mediator synthesis, including cytokines, chemokines, and leukotriene B_4 production (Figure 4.8), thus demonstrating that secondary mediator production occurs in RIG-I-dependent mechanism, while degranulation occurs in a RIG-I independent fashion (Figure 4.8).

RIG-I detects single stranded uncapped RNA in the cytoplasm. In the case of IAV, it recognizes vRNPs that have exited the nucleus after replication (26, 34-36). Once activated, RIG-I then interacts with MAVS to initiate downstream anti-viral responses through IRF3 and NF-κB (26, 34-36). RIG-I/MAVS interactions can also mediate signaling events through STING, CARD9, and STAT6, all which have been shown to be involved during VSV infections (37-39). CARD9 signaling in response to VSV results in NF-κB activation, but not IRF3 (37). STING is an endoplasmic reticulum localized molecule, enhances the RIG-I-dependent interferon response to VSV (38). During Sendai virus or VSV infections, STAT6 is recruited to the STING/RIG-I/MAVS complex to initiate cytokine production of CCL2 and CCL20, but not for IL-6 (40). For BMCMC production of secondary mediators in response to IAV, CARD9 is not required for their activation, while both STING and STAT6 play partial roles in driving mast cell activation

(Figure 4.8). Therefore, once RIG-I detects IAV in mast cells, it likely binds with MAVS and further forms a complex with STING, resulting in STAT6 activation and signaling, which fully activates mast cells to produce secondary cytokines and chemokines mediators.

Viruses have evolved numerous proteins to block particular signaling pathways that are critical in initiating the anti-viral immune responses. NS-1 is a viral protein that plays a critical role in limiting the host immune response during IAV infection. Not only does it regulate viral RNA synthesis, control mRNA splicing, and enhance viral mRNA translation, it also suppresses host immune responses by blocking anti-viral genes, including 2'-5'-oligadenylate synthetase (OAS) and PKR (41). More importantly, it plays a major role in antagonizing IFN-I production. IAV with deleted NS-1 can only successfully replicate in tissues that are lacking IFN-α/β signaling (42). IAV protein PB1-F2 interferes with MAVS by disrupting mitochondrial membrane potential (43-45). However, MAVS is not specifically located to the mitochondria, and has also been shown to localize to peroxisomes (46). Peroxisome-associated MAVS triggers rapid expression of interferon stimulating genes such as CXCL10, viperin, Ifit3, IL-33, and CCL4, as well IFN-γ from virus-infected fibroblasts, while mitochondrial-associated MAVS had delayed response consisting of IFN-I (46). As PB1-F2 inhibits mitochondrial associated MAVS, in addition to NS-1 suppression of IFN-I, and we observe minimal IFN-I expression from BMCMC, this leads us to hypothesize that peroxisome-associated MAVS is playing a role in RIG-I detection of IAV. However, it is doubtful that this signaling pathway is important in mast cell-induced disease observed during A/WSN/33

infections, as RIG-I signaling within mast cells is not required for pathology observed during infection (Figure 5.1)

RIG-I is also important in driving mast cell detection of other viruses. Human mast cells infected with Sendai virus upregulation in their expression of RIG-I and MDA-5 (47). Dengue virus infects mast cells and can directly activate RIG-I signaling to initiate the anti-viral response (33, 48). Similar to IAV, dengue virus infection of mast cells is not required for activation of a degranulation response (33). RLR family member MDA-5 recognizes vaccinia virus; however, it is the fusion of the viral envelope to the plasma membrane that initiates activation from mast cells (49-52). Thus, while RIG-I seems to play a major role in initiating *de novo* mediator response of mast cells to viral infections, these data also strongly imply that the *de novo* mediator response of mast cells is dispensable in viral-induced pathologies.

While secondary mediator production of cytokines, chemokines, and leukotrienes from mast cells in response to IAV is RIG-I-dependent, degranulation occurs through a RIG-I-independent mechanism. Since mast cell degranulation takes place early after A/WSN/33 stimulation, we next determined the viral components responsible for BMCMC degranulation. Degranulation of mast cells results in the release of pre-synthesized and stored mediators, including histamine, TNF-α, amines, β-hexosaminidase, serotonin, antimicrobial peptides, and proteases (tryptases and chymases) (8, 52-54). These mediators can be measured in activated mast cell supernatants. Along with measuring these specific mediators to assess mast cell degranulation, we also took advantage of annexin V binding to phosphatidylserine as a rapid detector of mast cell degranulation. When the mast cell granules fuse with the

plasma membrane, it exposes phosphatidylserine on the outer leaflet of the cell membrane, allowing annexin V binding occur (55). The annexin V binding profile during degranulation has a different profile from dead or dying cells, which bind more uniformly across the cellular membrane, while degranulation binding occurs at sites of secretory granule fusion (55).

Interestingly, we found that the sialic acid binding preference of viral HA plays a major role in regulating the mast cell degranulation response. A/WSN/33 preferentially binds to α2,6-linked sialic acids (56). In contrast, A/PR/8/34, another mouse-adapted H1N1 IAV strain commonly used in laboratories, preferentially binds to α2,3-linked sialic acids (57). Here we show that while A/WSN/33 could induce mast cell activation, A/PR/8/34 is not able to activate mast cells (Figure 4.5, Figure 4.6, and Figure 5.6). Thus, it appears that α2,6-preference IAV isolates can drive mast cell activation. The sialic acid binding pocket domain consists of a helix and two loops that determine sialic acid binding preference and mutations within these loops can switch preference (58). Domains that have been identified as important for α2,6-sialic acid binding preference for H1N1 strains are aspartic acid at the 190 and 225 positions (58). In the A/WSN/33 strain of IAV, mutating the aspartic acid at position 225 to glycine changed the sialic acid binding preference of this virus to α2,3-linked sialic acid residues (59). Using this viral mutant of A/WSN/33, we observed there was no activation of mast cells when compared to the wild-type A/WSN/33 (Figure 5.7). This provides strong evidence that the binding preference of HA for sialic acid linkages, rather than other difference in the A/PR/8/34 strain of IAV, is responsible for inducting mast cell degranulation. The ability of IAV isolates to cause mast cell degranulation also correlates strongly to the ability of the virus

to infect mast cells when we measured viral NS-1 expression and cytokine production (Figure 5.6, 5.7), which we demonstrated was highly dependent on mast cell infection by IAV. Since sialic acids are the major entry receptor for IAV, the pattern of sialic acid expression on mast cells might dictate the infectivity of these cells. Mast cells are known to express sialic acids (60), however the exact linkages expressed on mast cells are currently unknown. HA binding to sialic acid does not have a strong interaction; instead, it is the abundance of sialic acids on the cell that aids in efficient entry (61). Perhaps viruses that bind to α2,3-sialic acid linkages, such as A/PR/8/34 and A/WSN/33 D225G, are not able bind efficiently and subsequently fail to infect the cells because there is not sufficient expression of α2,3-sialic acid linkages on BMCMC, whereas A/WSN/33 is able to successful infect and activation mast cells due to α2,6-sialic acid expression levels.

HA is not the only protein expressed on the viral envelope with sialic acid binding activity. The role of NA in viral infections is mostly limited to the release of newly formed virions from the cell by binding and cleaving sialic acids on the cell surface. However, the role of NA has not been well studied in terms of its role in regulating mast cell activation. Our data shows that HA binding is important for mast cell degranulation, but the mechanism behind this process is unknown. In human mast cells, NA has been shown to enhance histamine release from human leukocyte suspensions (containing 2% basophils) stimulated with IgE or *Staphylococcus aureus* (62-65). Inhibition of NA decreased histamine levels (62, 63), while addition of *Vibrio cholera* NA produced similar histamine production as IAV (62, 64). A strain of A/WSN/33 containing a mutated NA with an added glycosylation site at amino acid residue 130 results in reduced sialic acid cleavage activity (66), was unable to induce mast cells to degranulate or

produce secondary mediators (Figure 5.8). This data demonstrate that the IAV NA plays a role regulating mast cell activation. We now hypothesize that HA binding could be important for successful binding of the IAV virion to the cell membrane, thus allowing the IAV NA to enzymatically cleave sialic acids from the cell membrane, which ultimately drive mast cells signaling cascades which lead to mast cell degranulation.

However, the cellular mechanism behind how mast cells degranulate in response to viruses remains unclear. Dengue virus is also known to induce mast cell degranulation, but as of yet, the receptor driving mast cell degranulation is unidentified (15). Vaccinia virus also stimulates mast cell degranulation. The first step of the vaccinia virus replication cycle is the fusion of the viral envelope to the mast cell membrane, which induces degranulation. Fusion of the membrane produces S1P and allows signaling through the S1PR2 that induces mast cell degranulation (52). Whether mast cell recognition and detection of IAV can signal through S1PR2 remains to be elucidated, but S1P antagonists are known to limit the IAV-induced immunopathology observed in mice (67). Other PRR, such as TLR-3 and TLR-7, are able to recognize IAV earlier in the viral replication cycle (20, 22, 29). However, polyI:C was not able to induce degranulation (68), and in MyD88/TRIF$^{-/-}$ BMCMC, we still observed production of histamine by BMCMC response to A/WSN/33 stimulation(Appendix A). Thus, while it appears that early events in the viral replication cycle are capable of triggering mast cell degranulation, the signaling mechanisms behind this event in response to IAV remains unknown.

Mast cells are strategically positioned to allow them to encounter viruses very quickly after infection. We and others have shown that mast cells do play a role during

IAV infections, as mice lacking mast cells are not as susceptible as wild-type mice (69) and the addition of mast cell stabilizers limit IAV-induced disease (70). RIG-I$^{-/-}$ BMCMC do not produce secondary mediators, but still undergo degranulation. This dichotomy in the response of RIG-I-deficient BMCMC is a great tool to understand whether mast cell degranulation or secondary mediator production is critical in the mast cell-induced disease observed following A/WSN/33 infection of mice. RIG-I$^{-/-}$ BMCMC are able to degranulate, and when B6.Cg-*kit*$^{W\text{-}sh}$ were reconstituted with these cells, the mice were still susceptible to A/WSN/33-induced disease, indicating that it is mast cell degranulation mediators rather than secondary *de novo* mediators which amplify the lung damage and disease during A/WSN/33 infection (Figure 5.1). Degranulation mediators are also known to modulate disease severity during dengue virus infections (71, 72). Not only have infected patients have detection of histamine and chymases in the blood or urine (72-74), there is a strong correlation between severity of dengue virus infection and chymases levels (72), suggesting that mast cell degranulation is critical in the immunopathological diseases associated dengue virus infections.

As the role of mast cells during viral infections is becoming more understood, a link between high pathologic viruses and mast cells is emerging. Mast cells have been implicated in enhancing the pathological damage seen during numerous highly pathological viruses, such as IAV and dengue virus infections in humans and Newcastle's disease virus (NDV) and infectious bursal disease virus (IBDV) in chickens. In IAV infections, primary human isolated viruses have been shown to activate mast cells (69), and humans experimentally infected with IAV have elevated histamine levels during infection (75, 76). Genetic analysis in the lungs of IAV infected mice show that mast

cells are present in the lungs during IAV infection (4). When these cells are absent, or treated with granule stabilizing drugs such as ketotifen and cromolyn, mice infected with IAV or dengue virus fare better than wild-type or untreated mice (70, 72). This is not observed in just human viruses, but in viruses that cause high economic impact on animals including poultry and swine. NDV and IBDV both cause high mortality in chickens. Treatment of chickens with ketotifen resulted in less virus-induced lesions, as well as lower mast cell recruitment to sites of infection (77-80). In swine, elevated levels of mast cell mediator's histamine and LTB_4 have been observed in highly pathogenic porcine reproductive and respiratory syndrome virus, but were absent in a lower pathogenic strains (81-83). Similarly, in Hantavirus infections, patients can present with elevated histamine levels (13), and mast cells have been shown to infected and activated by Hantavirus isolates (13). Finally, the pathogenicity of Sendai virus in rats correlates with mast cell activation *in vivo* (13). Thus, mast cells appear to have a detrimental role during numerous highly pathogenic viruses. By understanding how mast cells are contributing to damage during these virus infections, we can use these cells as targets for new therapeutics to limit severity of disease.

It appears that not all IAV isolates can activate mast cells (Figure 4.5). However, viruses that do not activate can still induce sustained disease during *in vivo* infection. Infection of mast cell deficient B6.Cg-$kit^{W\text{-}sh}$ mice with either A/PR/8/34 or A/WSN/33 D225G induced significant weight loss and inflammatory cellular infiltrates into the lungs, which was similar to what was observed in wild-type C57Bl/6 mice. This suggests that the disease pathology caused by the A/PR/8/34 strain of IAV was not dependent on mast cells, while during A/WSN/33 infection the pathology appears to be dependent on

mast cell degranulation mediators (Figure 5.1). As A/PR/8/34 and A/WSN/33 D225G were not able to activate BMCMC, this could be a possible explanation of why mast cells do not participate in the IAV-induced pathology, but raises the important observation that there is another inflammatory mechanism responsible for the increased pathology, which is observed during infection with these non-mast cell activating viruses.

There are many reports of neutrophils playing a role during IAV infection (84-88). However, neutrophil influx is not always beneficial and does play a detrimental role during some IAV infection models. During a lethal H1N1 IAV infection model, neutrophils created a feed-forward loop that was detrimental to the host (84). Our data show a stronger neutrophil recruitment following infection of C57Bl/6 mice with A/PR/8/34 or A/WSN/33 D225G when compared to A/WSN/33 infected mice (Figure 5.8).

Why viruses that have different sialic acid binding preference are able to recruit different inflammatory cells is unknown. Perhaps viral infection of mast cells is limiting neutrophil recruitment, thus understanding the downstream responses during infection is critical. Type I interferons (IFN-1) are one of the first cytokines produced after IAV infections and are important cytokines for mediating viral clearance. IFNAR signaling has been shown to be important in regulating monocyte and neutrophil recruitment during IAV infection (89). High level of IFN-I signaling amplifies Ly6C^{hi} monocyte recruitment to the lungs after IAV infection by enhancing CCL2 (MCP-1) production, while low/absence IFN-I signaling leads to higher production of neutrophil recruiting chemokines such as CXCL1 (KC) and subsequent neutrophil recruitment to the lungs during IAV infection (89). Our experiments with A/WSN/33infection resulted in lower

amounts of the neutrophil recruiting chemokines CXCL2 and CCL4 compared to mice infected with A/PR/8/34, which correlated with neutrophil influx to the airway of those mice (Figure 5.8, 5.9). Conversely, during A/WSN/33 infections, we detect higher amounts of CCL2 in the lungs of infected mice (Figure 5.9). How this correlates with IFN-I production to regulate inflammatory cellular recruitment, and the role that mast cells play in this system, warrants further exploration. Alternatively, α2,3-binding viruses could have enhanced infectivity to the epithelium resulting in increased neutrophil recruitment (87, 88).

The role that mast cells play during viral infections is only now becoming apparent. Mast cells can be directly activated by viruses *in vitro* causing them to degranulate as well as synthesize *de novo* mediators. *In vivo*, a strong correlation between mast cell activation and disease severity has been identified by us in IAV (Chapter 3) and others for dengue virus, IBDV, NDV, and Sendai virus (13, 17, 72, 77-80, 90-93). Therefore, understanding the mechanisms behind mast cell activation in response to viruses will aid in developing novel therapies to limit morbidity and mortality associated with numerous highly pathogenic viral infections.

Future Studies

Understanding the Cellular Mechanism Driving Mast Cell Degranulation

In this thesis we have shown that mast cell degranulation is dependent on the sialic acid binding preference of IAV. However, the exact mechanism for degranulation remains elusive. We have explored the role of many PRR to determine if they have a role

in this process and have found that in BMCMC that lack specific PRR signaling, degranulation is still occurring (Appendix A). Mast cell degranulation is dependent on sialic acid binding preference; therefore, it likely plays a role in the signaling events, which initiate mast cell degranulation. It has been proposed that the early events of the viral replication cycle, such as binding of HA to sialic acid, initiate clustering of tyrosine kinase receptors (RTK) (94, 95). When IAV binds to sialic acids, it induces lipid raft aggregation and clustering of RTK, resulting in signaling through these receptors. RTK signaling activates the phosphatidylinositol-3 kinase (PI3K) signaling pathway, which is important in endocytosis of IAV (95). Epidermal growth factor receptor (EGFR) and c-Met are two RTK receptors that have been shown to be involved in IAV uptake (95). RTK are present on mast cells, a major one is CD117 (c-kit), important for stem cell factor signaling for growth, proliferation, and differentiation of mast cells (96, 97).

Because blocking EGFR can decrease IAV infection (95), we used the drug genistein in a pilot study to determine if EGFR plays a role in mast cell degranulation. BMCMC were treated with 50μM genistein prior to and during infection with A/WSN/33. Mast cell degranulation, measured by annexin V binding, still occurred in response to A/WSN/33 stimulation in the presence of genistein (Figure 6.1). However, other RTK are known to play a role in IAV infections (98) and should still be explored for their role in regulating mast cell degranulation in response to IAV. The focal adhesion kinase (FAK) is a major component of local adhesion complexes, which serves as cellular adherences and to tether the actin cytoskeleton to the extracellular matrix (99). Integrins are a major family of proteins located in focal adhesions, and once engaged, integrins result in FAK phosphorylation, which results in downstream signaling (100, 101). In

Listeria monocytogenes (LM) infections, binding of the bacteria to the cell surface requires engagement of LM internalin B binding to RTK for entry into non-phagocytic cells (101). More specifically, LM internalin B binds to β1 and β3 integrins, inducing clustering on the cell membrane to form focal adhesions containing FAK (101). Peritoneal mast cells express α2β1 integrins, and binding of LM internalin B to these integrins results in interaction with c-Met, leading to mast cell activation, degranulation, and production of cytokine IL-6 (100). Thus, mast cells express integrins that can signal through focal adhesion associated FAK to initiate activation. Additionally, IgE stimulation of mast cells results in the aggregation of integrins on the cell surface leading to FAK phosphorylation (102-104). FAK-deficiency in the rat mast cell line, RBL-2H3, resulted in decreased histamine production when stimulated through the IgE receptor (102), suggesting that FAK is required for mast cell degranulation in response to FcεRI engagement. Further studies using $FAK^{-/-}$ embryonic derived mast cells demonstrated that mast cells were still able to produce histamine; however, as these cells developed in the absence of FAK signaling, they may have developed compensatory mechanisms for their activation (103). Regardless, engagement of FAK in mast cells during pathogen infections has not been previously observed. Inhibition of FAK phosphorylation during IAV infection lead disregulated actin meshwork and decreased IAV entry into lung epithelial line A549 and human bronchial epithelial cells (98). Because mast cells are known to express FAK, it would be interesting to test if inhibiting FAK signaling would lead to decreased degranulation in response to IAV stimulation or other viruses that are known to activate mast cells.

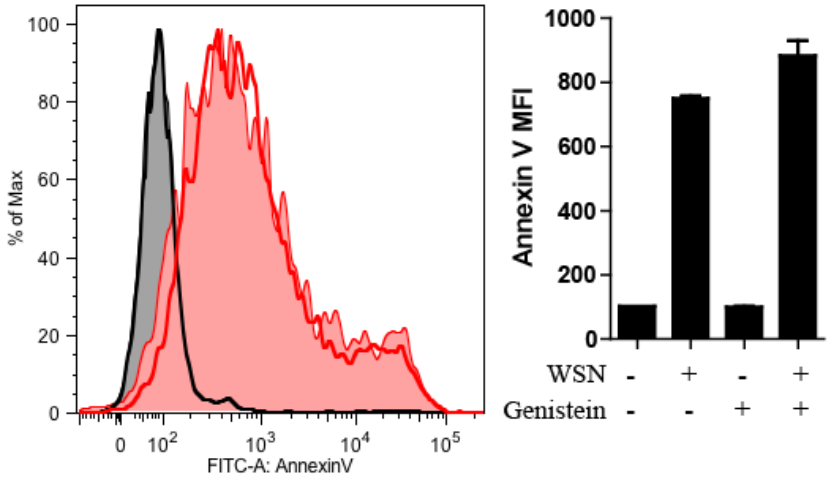

Figure 6.1 EGFR inhibited mast cells still degranulate. A total of $2.5x10^5$ BMCMC were pre-treated with 50μM genistein, an inhibitor of epithelial growth factor receptors (EGFR) and then treated with media or A/WSN/33 with our without 50μM genistein for 30 minutes. Cells were then washed and stained with annexin V to determine degranulation. Black shaded graph – cells treated with media alone; Red shaded graph – cells treated with A/WSN/33 alone; Black graph – cells treated with media and genistein; Red graph – cells treated with A/WSN/33 and genistein.

Sialic Acid Expression on Mast Cells

Our data strongly supports that mast cell degranulation in response to IAV is dependent on sialic acid binding by the HA molecule. A/WSN/33 preferably binds to α2,6-sialic acid linkages and can activate mast cells to degranulate and produce secondary mediators (Chapter 5). Conversely, A/PR/8/34 and the A/WSN/33 D225G, both of which are known to primarily bind to α2,3-linked sialic acids (57, 59), cannot activate or infect mast cells (Chapter 5). Mast cells express sialic acids (60), but the specific linkages that are expressed on mast cells has yet to be been clearly identified. Sialic acid linkage expression can be determined using plant lectins. *Sambucus nigra* (SNA) binds to α2,6-sialic acid linkages while *Maackia amurensis* (MAA) binds to α2,3-sialic acid linkages (105). These linkages can be conjugated to fluorescent antibodies and

used in either flow cytometry or fluorescent microscopy to determine if these linkages are expressed on mast cells. As sialic acids with a α2,6- linkage appear to lead to mast cell activation, we hypothesize that these are expressed on mast cells. Knowing the sialic acid binding preference is important for understanding novel IAV strains. Avian influenzas preferentially recognize α2,3-linked sialic acids. However, humans have higher expression of α2,6-linked in their upper respiratory tract (106), limiting infections from avian IAV. In order to become pathogenic to humans, these α2,3-sialic acid binding viruses must either infect deep into human lungs, where α2,3-linkages are expressed, or mutate to preferably bind α2,6-linked sialic acids (107). We have shown that viruses that bind to α2,6-linked sialic acids are capable of activating mast cells, which enhances pulmonary damage during infection. Therefore, by understanding if mast cells express these linkages, and by knowing the sialic acid binding preference of emerging IAV strains, we can predict which viruses will activate these cells and prepare pharmacological drugs that target activation mediators to limit pulmonary damage, consequently decreasing the morbidity associated with infection.

Further Explore the Role of the IAV NA in Driving Mast Cell Degranulation

There are three viral proteins located on the IAV envelope: HA, NA, and M2. We have shown a critical role for the sialic acid binding preference of HA in regulating mast cell degranulation (Chapter 4). However, the involvement of NA or M2 in mast cell activation has not been determined. M2's major function occurs within the cell and is required for viral genome release from the endosome to the cytoplasm. However, NA cleaves sialic acids that are important for the release of newly formed virions from a cell.

Activation of mast cell to undergo a degranulation response appears to occur at the cell surface (Figure 5.8), we hypothesize that activation of mast cells to degranulate may require more than binding of the HA to sialic acids, and requires NA enzymatic activity of cleaving sialic acids as well. Using a viral A/WSN/33 mutant that has an extra glycosylation site in the NA at position 130, which is unable to cleave sialic acids as effectively (66), we can test and determine if NA play a role in BMCMC activation, as HA will still bind to α2,6- sialic acid linkages. Interestingly, we see that A/WSN/33 NA130 does not cause activation in BMCMC as detected by annexin V binding expression or cytokine or chemokine expression (Figure 5.8). However, when confirming if A/WSN/33 NA130 could infect BMCMC, we found that A/WSN/33 NA130 had NS-1 expression (Figure 6.3). Therefore, A/WSN/33 NA130 is able to infect BMCMC but not able to induce cells to degranulate. Thus, IAV NA appears to be playing a role in degranulation of mast cells, but the mechanism behind this activation needs further exploration.

NA cleavage can be inhibited by using drugs currently used to treat IAV symptoms. These drugs prevent NA activity by serving as a competitive inhibitor of NA, therefore blocking the activity of the enzyme (1). If NA is playing a role in mast cell activation, these NA inhibiting compounds may help limit the effect of mast cell-induced disease during IAV infections. In human leukocyte suspensions containing >2% basophils, IAV NA enhanced production of histamine with cells were stimulated with IgE or Staphylococcus aureus (62, 64, 65, 108). IAV NA inhibiting compounds already exist on the market, as they are used to limit IAV spread during infection, reducing IAV-induced disease. Using these pharmacological compounds to prevent NA activity, we can

test the role of NA cleavage of sialic acids on mast cell degranulation. The ability of HA alone to be sufficient for mast cell degranulation, and the role that NA plays in this process, will provide interesting insights to mast cell degranulation responses to viruses.

Another way to test which IAV envelope proteins are important for driving mast cell degranulation is the use of nanoparticles. Virus like particles (VLP) are protein cages, and can have viral proteins expressed on the interior or exterior surface. VLP with H5N1 IAV HA on the surface have already been produced (109). P22 is a non-replicative bacteriophage capsid and using this technology, A/WSN/33 HA could be expressed on the surface of the VLP (110). Using VLP only expressing A/WSN/33 HA on the surface and no A/WSN/33 NA will further confirm the role of HA in activating mast cells. Additionally, A/WSN/33 NA alone can be expressed on the VLP surface to further confirm the role of NA in mast cell degranulation. To confirm that only the IAV surface proteins HA and NA are important in driving mast cell degranulation, both proteins can be expressed on VLP, and this VLP-HA/NA complex can be treated with BMCMC to determine mast cell degranulation. IAV viral proteins are attached to the surface of VLP P22 using dec. To confirm that the P22-Dec complex does not activate mast cells to degranulate, dec alone and P22-Dec was added to BMCMCs at MOI of 0.1, 1, and 10 for one hour, and cells were subsequently stained with annexin V to determine mast cell degranulation. Reassuringly, mast cells do not degranulate in response to dec alone or the P22-Dec complex (Figure 6.3). Furthermore, it will be interesting to determine if A/WSN/33 proteins attached to a VLP would be able to induce degranulation, as a VLP will be more similar to the size of IAV.

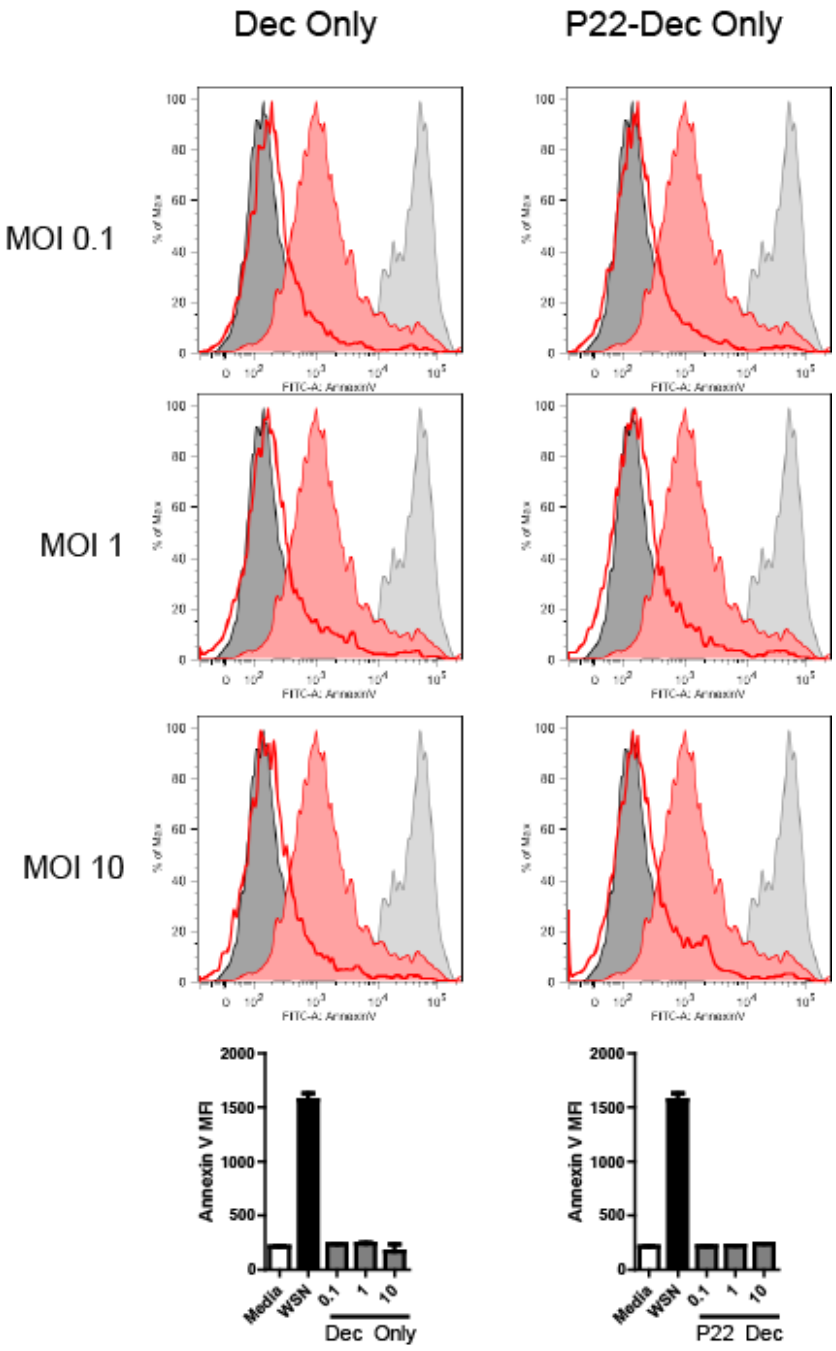

Figure 6.2 Dec and P22-Dec do not Induce BMCMC Degranulation. A total of $2.5x10^5$ BMCMC were treated with P22 or P22-Dec at MOI of 0.1, 1, or 10 particles/mL for 1 hour. Annexin V binding was used to determine degranulation. Black shaded graph – BMCMC treated with media alone; Red shaded graph – BMCMC treated with A/WSN/33 alone; Gray shaded graph – calcium ionophore treated BMCMC; Red graph – Dectin or P22-Dec treated BMCMC.

Role of Mast Cells in the
Innate Immune Response to IAV

By using RIG-I$^{-/-}$ BMCMC in our mast cell deficient B6.Cg-*kit*$^{W\text{-}sh}$ mouse model, we concluded that production of cytokines and chemokines from mast cells was not required to mediate mast cell-induced lung pathology and disease during A/WSN/33 infections (Figure 5.1). Moreover, the mast cell stabilizer, ketotifen, is able to reduce IAV-associated symptoms during infection with H5N1 IAV (70). Similar results were obtained during dengue infection; when mice were treated with cromolyn sulfate, they displayed less severe dengue-induced disease compared to untreated mice (72). Therefore, degranulation of mast cells appears to be an important contributor to mast cell-induced disease. We can further solidify these findings using the mast cell reconstitution model with BMCMC that are incapable of degranulating in response to viral infections. Mice that lack heparin sulfate (N-deacetylase/N-sulfotransferase-2 (NDST-2)) cannot effectively store histamine and protease in their granules, thus their degranulation response is non-inflammatory (111, 112). Reconstituting NDST2-deficient mast cells into B6.Cg-*kit*$^{W\text{-}sh}$ mice will formally test if the mast cell mediators stored in these granules are important for initiating mast cell-induced disease during A/WSN/33 infections.

The mast cell reconstitution model can also utilize BMCMC from other knock-out mice in a similar manner to more specifically define the cause(s) of A/WSN/33-induced disease. Histamine is a major component of the mast cell degranulation response, and therefore, we can assume that histamine might play a major role A/WSN/33-induced disease. Histadine decarboxylase (HDC) is the enzyme required for histamine synthesis. Mast cells found in mice lacking the HDC enzyme have reduced granular contents and

because of that, lack histamine (113). BMCMC derived from HDC-deficient mice can be reconstituted back into B6.Cg-*kit*$^{W\text{-}sh}$ mice to help determine if histamine plays a role in the immunopathological response observed during A/WSN/33 infection. TNF-α is another pre-synthesized component found in mast cell granules that can be released during mast cell degranulation. This cytokine is associated with inflammatory diseases, including asthma, and has been found in the BALF of mice undergoing dinitrofluorobenzene (DNFB)-induced non-IgE-mediated pulmonary hypersensitivity reaction (114). Mice lacking mast cells not only had decreased TNF-α with the BALF, but had a decreased reaction during DNFB challenge (114), indicating that TNF-α has a role during this response. During A/WSN/33 infections, TNF-α was decreased in the BALF of infected B6.Cg-*kit*$^{W\text{-}sh}$ mice correlating with decreased disease severity, and weight loss, as TNF-α is a major cachexia, suggesting that TNF-α might have a role in regulating the immunopathological response during A/WSN/33 infection (Figure 4.3). If TNF-α is produced from A/WSN/33 activated mast cells within an hour during degranulation response, correlating with histamine production and annexin V staining (Figure 4.4 and Figure 5.2), TNF-α$^{-/-}$ mast cells could be reconstituted into B6.Cg-*kit*$^{W\text{-}sh}$ mice and disease susceptibility can be observed. By understanding degranulation mediators critical in A/WSN/33-induced disease, we can target these components to reduce severity during infection.

Explore the Different Inflammatory Milieus Induced by α2,3- and α2,6- Sialic Acid Binding Influenza A Viruses

We have found that A/PR/8/34 infection induces neutrophil recruiting chemokines, which correlates with a robust influx of neutrophils into the lungs observed during A/PR/8/34 infection. During A/WSN/33 D225G infections, we also see a strong influx of neutrophils to the lungs during infection. However, the cytokine/chemokine profile has not been examined within airways during infection with this mutant strain of IAV. As there appear to be many similarities between the A/PR/8/34 and A/WSN/33 D225G virus, which correlate with their ability to bind α2,3-linked sialic acids, we would expect to see a similar trend in the production of inflammatory cytokines and chemokines, particularly in regard to the increased levels of neutrophil recruiting chemokines.

The regulation of the immune response downstream of mast cells during IAV remains unknown. During A/WSN/33 infection, there is a trend towards higher expression of CCL2 in the lungs when compared to A/PR/8/34 infection. Signaling through the IFN-I receptor IFNAR induces high influx of $Ly6C^{hi}$ monocytes through CCL2 signaling (89). Testing the role of IFN-I during A/WSN/33 and A/PR/8/34 infections can be done by using $IFNAR^{-/-}$ mice or using monoclonal antibodies, which specifically block IFN-I, and determine mouse susceptibility. Subsequently, the inflammatory cytokines and chemokines produced can be measured. Thus, identifying if this is important during A/WSN/33 infections will help determine how mast cells contribute to disease during IAV infections.

Understanding the Link Between Asthma, Mast Cells, and IAV

Mast cells are well recognized for their role in allergic reactions and asthma. The prevalence of asthma among children has been on the rise in recent years. The most common form is atopic asthma, where exposure to inhaled antigens results in specific immune responses (115). Asthma patients have been shown to have increased mast cell numbers within their respiratory tract (116) and constant activation of these cells results in a disease that is characterized by airway restriction, shortness of breath, wheezing, coughing, chronic lung inflammation, and tissue remodeling (116, 117). Respiratory viruses are known to exacerbate the symptoms of asthma, leading to potential hospital admission (118-123). During the 2009 H1N1pdm, asthma has the most common co-morbidity among hospitalized patients (124-127). Thus, it is crucial we not only understand the role mast cells are playing during viral infections, but also the contribution of mast cell activation by viruses resulting in increased airway hyperresponsiveness in asthma patients.

While mast cells play a large role in asthma hyperresponsiveness, there are relatively few asthmatic mouse models that have increased mast numbers due to inhaled antigen (115, 128). Recently, a mouse model has been developed using house dust mite (HDM) sensitization over three weeks that results in mast cell expansion (129). HDM-induced mast cell expansion not only increased bronchoconstriction upon challenge, but had an increased production of IL-4, IL-5, IL-13, IL-17, and IFN-γ. Bronchoconstriction in this model of asthma was mast cell-dependent, as mast cell deficient mice had no HDM-induced disease (129). Using this model, we can more closely examine the effects

of mast cell-induced asthma on the outcomes of IAV infections in an asthmatic population. A/WSN/33 induces mast cell activation and leads to mast cell-induced pulmonary damage during infection (69). We hypothesize that in this HDM mouse model of asthma that increased mast cell numbers in the respiratory tract due to asthma will lead to more severe IAV-induced disease. IAV will be readily able to interact with and infect mast cells and cause them to activate, releasing mediators including histamine, proteases, cytokines, chemokines, and leukotrienes. Mast cell degranulation mediators are especially important to study in an asthma mouse model, as these mediators are known to cause vascular permeability, vasodilation, and smooth muscle constriction (9, 130, 131), all which can exacerbate asthma symptoms including wheezing, coughing, and shortness of breath. Cytokine, chemokine, and leukotriene production recruit inflammatory cells, which would aid in tissue remodeling (132, 133)2(71, 134). Therefore, it is crucial to understanding the mast cell interaction with viruses in both asthmatic and non-asthmatic mice to help us understand how to limit mast cell-induced disease in asthma patients during the IAV season.

Conclusion

We have shown that mast cells play a pathological role during IAV infections. Some viruses are able to induce mast cells to activate, and this activation is dependent on the sialic acid binding preference of the viruses studied. A/WSN/33, which preferably binds to α2,6-linked sialic acids, causes mast cells to degranulate, releasing pre-synthesized *de novo* mediators, as well as produce secondary mediators. While the signaling mechanism behind mast cell degranulation remains elusive, sialic acid binding

preference and NA activity was shown to be important. Secondary mediator production upon IAV stimulation is RIG-I-dependent (Figure 4.7), although it does not have a role in A/WSN/33-indcued pathology during infection of mice. *In vivo*, α2,6-linked sialic acid binding A/WSN/33 induces disease that is mast cell dependent. Conversely, α2,3- linked sialic acid binding viruses, such as A/PR/8/34 and A/WSN/33 D225G, induce higher neutrophil influx during infection compared to A/WSN/33 that correlated with the inflammatory cytokine and chemokine signature observed within the lungs (Figure 6.5).

Understanding the role that mast cells play during IAV will be beneficial to reducing IAV-related morbidity and mortality, especially during future IAV pandemics. By comprehending whether mast cells are activated by the newly emerging IAV, we may be able to treat patients with mast cell stabilizing drugs for example, which are already available on the market due to mast cell involvement in diseases such as allergy and asthma, in order to minimize the morbidity and mortality associated with the emerging IAV. As there is a strong link with sialic acid binding preference and mast cell activation, we can rapidly test for which viruses induce mast cell activation, specifically degranulation, as this process appears to play a major role in mast cell-induced disease during IAV infection. The next strain of IAV that will cause a pandemic is impossible to predict. Morbidity and mortality associated with IAV pandemics is not always through viral replication, but is associated with immune response damage as well (2, 3). Thus, having the knowledge of how to reduce the damage observed during infections by controlling the host immune response, we can potentially decrease the morbidity and mortality associated with future IAV pandemics, as well as in other high pathogenic viruses.

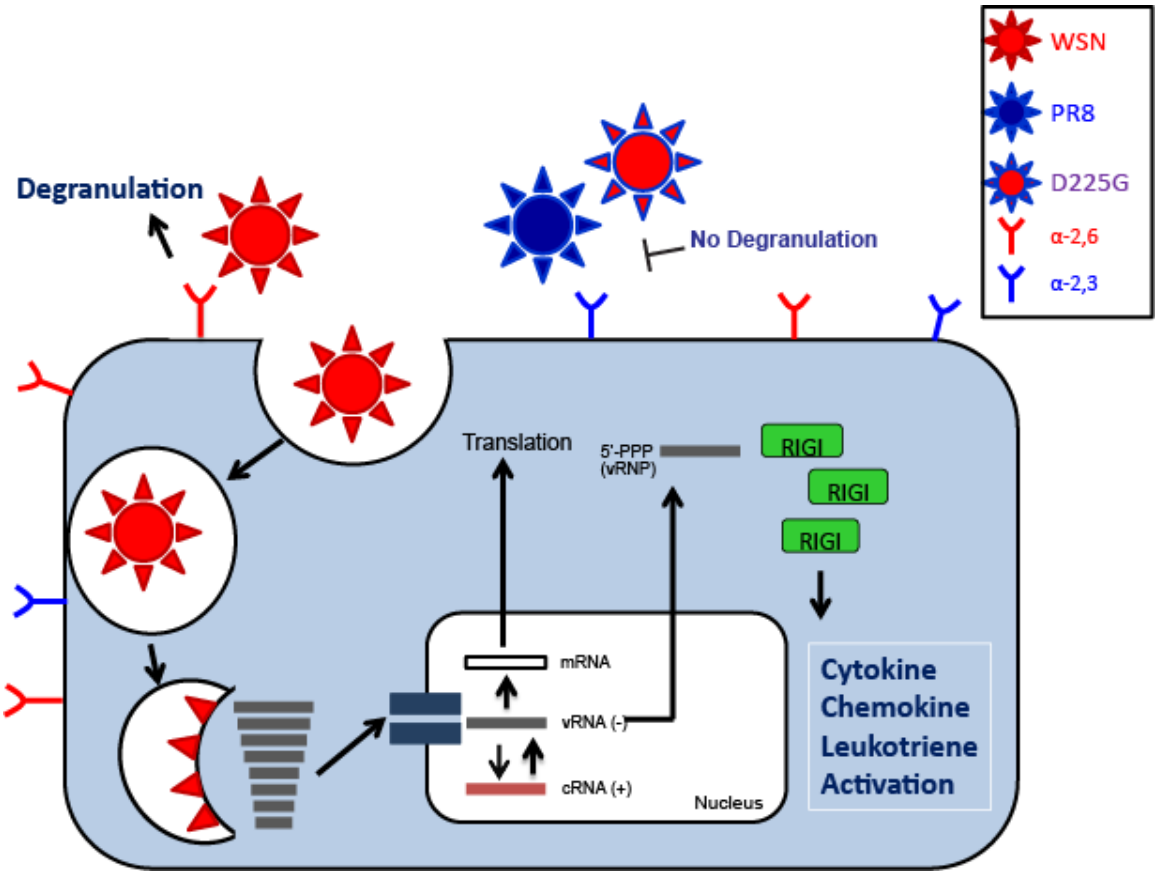

Figure 6.3 Mast cell activation by IAV. IAV HA binds to sialic acids located on the surface of cells. A/WSN/33 preferably binds to α2,6 –linked sialic acids and induces degranulation. A/PR/8/34 and A/WSN/33 mutant A/WSN/33 D225G bind to sialic acids with a α2,3- linkage, and this does not result in degranulation. After binding to sialic acids, the virus is endocytosed, through clathrin mediated or non-clathrin mediated endocytosis, into an endosome. Via M2, the protons will enter the virion and HA will fuse to the endosome, allowing the vRNP to enter the cytoplasm. The vRNP will translocate to the nucleus, where the negative sense single stranded RNA will transcribe to positive sense cRNA or mRNA. cRNA is used to create more copies of vRNA, while mRNA will move back into the cytoplasm to be translated. The viral RNA is recognized by cytosolic RIG-I, which signals through MAVS and leads to activation of secondary mediators from mast cells, including cytokines, chemokines, and leukotrienes. A/WSN/33 infection of mast cells does not result in new virions being released from the cell.

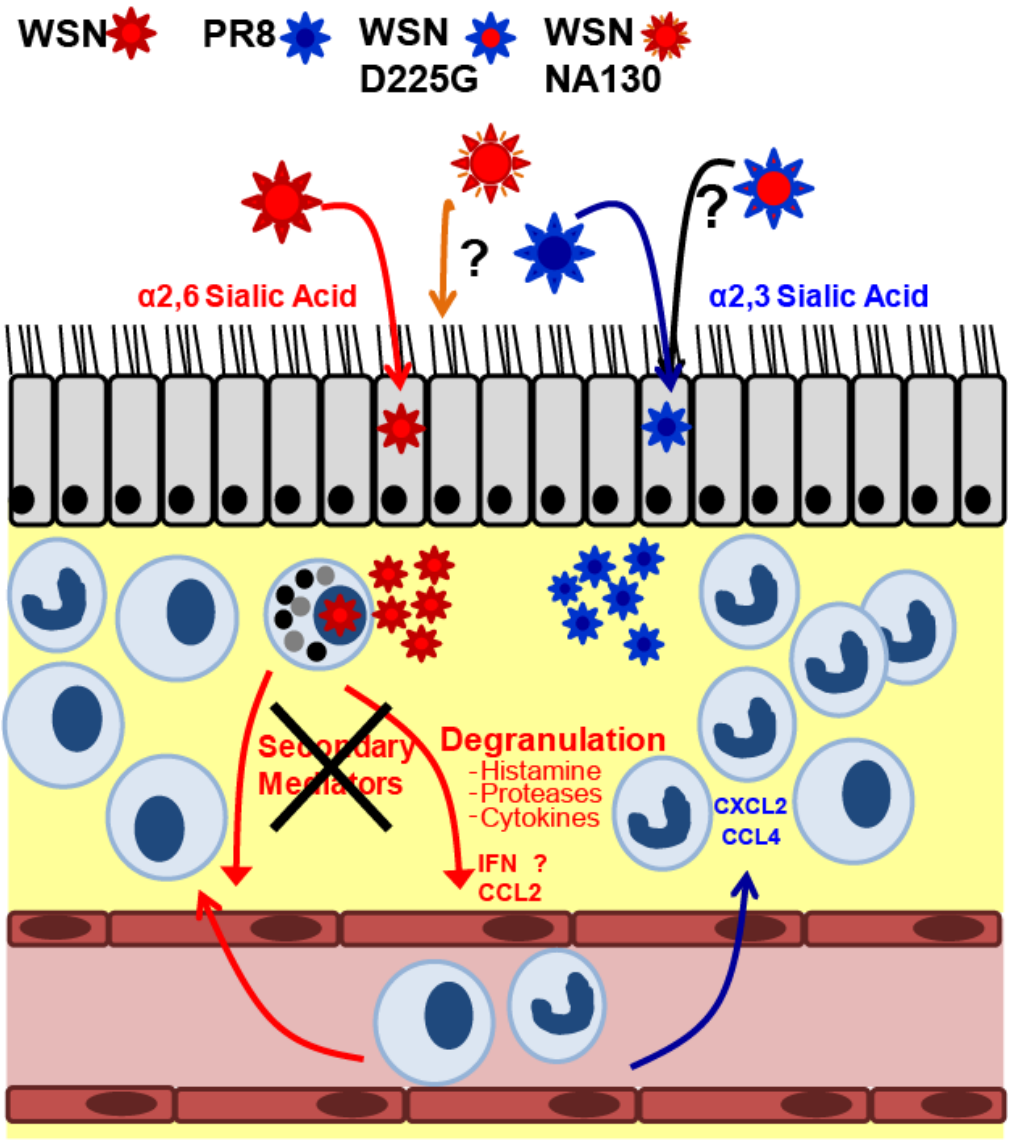

Figure 6.4 The role of mast cells in the lung during IAV infections. A/WSN/33 enters the lung and binds to α2,6 –linked sialic acids. A/WSN/33 is able to infect mast cells and cause mast cell activation. While mast cells produce mediators that have a delayed release, the mediators do not contribute to mast cell-induced disease during infection. However, degranulation mediators could be playing a role. A/WSN/33 infections induce elevated amounts of CCL2, which has been shown to be in response to IFN-I production and signaling and promotes monocytic cellular influx during infection (89). Conversely, A/PR/8/34 and A/WSN/33 D225G preferably bind to α2,3- linked sialic acids. These viruses do not infect mast cells or cause mast cells to degranulate. During infection of these viruses, there is a higher amount of neutrophil recruiting chemokines CXCL4 and CCL4, which results in higher neutrophil influx during infection.

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APPENDIX A

SUPPORTING INFORMATION FOR DISCUSSION

APPENDIX A

Mast cells, located in tissues exposed to the environment are in a prime region to be one of the first responders to infections (1-4). Activation of mast cells can be defined in two ways: 1) degranulation of pre-synthesized compounds, and 2) production and release of *de novo* synthesized mediators. *In vitro*, degranulation can be detected within 30 minutes of stimulation by measuring histamine release, β-hexoaminidase, and extracellular annexin V expression. *De novo* synthesis of mast cells can be detected by cytokine, chemokine, and leukotriene production *in vitro* at 4 hours after stimulation.

In terms of viral infections, *in vivo* murine models have demonstrated that mast cells play a role during systemic dengue virus infection, as well as local skin infections with vaccinia virus (5-8). *In vitro* it has been shown that mast cells can be activated by a multitude of viruses (9-12). Mast cell mediators are important for recruiting effector cells, such as neutrophils, macrophages, and lymphocytes, to the site of infection and mast cell activation (13).However, the role and function of mast cells during influenza infection has been not been studied. As our preliminary data demonstrate that mast cells play a critical role during A/WSN/33 IAV- induced pathology, we want to determine how these mast cells are being activated in response to A/WSN/33.

Pattern recognition receptors (PRR) play an essential role in innate immunity by detecting conserved pathogen associated molecular patterns. PRRs are located both on the cell surface to detect extracellular pathogens, as well as located within a cell to sense intracellular invading pathogens (14). Immune recognition of IAV has been shown to be mediated by numerous PRR, including toll-like receptors (TLR) 3, TLR7, NLRP3, Nod2,

MAVS, and RIG-I (15-25). Each of these PRR has been shown to be expressed and functional in mast cells (7, 12, 26, 27).

Mast cells can be activated through numerous pathways, and express a wide variety of innate immune receptors, many of which are able to detect viruses in other cell types (15-25).

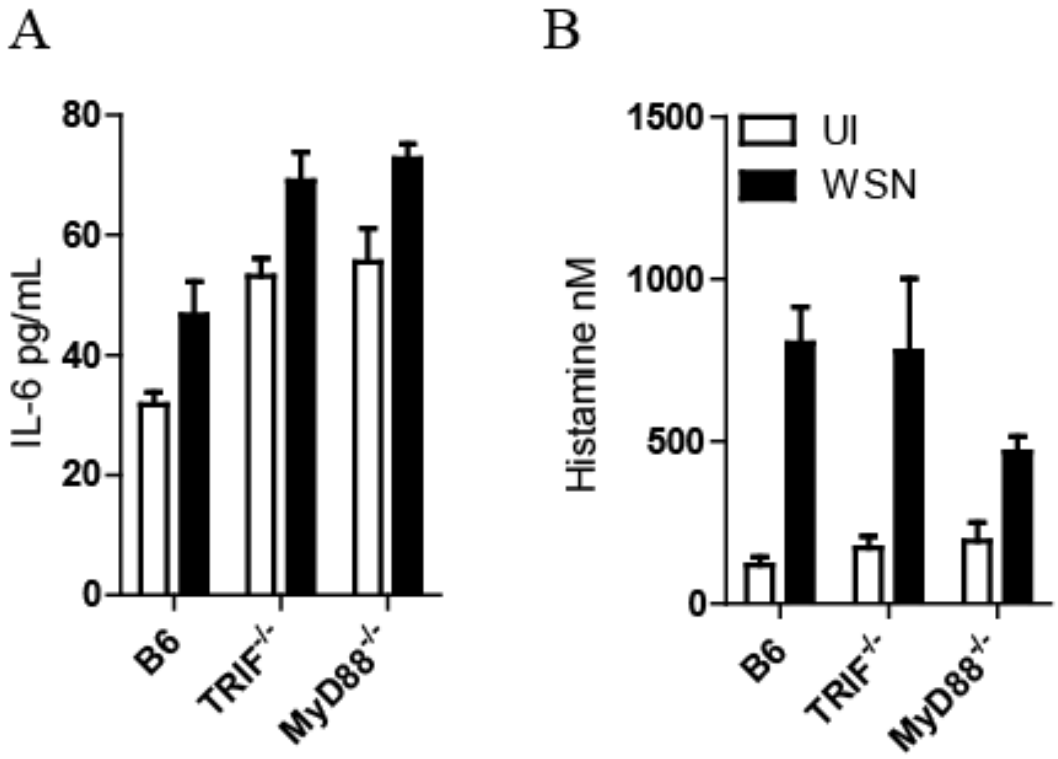

Figure A.1 Mast cell activation by A/WSN/33 is not dependent on TLR. BMCMC were generated by culturing total bone marrow from either C57Bl/6 (B6) or MyD88$^{-/-}$, TRIF$^{-/-}$, MyD88/TRIF$^{-/-}$, TLR2$^{-/-}$, or TLR4$^{-/-}$ mice with IL-3 for 5 wk and supplementing with stem cell factor for the last 2 wk. A total of 2.5x10^{5} FcRε1^{+} and CD117^{+} mast cells were treated with media or A/WSN/33 (WSN). Virus was added at an MOI of 1. Six hours after inoculation CCL4 or IL-6 (A), or histamine (B) levels were measured by Milliplex™ multiplex analysis or EIA. Similar data were observed for CCL2, CCL4, or IL-6 expression. Data are representative of two to four independent experiments.

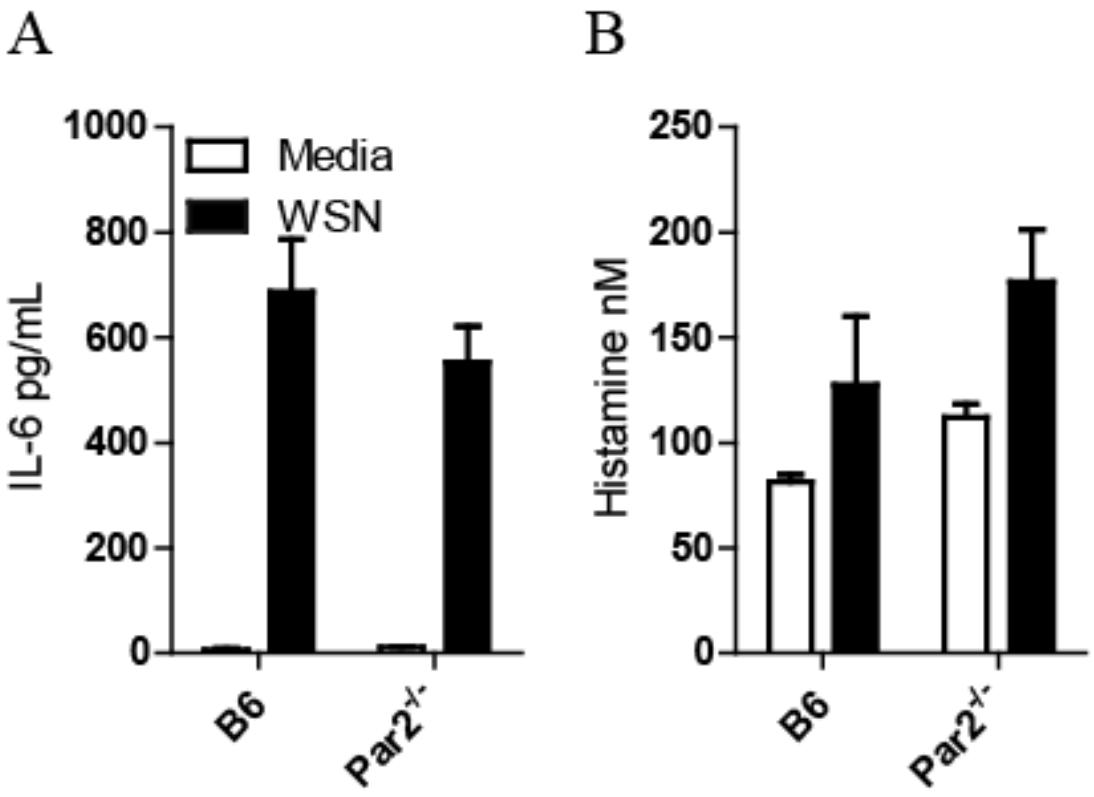

Figure A.2 Mast cell activation by A/WSN/33 is not dependent on Par2. BMCMC were generated by culturing total bone marrow from either C57Bl/6 (B6) or $Par2^{-/-}$ mice with IL-3 for 5 wk and supplementing with stem cell factor for the last 2 wk. A total of $2.5x10^5$ $FcR\varepsilon1^+$ and $CD117^+$ mast cells were treated with media or A/WSN/33 (WSN). Virus was added at an MOI of 1. Six hours after inoculation IL-6 (A), or histamine (B) levels were measured by Milliplex™ multiplex analysis or EIA. Similar data were observed for CCL2 and CCL4 expression. Data are representative of two to four independent experiments.

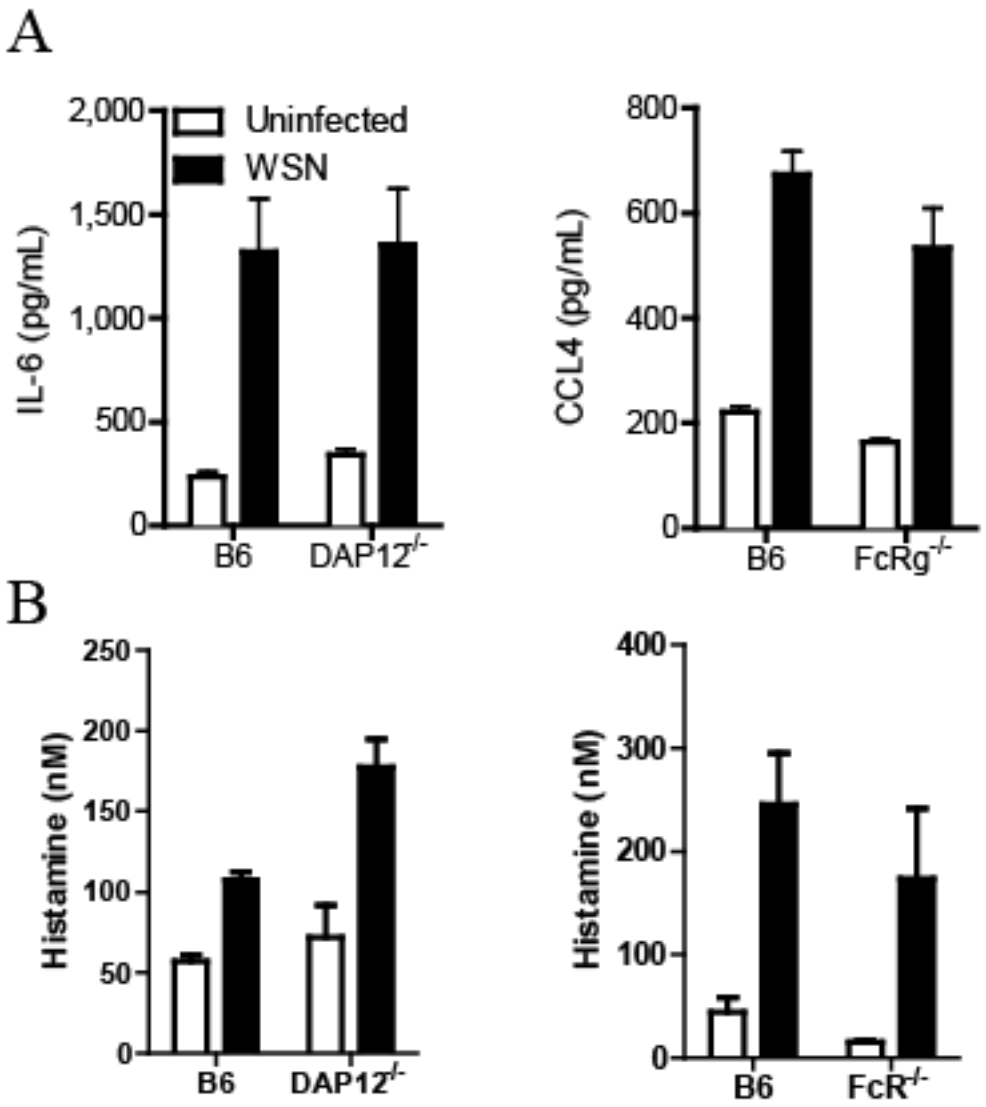

Figure A.3 Mast cell activation by A/WSN/33 is not dependent on CLR. BMCMC were generated by culturing total bone marrow from either C57Bl/6 (B6), DAP12$^{-/-}$, or FcR$\gamma^{-/-}$ mice with IL-3 for 5 wk and supplementing with stem cell factor for the last 2 wk. A total of 2.5x10^5 FcRε1$^+$ and CD117$^+$ mast cells were treated with media or A/WSN/33 (WSN). Virus was added at an MOI of 1. Six hours after inoculation IL-6 or CCL4 (A), or histamine (B) levels were measured by Milliplex™ multiplex analysis or EIA. Similar data were observed for IL-6, CCL2, and CCL4 expression. Data are representative of two to four independent experiments.

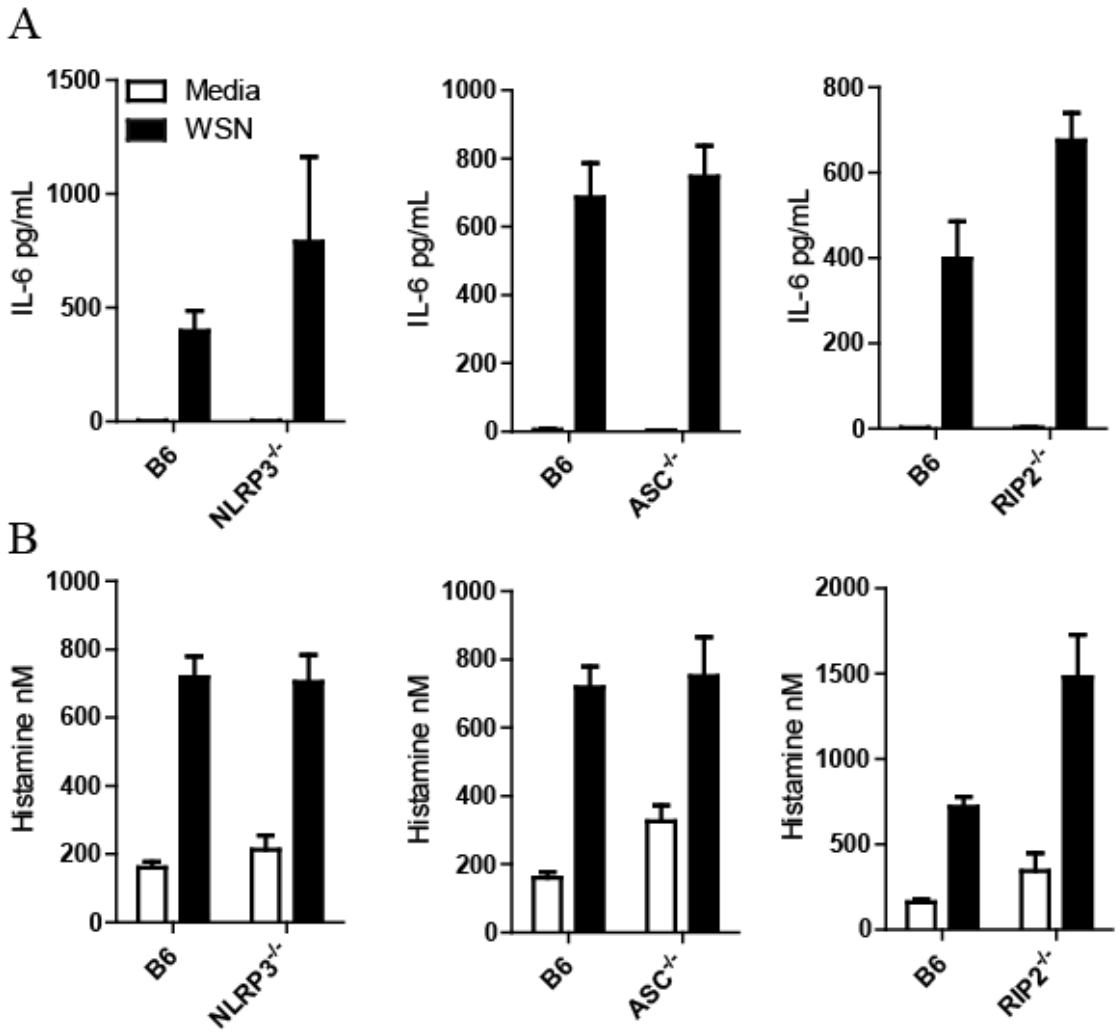

Figure A.4 Mast cell activation by A/WSN/33 is not dependent on NLR. BMCMC were generated by culturing total bone marrow from either C57Bl/6 (B6) or NLRP3$^{-/-}$, ASC$^{-/-}$, or RIP2$^{-/-}$ mice with IL-3 for 5 wk and supplementing with stem cell factor for the last 2 wk. A total of $2.5x10^5$ FcRε1$^+$ and CD117$^+$ mast cells were treated with media or A/WSN/33 (WSN). Virus was added at an MOI of 1. Six hours after inoculation CCL4 or IL-6 (A), or histamine (B) levels were measured by Milliplex™ multiplex analysis or EIA. Similar data were observed for CCL2, CCL4, or IL-6 expression. Data are representative of two to four independent experiments.

Conclusions

While mast cells have been shown to express these PRR, and these PRR and important in the detection of viruses (15-25), in the case of IAV these PRR are not playing a role. In these studies, mast cells were cultured with IAV and measure for both degranulation (by histamine production) and secondary activation mediators (cytokine or chemokine production). However, when these signaling pathways were knocked out, mast cells were still able to become activated. This suggests that BMCMC are not able to detect IAV through these mechanisms. An alternative explanation is that mast cells are able to detect IAV through redundant pathways.

We have concluded and published that BMCMC are able to recognize IAV through RIG-I, and this is important for activation and production of secondary mediators from mast cells (Chapter 4). As for degranulation, binding of viral hemagglutinin to α2,6-linked sialic acids is important (Chapter 5).

This appendix is to provide data to support conclusions in previous chapters.

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