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Antiviral and Cytokine Responses of Human Mast Cells to Influenza A Virus Infection

by

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Dedication

This thesis is foremost dedicated to my husband, Marcelo Marcet Palacios. You are my partner in life and inspire me to dream, to create and to persevere in everything that I do. From you, I learned that “no effort goes unrewarded”. I also want to dedicate this thesis to my son, Camilo, who brings meaning to my work with a smile when I get home.

Abstract

Mast cells are immune cells important in innate immunity. Besides their role in asthma and allergies, mast cells are critical effector cells against various pathogens. Mast cells are established to be protective against bacterial infections, but little is known about their functions in viral infections.

Mast cells are abundant in the lungs where influenza A virus (FluA) enter the host. We measured mRNA transcription, protein translation, and synthesis of new viral particles in FluA-treated mast cells. While expression of FluA mRNA and proteins followed similar time courses in both mast cells and epithelial cells, mast cells released a near absence of FluA.

We also studied mast cell cytokine release in response to FluA and other viral-associated stimuli such as TLR ligands and type I interferons. Mast cells released the cytokines IL-6 and TGF-β, and chemokines CXCL8 and CCL5 in response to various viral stimuli. However, different stimuli were capable of inducing release of different mediator subsets, demonstrating the specificity of mast cell responses.

Since FluA infection of mast cells produce little new FluA virions, we investigated whether FluA induces expression of antiviral proteins in mast cells. FluA treatment resulted in mast cell expression of antiviral proteins, namely

myxovirus resistance protein A, protein kinase R, interferon stimulated gene 15, viral stress inducible protein 56, and endothelial nitric oxide synthase.

Next, we performed co-culture experiments using FluA-infected epithelial cells with or without the addition of mast cells. Our results showed that mast cells in co-culture inhibited the expression of the viral hemagglutinin protein in FluA-infected epithelial cells. Also, preliminary results showed that addition of mast cells protected epithelial cells from FluA infection by limiting the release of FluA particles and reducing epithelial cell death.

Our discovery that mast cells produce little virus and express antiviral proteins suggest that mast cells have antiviral mechanisms to restrict FluA infection. This concept was further supported by evidence that mast cells are protective against FluA infection in epithelial cells. This research provides a better understanding of mast cells in innate immunity and may reveal unique antiviral mechanisms valuable in the development of antiviral therapeutics.

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Abbreviations

AHR - airway hyperresponsiveness

AIDS - acquired immunodeficiency syndrome

AngII - angiotensin II

APC - antigen presenting cell

BAL - bronchoalveolar lavage

BCA - bicinchoninic acid

BDNF - brain-derived neurotrophic factor

BMMC - bone marrow-derived mast cell

C3 – complement 3

C3a – complement 3a

C4 – complement 4

C5a - complement 5a

CaI - calcium ionophore

CCL1 - CC chemokine ligand 1or I-309

CCL2 - CC chemokine ligand 2 or monocyte chemotactic protein (MCP)-1

CCL3 - CC chemokine ligand 3 or macrophage inflammatory protein (MIP)-1α

CCL4 - CC chemokine ligand 4 or macrophage inflammatory protein (MIP)-1β

CCL5 - CC chemokine ligand 5 or regulated on activation, normal T cell expressed and presumably secreted (RANTES)

CNS - central nervous system

COPD - chronic obstructive pulmonary disease

CPA - carboxypeptidase A

CpG - cytosine phosphate guanine

CR – complement receptor

CTMC - connective tissue-type mast cell

CXCL8 - CXC chemokine ligand 8 or interleukin (IL)-8

CXCL9 - CXC chemokine ligand 9 or monocyte induced by γ-interferon (MIG)

CXCL10 - CXC chemokine ligand 10 or interferon-inducible protein (IP)-10

DAPI - 4',6-diamidino-2-phenylindole

DC - dendritic cell

DIC - differential interference contrast

eIF2a - eukaryotic translation initiation factor 2a

eIF2AK2 - eukaryotic translation initiation factor 2a kinase 2

ER - endoplasmic reticulum

ERK1 - extracellular signal-regulated protein kinases 1

ERK2 - extracellular signal-regulated protein kinases 2

ET1 - endothelin-1

ETA - endothelin type A receptor

FcR - Fc receptors

FGF - fibroblast growth factor

FluA - influenza A virus

FMLP - formyl-methionyl-leucyl-phenylalanine

FSMC - fetal skin-derived cultured mast cells

GM-CSF - granulocyte-macrophage colony stimulating factor

H1- histamine receptor 1

H2 - histamine receptor 2

HA - hemagglutinin

HAU - hemagglutinating unit

HIV - human immunodeficiency virus

HMC - human mast cell line

HPIV - human parainfluenza viruses

HSP - heat shock protein

ICAM - inter-cellular adhesion molecule

IFN - interferon

IFNAR - IFNα receptor

IL - interleukin

IRF - interferon regulatory factor

ISG - IFN-stimulated genes

ISGF - ISG factor

LAD 1 - laboratory of allergic diseases mast cell line 1

LAD 2 - laboratory of allergic diseases mast cell line 2

LAIV - live attenuated influenza vaccine

LPS - lipopolysaccharide

LRTI - lower respiratory tract infection

LT - leukotriene

M1 - matrix protein 1

M2 - matrix protein 2

MCc - chymase single-positive mast cell

MC_T - tryptase-positive mast cell

MC_{TC} - tryptase/chymase-positive mast cell

MDCK - Madine-Darby Canine Kidney cell

MHC - major histocompatibility complex

MMC - mucosal mast cell

mMCP-1 - mouse mast cell chymase

MMP - matrix metalloproteinase

Mx1 - mouse homologue of human MxA

MxA - myxovirus resistance protein A

NA - neuraminidase

NEP - nuclear export protein

NES - nuclear export signal

NGF - nerve growth factor

NLS - nuclear localization signal

NO - nitric oxide

NOS - NO synthase

NOS1 - neuronal NOS or nNOS

NOS2 - inducible NOS or iNOS

NOS3 - endothelial NOS or eNOS

NP - nucleoprotein

NS1 - nonstructural protein 1

NS2 - nonstructural protein 2

OAS - 2’5’ oligoadenylate synthetase

OVA - ovalbumin

PA – acidic polymerase proteína

PAF - platelet activating factor

PAI-1 - plasminogen activator inhibitor-1

PAMP - pathogen-associated molecular pattern

PAR-2 - protease-activated receptor-2

PB1 - basic polymerase protein 1

PB2 - basic polymerase protein 2

PBS - phosphate buffered saline

PDGF - platelet-derived growth factor

PG - prostaglandin

PGN - peptidoglycan

PolyI:C - polyinosinic:polycytidylic acid

PKR - dsRNA protein kinase

PRR - pattern recognition receptors

PrX - malarial protein peroxiredoxin

Ps - polymerase proteins

PVDF - polyvinylidene fluoride

RBC - red blood cell

RBL - rat basophilic leukemia cell line

ROS - reactive oxygen species

RSV - respiratory syncytial virus

RT - room temperature

SCF - stem-cell factor

SDS - sodium dodecyl sulfate

SLPI - secretory leukocyte protease inhibitor

SP - substance P

ST - safratoxin

TGF-β - transforming growth factor-β

Th1 - T helper cell 1

Th2 - T helper cell 2

TIV - trivalent inactivated influenza vaccine

TLR - toll-like receptor

TNF - tumor necrosis factor

TSLP - thymic stromal lymphopoietin

TTBS – tween tris buffered saline

URTI - upper respiratory tract infections

VCAM - vascular cell adhesion molecule

VEGF - vascular endothelial growth factor

vRNP - viral ribonucleoprotein

Chapter I: Introduction

1. Mast Cells

Since human mast cells were used exclusively for the research in this thesis, the introduction will focus mainly on the biology of human mast cells, with information on rodent mast cells where relevant.

A. Mast Cell Biology

In 1878, Paul Ehrlich first described mast cells based on their unique staining properties and prominent granules (1). At that time, he named them "mastzellen", denoting well-fed cells, assuming incorrectly that the granules stored nutrients to nourish the surrounding tissues. Currently, mast cells are recognized as immune secretory cells that provide homeostatic functions in health and are also involved in various disease states as critical sentinel and effector cells (Figure 1.1) (2). Detailed information on mast cell biology, heterogeneity, functions in health and disease, role in innate and acquired immunity, and other topics are available in various reviews (3-12)

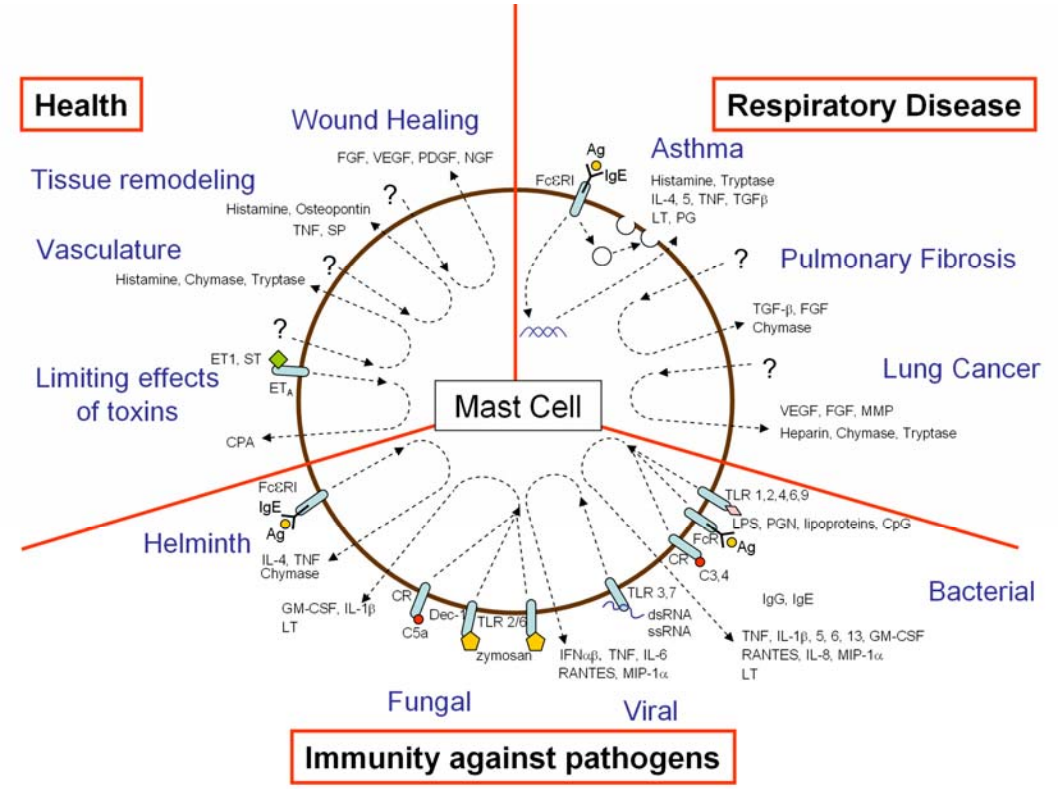

Figure 1.1: Mast cell responses in health, immunity and disease (original figure). Mast cell responses are divided according to their roles in health, respiratory disease, and immunity against pathogens. The mast cell mediators illustrated in this figure are not comprehensive, but instead demonstrate the ability of mast cells to respond in a specific and fine-tuned manner to different stimuli (please refer to text for more details and references). In health, mast cells contribute to wound healing, tissue remodeling, effects on vasculature, and limiting the effects of toxins. Mast cells secrete fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF), which have important effects in wound healing. In tissue remodeling, mast cell mediators such as tumor necrosis factor (TNF), substance P (SP), histamine, and osteopontin have potential effects in hair follicle recycling

and bone remodeling. Mast cells respond to the toxins endothelin-1 (ET1) and safratoxin (ST) through the endothelin type A (ET_A) receptor by producing carboxypeptidase A (CPA) to limit toxic effects. Mast cells are involved in respiratory diseases such as asthma, pulmonary fibrosis, and lung cancer. Mast cells recognize antigen-specific IgE through high-affinity FcεRI receptors. They respond to antigen-specific IgE by releasing histamine through degranulation and cytokines such as interleukin (IL)-4 and 13, TNF, as well as *de novo* synthesized leukotrienes (LT) and prostaglandins (PG). Mast cells contribute to tissue remodeling in asthma by releasing tryptase, transforming growth factor (TGF)-β, and TNF. Mast cell effects in pulmonary fibrosis could be due to the release of chymase and the fibrogenic cytokines TGF-β and FGF. Mast cells are implicated in promoting angiogenesis in lung cancer, possibly through the effects of VEGF, FGF, matrix metalloproteinases (MMP), and granule-associated heparin, tryptase, and chymase. Evidence for mast cells in immunity against pathogens includes their roles in helminth, fungal, viral, and bacterial infections. Helminth infections increase total and pathogen-specific IgE that activate mast cells through FcεRI receptors and induce release of IL-4, TNF, and chymase. Fungal infections activate mast cells through the fungal cell wall component zymosan by binding toll-like receptor (TLR)2/6, dectin-1, or through the release of complement 5a (C5a) activating complement receptors (CR). Mast cells respond to fungal stimuli by releasing granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1β, and LT. Viral stimuli such as single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) activate mast cells through TLR3 and 7 to release

interferon (IFN)α, β, TNF, IL-6, and the chemokines CCL5 (RANTES) and CCL3 (MIP-1α). Bacterial components activate mast cells through TLR, Fc receptors (FcR), and CR. In mast cells, bacterial lipopolysaccharide (LPS) activates TLR4, peptidoglycan (PGN) activates TLR2/6, bacterial lipoproteins activate TLR2/1, and unmethylated CpG DNA activates TLR9. Bacterial infections also result in the release of the complement components C3 and 4 that activate mast cell CR and the production of IgE and IgG, which acitvate mast cell FcR (FcεRI, FcγRI, II, and III). Mast cells release TNF, IL-1β, 5, 6, 13, GM-CSF, the chemokines CCL5, CXCL8 (IL-8) and CCL3, LT, and tryptase in response to bacteria-associated stimuli.

i. Origin and Differentiation

Mast cells and basophils are granulocytes that share the same origin with pluripotent CD34-positive hematopoietic stem cells from the bone marrow (13). Although mast cells and basophils have some common phenotypic and functional properties, they are distinct in their morphology, differentiation, location in the body, repertoire of mediators, and biological functions. Unlike basophils, mature mast cells do not circulate in the blood; instead, circulating CD34-positive hematopoietic progenitors migrate into peripheral tissues to differentiate into mature mast cells. Rodent mast cells require interleukin (IL)-3 as a growth factor for differentiation, whereas human mast cells depend predominantly on stem-cell factor (SCF, also known as Kit ligand and steel factor) as a determinant for

differentiation (14, 15). Accordingly, mature human lung mast cells do not express IL-3 receptors, but retain expression of c-Kit (CD117), the receptor for SCF (16). Anatomically, mast cells are located in vascularized tissues throughout the body, and are particularly abundant at sites that interface the environment, such as the skin and mucosal surfaces of the respiratory and gastrointestinal tracts. Mast cells can dwell in tissues for long periods, up to years, and reside close to blood vessels, nerves, epithelial cells, and mucous-producing glands.

ii. Morphology

Morphologically, human mast cells have a variable shape with a non-segmented nucleus that is round to oval in shape (13). Human mast cells exhibit long thin projections on the cell surface. In the cytoplasm, human mast cells contain numerous prominent granules, and within the granules are variable patterns of swirls, scrolls, and lattices that are evident under an electron microscope (17). The cytoplasm also contains mitochondria, rough endoplasmic reticulum, and electron-dense lipid bodies, with few visible ribosomes. Upon activation, mast cell degranulation occurs, with granules fusing to create convoluted degranulation channels that later fuse with the plasma membrane to release its granule contents (18). The morphology of mast cells is distinguishable from that of basophils, which show few short projections on their surface, a segmented nucleus, fewer and larger granules that lack the distinct patterns of mast cells, and granules that individually fuse with the plasma membrane (19).

iii. Activation and Mediators

Most well-known for their association with T-helper 2 (Th2) cells and IgE in allergy, mast cells are activated via crosslinking of IgE antibodies on FcεRI receptors on the cell surface (20). However, many other stimuli, such as IgG, complement, pathogens and their products, neuropeptides, cytokines, hormones, and venoms, are capable of activating mast cell (see table of mechanisms that activate mast cells in published review (4)). Mast cells respond in a fine-tuned manner depending on the type and strength of stimuli, and do not necessarily release their granule contents, but can release selected mediators through other pathways. Activation of high-affinity FcεRI receptors results in mast cell degranulation, a rapid release of preformed mediators stored in granules including: proteases such as tryptase, chymase, and carboxypeptidase, vasoactive amines such as histamine, and proteoglycans such as heparin (21). In addition to stored factors, mast cells also release *de novo*-synthesized eicosanoids generated from arachidonic acid metabolism, namely platelet-activating factor (PAF), leukotrienes (LTB_4 and LTC_4) and prostaglandins (PGD_2 and PGE_2) (22). Cytokines produced by mast cells include those that are classified as proinflammatory, immunomodulatory, Th2-associated, or Th1-associated (Table 1.1 and reviews (9, 21)). Also, mast cells release a variety of chemokines with chemotactic abilities to direct movement of immune cells and promote cell recruitment into tissues (23). As part of the innate immune system, mast cells can generate different antimicrobial products such as cathelicidin, an antimicrobial

peptide, or nitric oxide (NO) and reactive oxygen species (ROS) such as superoxide that have bactericidal and antiviral properties (24-26).

Mediators	Examples of function
Granule-associated	
Histamine, serotonin, chymase and tryptase	Alter vascular permeability
Heparin and/or chondroitin sulphate PGNs	Enhance chemokine and/or cytokine function and angiogenesis
Tryptase, chymase, carboxypeptidase and other proteases (MMP)	Remodel tissue and recruit effector cells
TNF, VEGF, PDGF, NGF and FGF2	Recruit effector cells, enhance angiogenesis and promote wound healing
Lipid-derived	
LTC_4, LTB_4, PGD_2 and PGE_2	Recruit effector cells, regulate immune responses, and promote angiogenesis, oedema and bronchoconstriction
Platelet-activating factor	Activates effector cells, enhances angiogenesis and induces physiological inflammation
Cytokine	
TNF, IL-1α, IL-1 β, IL-6, IL-18, GM-CSF, LIF, IFNα and IFNβ	Induce inflammation
IL-3, IL-4, IL-5, IL-9, IL-13, IL-15 and IL-16	Functions of T helper 2-type cytokines
IL-12 and IFNγ	Functions of T helper 1-type cytokines
IL-10, TGF-β and VEGF	Regulate inflammation and angiogenesis
Chemokine	
CCL2, CCL3, CCL4, CCL5, CCL11 and CCL20	Recruit effector cells, including dendritic cells, and regulate immune responses
CXCL1, CXCL2, CXCL8, CXCL9, CXCL10 and CXCL11	Recruit effector cells and regulate immune responses
Other	
Nitric oxide and superoxide radicals	Bactericidal
Antimicrobial peptides (Cathelicidin)	Bactericidal
Osteopontin	Tissue remodeling

Table 1.1 Mediators secreted from mast cells and examples of mediator functions. Modified from Table 1 from reference (9).

iv. Heterogeneity

Mast cells represent a heterogeneous population that differs in morphology, granule contents, and functional responses to various stimuli. There are variations in mast cell phenotype depending on factors such as species, age, anatomical site, and microenvironmental conditions during development or residence in tissues (27). It is hypothesized that this heterogeneity allows distinct populations of mast cells to function with diverse and malleable responses depending on the requirements of the microenvironment (21).

- ***Classification of Murine Mast Cells***

In rodents, mast cells are classified as mucosal mast cells (MMC) or connective tissue-type mast cells (CTMC), and can be differentiated using the Alcian blue/safranin stain (28). MMC granules contain chondroitin sulphate that stains with Alcian blue, while CTMC granules contain heparin that stains with safranin. Other characteristics such as protease content, arachidonic acid metabolism, and response to the secretagogue compound 48/80, also differ between MMC and CTMC (27).

- ***Classification of Human Mast Cells***

Although human mast cells cannot be easily discriminated based on staining with dyes, immunohistochemical staining of granule proteases distinguishes two subsets, tryptase-positive mast cells (MC_T) and

tryptase/chymase-positive mast cells (MC_{TC}), which are analogous to MMC and CTMC, respectively (29). Although human mast cells were originally classified into the two subsets, chymase single-positive mast cells (MCc) were later detected in human tissues (30). MC_T are found in high proportions in the lungs and small intestinal mucosa while MC_{TC} predominate in the skin and submucosa (31). Functionally, compound 48/80, morphine, and substance P (SP) elicit a degranulation response from skin mast cells of the MC_{TC} phenotype; in the same study, lung mast cells of the MC_T phenotype do not respond to these stimuli (32). The occasional MC_C exists in the lungs and gastrointestinal tract, with the largest numbers of MC_C in the intestinal submucosa (30).

- ***Human Lung Mast Cell Heterogeneity***

As the focus of this PhD thesis involves research on a human respiratory pathogen, heterogeneity within human lung mast cells is particularly relevant. The classification of MC_T as mucosal mast cells and MC_{TC} as connective tissue mast cells has placed human lung mast cells in the MC_T category, and indeed, lung mast cells demonstrate a predominantly MC_T phenotype. However, both MC_T and MC_{TC} exist in all compartments of the lungs, indicating that this classification does not adequately define the complexity of mast cell heterogeneity (33). In addition, the proportion and distribution of the two mast cell subtypes changes in disease states such as asthma, implying that important phenotypic changes occur with different local microenvironments (33). A recent study examined the morphometric and molecular characteristics of mast cell populations from

bronchial and transbronchial biopsies of healthy human lungs using immunohistochemistry (34). In this study, they found that while both MC_T and MC_{TC} exist in all compartments of the lungs, MC_T predominate the bronchi, bronchioles, and alveolar parenchyma, and MC_{TC} predominate the pulmonary vessels and pleura. Additionally, each mast cell subset can be further distinguished within the specific compartments based on size, and expression of IgE receptor, IL-9 receptor, renin, histidine decarboxylase, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), 5-lipoxygenase, and LTC_4 synthase. For example, MC_{TC} were larger while MC_T were smaller in pulmonary vessels than small airway walls. Also, MC_T expressed more histidine decarboxylase in the bronchi than the alveoli. Thus, when building conceptual models of how human lung mast cells react to a particular stimulus, it is important to consider responses of MC_T and other subtypes. It is critical to understand that complex mast cell heterogeneity exists within the lungs and that characterization of site-specific populations of mast cells may be warranted when exploring the role of mast cells in pulmonary diseases.

iv. Tools to Study Mast Cell Biology

As mast cells have roles in many diverse physiological and pathophysiological responses, it is important to study the function of mast cells in context with the biological process under investigation. Since human mast cells reside within tissues, it is difficult to obtain sufficient human tissue mast cells for studies *in vitro*. Thus, numerous approaches have been developed in both human

and murine models to obtain both *in vitro* and *in vivo* data that when taken together, provide a more detailed picture of mast cell function. A recent review summarizes the various tools used to study mast cells and differences between data obtained from murine and human models (35).

- ***Murine Mast Cells***

Mast cells from rats and mice have been used extensively to study mast cell function because of easy accessibility and the numbers of cells that can be harvested. Although murine peritoneal mast cells yield large quantities of mast cells that are easily purified, the relevance of this cell type is questionable since humans normally have few mast cells in the peritoneal cavity (36). Since the use of primary mast cells from murine tissues is limited by low yields, generation of mast cells by culturing progenitors from the bone marrow of mice with IL-3 (bone marrow-derived mast cells, BMMC) is the preferred method for studying murine mast cells (37). The transformed rat basophilic leukemia cell line RBL-2H3 has also been used as an analogous cell type to study mast cell function; however, like all transformed cells, RBL-2H3 have markedly altered responses compared to primary cells.

- ***Mast Cell-Deficient Mice***

Mice provide a relatively simple tool to study biological functions *in vivo*. The c-kit mutant mice WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ (W/W^v) and C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$

(W^{sh}/W^{sh}) are the most widely used models and both exhibit an almost complete lack of mast cells (38, 39). However, the c-kit mutation in the W/W^{v} mice also results in infertility and phenotypic abnormalities such as anemia, neutropenia, decrease in the number of interstitial cells of Cajal in the gastrointestinal tract, and a lack of cutaneous melanocytes (27). The W^{sh}/W^{sh} mice are becoming increasingly popular because they demonstrate milder abnormalities compared with W/W^{v} mice, as W^{sh}/W^{sh} mice are not sterile, anemic or neutropenic (39). Both W/W^{v} and W^{sh}/W^{sh} mice can be reconstituted with wildtype or mutant mast cells in "knock-in" models to assess the extent to which differences in the c-kit mutants can be attributed to the mast cell deficiency (39, 40).

- ***Human Mast Cell Lines***

Although results from murine studies provide a tremendous wealth of information on mast cell biology, especially in *in vivo* settings, these results need to be confirmed in human cells since many functional differences are present between species. Overall, it is important not to generalize results from one particular mast cell source to responses of mast cells as a whole since heterogeneity exists among mast cells from different species, anatomical sites, and maturation stages. There are many tools to study human mast cells, all with certain limitations. The human mast cell line HMC-1, first developed from a leukemia patient, has a c-kit mutation that makes the receptor active in the absence of SCF (41). HMC-1 are immature mast cells that do not express FcεRI receptors and are not heavily granulated, thus making it a difficult model to

properly study allergic responses (42). The laboratory of allergic diseases (LAD)-1 and -2 mast cell lines were established from bone marrow aspirates of a mast cell sarcoma-leukemia patient. LAD1 and 2 do not have c-kit mutations and closely resemble primary cultured mast cells in that they require SCF for survival, are heavily granulated, express FcεRI, and demonstrate IgE-dependent degranulation. Since LAD cell lines persist in long term culture, this provides an invaluable mast cell source with similar characteristics to primary cultured mast cells. However, LAD cells grow much slower than HMC-1 and require SCF-supplemented media, making it difficult and expensive to maintain large numbers of LAD cells for experimentation.

- ***Primary Human Mast Cell***

Prior to the discovery of the LAD cell lines, it was established that CD34-positive progenitors from human peripheral blood and umbilical cord blood can be cultured into human mast cells in the presence of SCF (43, 44). Although primary cultured cells are preferred over cell lines to study mast cell biology, current culture protocols are unable to achieve full mast cell maturation as determined by functional studies and expression of cell surface markers (34, 45, 46). Other drawbacks are the time and cost of culturing progenitors for 6-12 weeks with the addition of a cocktail of cytokines, only to attain small numbers of mast cells per culture. Human mast cells can also be isolated from tissues and cultured for up to 3 months in media supplemented with SCF (47). Although these cells are highly desirable since they reflect *in vivo* mast cells, this approach is

hampered by laborious isolation and purification procedures that require the availability of fresh tissue specimens and yield only $10^4 – 10^5$ mast cells per gram of tissue (48). Moreover, it is critical to carefully assess the effects of the isolation procedure on the characteristics of mast cells, particularly if cells are used immediately after isolation.

Finally, human mast cells can be examined *in vivo* by measuring mast cell mediators in bodily fluids, histology and immunohistochemistry of tissue biopsies, and determining the physiological effects of drugs with specificity for mast cells. However, these techniques are unable to provide the detailed information on mast cell function and mechanisms that some other mast cell models offer.

B. Mast Cells in Respiratory Disease

As the research in this thesis pertains to a respiratory virus, the evidence for mast cell involvement specifically in respiratory diseases will be discussed.

i. Asthma

Mast cells are best known for their role as key effector cells in asthma, a multigenic and heterogeneous allergic disorder of the airways. Asthma is an inflammatory illness characterized by variable constriction of the airways, airway hyperresponsiveness (AHR), and symptoms of dyspnea, chest tightness, wheezing, and cough. Evaluation of mast cell distribution revealed that mast cell numbers are increased near smooth muscle cells of both small and large airways in

asthmatic patients (49). Also, mast cell degranulation correlates with asthma severity (50). Chymase-positive mast cells in the small airways correlates with improved lung function in severe asthma patients (33). Mast cells are associated with both atopic and non-atopic asthma, where atopy is defined in patients with a positive skin prick test to common allergens and characterized by elevated serum levels of total and allergen-specific IgE (51). In a murine model of non-atopic asthma, AHR of mast cell-deficient mice was decreased compared with control mice (52). Further investigation suggests that immunoglobulin (Ig) free light chains could be responsible for activation of mast cells in non-atopic asthma (53).

In classical atopic asthma, individuals exposed previously to an allergen are sensitized to develop an IgE-dependent allergic response upon secondary challenge. Crosslinking of allergen-bound IgE molecules on FcεRI receptors causes mast cells to release a variety of preformed and newly synthesized mediators. Histamine from granules and newly formed eicosanoids (LTC4, LTB4, and PGD2) cause potent smooth muscle contraction resulting in bronchoconstriction in the allergic response (3). Mast cell mediators have pro-inflammatory effects in the lungs, including the recruitment of immune cells through release of chemokines and the upregulation of adhesion molecules from the vascular endothelium, activation of the epithelium, increase in vascular permeability, and increase in mucous production. For example, mast cells produce the key Th2 cytokines IL-4 and IL-5, which are important for differentiation of T-cells to a Th2 phenotype, and recruitment and maturation of eosinophils, respectively (54). In an allergic response, mast cells also release tumor necrosis

factor (TNF), a pro-inflammatory cytokine with effects on cell recruitment and the vasculature (55). In addition, mast cells contribute to the tissue remodeling and fibrosis seen in asthmatic individuals by releasing tryptase, transforming growth factor (TGF)-β, and TNF, which induce fibroblast proliferation and collagen deposition (56). Mast cell responses are subject to influence by various factors in the microenvironment, and an ability to regulate mast cell responses in patients would appear to be another step towards the effective treatment of asthma.

The vast majority of studies in various model systems reveal that IgE-associated biological responses are mast cell-dependent. In a mouse model of asthma, wildtype mice develop AHR to ovalbumin (OVA) challenge, but mast cell-deficient W/W^v mice fail to develop AHR (57). This study also showed that W/W^v mice reconstituted with BMMC from wildtype mice are capable of developing AHR, indicating that mast cells are essential for this response. Another study showed that while W/W^v mice showed reduced AHR and numbers of lung eosinophils in an adjuvant-free asthma model, these mice had similar responses to wildtype mice when adjuvant was used in sensitization, i.e. mast cell-independent asthma (58). Thus, it is important to recognize that different results can be obtained between different mouse asthma models and to take this into consideration when evaluating the role of mast cells in asthma.

ii. Pulmonary Fibrosis

Pulmonary fibrosis is a chronic disease characterized by inflammation and scarring of the alveoli and interstitial tissues of the lungs. Although the evidence

for mast cells in the development of pulmonary fibrosis is unclear, increased numbers of mast cells were seen in the lungs of pulmonary fibrosis patients (59). In this study, mast cells are present near abnormal epithelial cells with mesenchymal features and accumulate in areas where alveolar septa have thickened. Also, mast cells from fibrotic lungs demonstrate morphological changes compared to normal lungs, with reduced granule numbers and more disorganized granules. Rats with bleomycin-induced pulmonary fibrosis demonstrated hyperplasia of connective tissue type mast cells in the lungs parenchyma and increased histamine levels; in contrast, normal rats had higher densities of both mucosal and connective tissue type mast cells in the trachea and lower densities of only connective tissue type mast cells in the parenchyma (60). In mice with bleomycin-induced pulmonary fibrosis, mast cell chymase was shown to be important for the pathogenesis of fibrosis through the activation of TGF-β (61). Activation of mast cells can result in the release of basic fibroblast growth factor (FGF) that is mitogenic for fibroblasts and TGF-β that induces the deposition of extracellular matrix proteins (62, 63). Also, mast cell histamine induces proliferation of human adult lung fibroblasts, which could contribute to fibrosis (64). The role of mast cells in pulmonary fibrosis still needs to be clarified, but the evidence suggests a potential involvement of lung mast cells in this inflammatory disease.

iii. Lung Cancer

Mast cells are associated with angiogenesis in a variety of malignancies, including lung adenocarcinoma, which demonstrates a higher number of vessels in the tumors compared with other lung cancers such as squamous cell carcinoma (65). High mast cell numbers are correlated with a worse prognosis in patients with lung adenocarcinomas, suggesting a detrimental role for mast cells in this type of malignancy (66). For tumors to grow, angiogenesis must be induced to create a new blood supply to the tumor tissues. Mast cells are suggested to play a role in tumor angiogenesis as they accumulate in areas of angiogenesis and secrete various products that promote angiogenesis. Heparin from mast cells is suggested to induce vascularization through stimulation of endothelial cell (EC) migration to form new blood vessels (67). Mast cell tryptase stimulates vessel tube formation and enhances the growth of microvascular EC, and mast cell chymase promotes angiogenesis through the effects of angiotensin II (AngII) (68, 69). Mast cells are a source of FGF, VEGF, and matrix metalloproteinases (MMP), which all have angiogenic effects (70). Also, the mast cell-deficient W/W^v mice demonstrate decreased angiogenesis at tumor sites and decreased rates of hematogenous metastasis compared with wildtype mice (71). Taken together, mast cells appear to be important in the development of certain lung cancers through their involvement in tumor angiogenesis. However, increased mast cell numbers in tumors of non-small cell lung cancer have also been associated with enhanced survival of patients (72). Although mast cells may have a protective role

in certain lung cancers, whether mast cells are directly involved in killing tumor cells needs to be investigated.

C. Mast Cells in Health

Although the role of mast cells in immune defense relates to the maintenance of health, the topic of mast cells in innate immunity will be discussed in section D. The physiological roles of mast cells are shown in Figure 1.2.

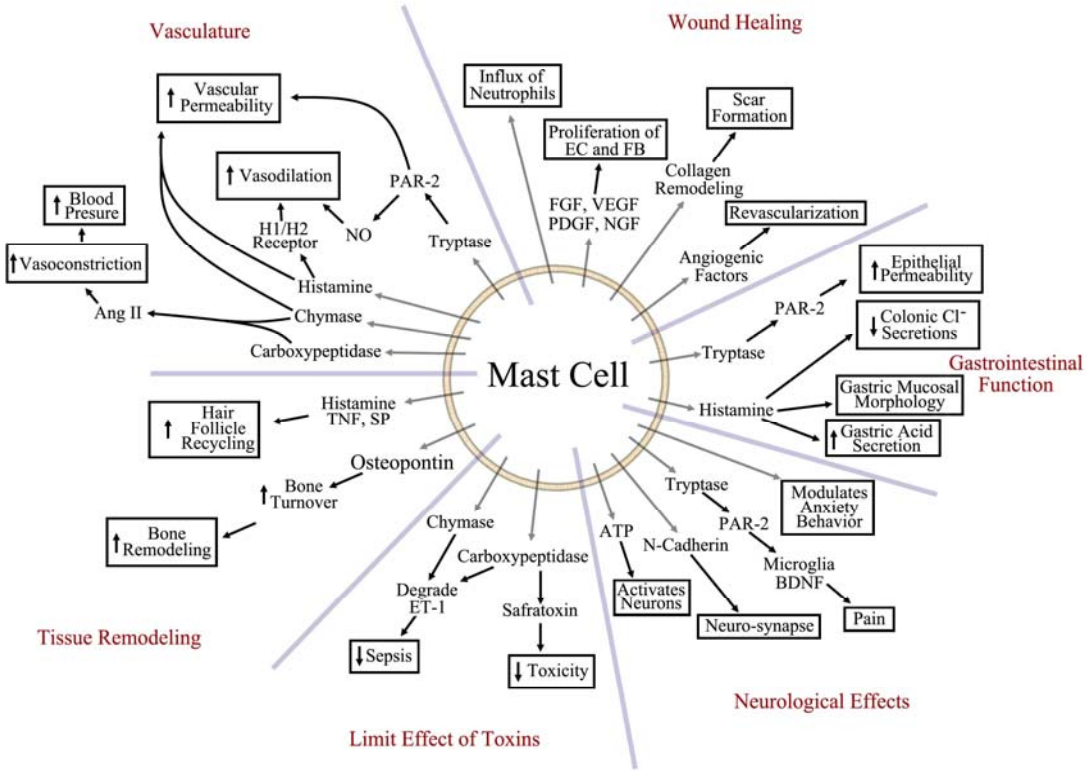

Figure 1.2: Physiological roles of mast cells (original figure). Six major physiological processes are illustrated (underlined). Vasculature - Mast cell tryptase activates PAR-2 and subsequent nitric oxide (NO) production; PAR-2 increases vascular permeability whereas NO increases vasodilatation. Mast cell

histamine acts on histamine 1 and 2 (H1/H2) receptors leading to vasodilatation. Both histamine and chymase increase vascular permeability. Chymase and carboxypeptidase activate angiotensin II (Ang II) to cause vasoconstriction and increased blood pressure. Wound Healing – Mast cells are involved in wound healing by promoting the influx of neutrophils. Mast cells induce the release of FGF,VEGF, PDGF and NGF, which have effects on epithelial cell and fibroblast proliferation. Mast cell involvement in collagen remodeling, scar formation and release of various angiogenic factors that induce revascularization all contribute to wound healing. Gastrointestinal Function – Mast cell tryptase increases epithelial permeability through activation of PAR-2. Histamine causes a decrease in colonic chloride secretions, change the morphology of gastric mucosa and increase gastric acid secretion. Neurological Effects - Mast cells modulate anxiety responses in studies of mast-cell deficient mice. The tryptase/PAR-2 axis is involved in pain elicitation by inducing release of microglia brain-derived neurotrophic factor (BDNF). Mast cells interact with neurons through homophilic N-cadherin interactions to form neuro-synapses. Mast cell-derived ATP results in activation of neurons. Limit Effect of Toxins - Both chymase and carboxypeptidase degrade endothelin-1 (ET-1), which results in a reduction of sepsis. Carboxypeptidase decreases the toxicity of ST protein through direct degradation. Tissue Remodeling - Mast cells stimulate hair follicle recycling through the release of histamine, TNF and SP. Mast cells also stimulate bone remodeling through the release of osteopontin, which increases bone turnover.

i. Effects on Vasculature

Mast cell mediators, including tryptase, chymase, and histamine, have important effects on the vasculature such as increasing vascular permeability and vasodilatation. Human tryptase increases vascular permeability through cleavage and activation of protease-activated receptor (PAR)-2 expressed on endothelial cells (73). Tryptase also causes vasodilatation of the aorta in the rat, likely through a PAR-2 and NO-dependent pathway (74). Chymase and histamine are both capable of increasing microvascular permeability in the skin, with histamine having more potent effects (75). Histamine induces vasodilatation through activation of both H1 and H2 receptors on endothelial cells (76). Interestingly, mast cell chymase and carboxypeptidase A both promote the generation of AngII, a potent vasoconstrictor that increases systemic blood pressure in humans (77).

ii. Gastrointestinal Function

The intestinal mucosa is an important barrier that is tightly regulated to allow absorption of important nutrients while preventing the entry of infectious and toxic agents. Mast cells are not only important in host defense of the intestine, but also regulate functions of various cells in the gut to maintain a homeostatic equilibrium. For example, mast cells and histamine are involved in the physiological chloride secretion of human colonic mucosa mediated by SP (78). Mast cell tryptase regulates the permeability of the epithelial barrier by activating PAR-2 through a beta-arrestin and ERK1/2-dependent pathway (79). Also, studies

show that histamine is important in secretion of gastric acid as well as maintenance of the morphology of gastric mucosa (80, 81).

iii. Neurological Effects

Research on neuroimmune interactions reveal the presence of bidirectional signals between mast cells and neurons. The direct effect of mast cells on the nervous system was demonstrated by the ability of mast cells, by releasing ATP, to activate calcium signaling in neuronal projections (82). Mast cells and nerves have been shown to form a special synapse, though the homophilic interaction of the adhesion molecule N-cadherin (83). The presence of mast cells in brain areas involved in behavior, such as the thalamus and hippocampus, prompted a study that showed an involvement of mast cells in modulating anxiety behavior using mast cell-deficient mice (84). Mast cells are known to be related to pain perception in situations such as migraine headaches and cystitis pain (85, 86). A recent study showed that activation of PAR-2 by mast cell-derived tryptase induces microglia secretion of BDNF, which is involved in pain elicitation (87).

iv. Wound Healing

Mast cells demonstrate diverse functions in regulating health that extend beyond their involvement in disease pathogenesis. One of these functions is the involvement of mast cells in wound healing. Mast cells play a role in all three stages of wound healing: the acute inflammatory phase, the proliferative phase,

and the remodeling phase (88). In the acute phase, mast cells promote the influx of immune cells to the site of injury as demonstrated by lower neutrophil counts in the wounds of mast cell-deficient W/W^v mice (89, 90). Re-epithelialization and angiogenesis are typical features of the proliferative phase. As discussed in the section B.iii on mast cells in lung cancer, mast cells release many angiogenic factors, which have to ability to induce revascularization of damaged tissues. Mast cells also produce various mediators that induce proliferation of epithelial cells and fibroblasts, including FGF, VEGF, platelet-derived growth factor (PDGF), and nerve growth factor (NGF) (2). Mast cells are required in proper wound healing, as W/W^v mice had fewer proliferating keratinocytes in the wounds and took longer time for the wound to close (90). As the fibroblasts expand in the proliferative phase, they deposit collagen and other extracellular matrix proteins and remodel these proteins into scar tissue. Evidence suggests that mast cells are also involved in collagen remodeling and scar formation with less tightly interwoven collagen in mast cell-deficient mice (91). Also, increased mast cell numbers are associated with hypertrophic scars in humans (92).

v. Tissue Remodeling

Although mast cells contribute to airway remodeling that is detrimental in asthma, mast cells are also involved in physiological processes of remodeling such as hair follicle recycling and bone remodeling. Mast cell-deficient mice demonstrate impaired hair follicle recycling, where hair growth and regression continuously occur during periods of growth and rest (93). Mast cell histamine,

TNF, and SP are thought to contribute to the remodeling events such as that of hair follicle recycling (94). Evidence for mast cells in bone remodeling shows that mast cell-deficient mice have more fragile bones, with femurs that are lighter and thinner than wildtype mice, although these results need to be confirmed with mast cell-reconstituted mice (95). In mastocytosis, where there is an increase in mast cell proliferation, bone turnover is accelerated, resulting in enhanced bone loss (96). Mast cells are a source of osteopontin, a glycoprotein component of bone extracellular matrix that contributes to bone resorption and calcification, suggesting a potential mechanism for mast cells in bone remodeling (97).

vi. Inhibition of Toxins

Mast cells are involved in a variety of homeostatic mechanisms, one of which involves the degradation of toxins in the host. Endothelin-1 (ET-1) is a potent vasoconstrictor produced endogenously that mediates the toxic effects of sepsis, as demonstrated in a cecal ligation and puncture model in mice (98). When activated by ET-1, mast cells limit the toxicity of ET-1 by releasing chymase and carboxypeptidase, which degrade this molecule (98, 99). Mast cells are also protective against the deadly effects of safratoxin (ST) 6b, the most toxic component of the venom from the snake *Atractaspis engaddensis*, which is highly homologous in amino acid sequence to ET-1 (100). The protective effects of mast cells were also shown for resistance against other snake venoms as well as a honeybee venom (94). These detoxifying effects of mast cells can be attributed to

its proteases, particularly carboxypeptidase A, by removal of a single amino acid from the toxin (101).

D. Mast Cells in Innate and Acquired Immunity Against Pathogens

S. Wasserman postulated in 1980 that mast cells could be involved in pulmonary defenses due to their strategic location at "portals of entry" to act as sentinels poised to respond rapidly to insults by mediating a local inflammatory response (102). Equipped with a large inventory of mediators, mast cells respond to different pathogens by selectively releasing mediators at varying durations and doses. When activated by pathogens or pathogen products, mast cells secrete granule-associated preformed mediators, *de novo* synthesized lipid mediators, and a spectrum of cytokines and chemokines that enhance vascular permeability, recruit effector cells, and mediate acquired immunity. Although mast cells have been most extensively studied in the context of allergic diseases, a role for mast cells in innate and acquired immunity against fungal, bacterial, and viral pathogens has more recently been established (9). As the subject of this PhD thesis focuses on the innate response of mast cells to influenza A virus, the section on mechanisms of mast cells in innate immunity, with a special focus on toll-like receptors, and the section on mast cells in viral infections will be discussed in the greatest detail.

i. Mechanisms in Innate Immunity

As the innate immune system is the first line of defense against pathogens, tissue-resident mast cells are in prime locations to detect and initiate responses against invading microbes. Various pathogens and their products activate mast cells through different receptor systems, demonstrating mechanisms for mast cells to directly react to pathogenic stimuli.

- ***Toll-Like Receptors***

Much progress in understanding mechanisms of the innate immune system has come from the work on toll-like receptors (TLR), a subset of pattern recognition receptors (PRR) that recognize pathogen-associated molecular patterns (PAMP). Different TLR recognize specific PAMP such as components of yeast cell wall, bacterial cell wall, and viral RNA. Humans express TLR 1-10 either on the cell surface or in endosomes, which recognize different pathogen motifs (103). Human mast cells express all TLR except TLR10 and have been shown to respond to the ligands of most of these receptors (104, 105).

Most extensively studied is the activation of TLR4 by its classical ligand bacterial lipopolysaccharide (LPS), although TLR4 on mast cells can also be activated by the malarial protein peroxiredoxin (Prx), the extra domain A of fibronectin, and heat shock protein (HSP) 70 (106-108). In contrast to monocytic cells, mast cells do not express the TLR4 coreceptor CD14 and thus require exogenous soluble CD14 for effective LPS responses (109). Although LPS does not induce mast cell degranulation, LPS activates mast cells to release various

mediators (110). LPS activates murine mast cells to release the proinflammatory cytokines TNF and IL-6 through TLR4 (111, 112). However, human mast cells require priming with interferon (IFN)γ or IL-4, which upregulate TLR4 expression, for these cytokine responses (109, 113). In addition to proinflammatory cytokines, a study using oligonucleotide gene chip arrays showed increases in mast cell expression of Th2 cytokines such as IL-5 and IL-13, and chemokines such as CXCL8 (IL-8) and CCL5 (RANTES) (113). The role of TLR4 *in vivo* was demonstrated in a mast cell "knock-in" model where W/W^v mice reconstituted with TLR4-mutated BMMC had higher mortality than mice reconstituted with wildtype BMMC (112).

TLR2 forms functional heterodimers with TLR1 and TLR6, with the suggestion that the TLR2/TLR1 complex responds to bacterial lipoproteins such as Pam_3CSK_4, while the TLR2/TLR6 complex responds to the gram-positive bacteria cell component PGN and the yeast cell wall component zymosan (114, 115). PGN has been the most extensively studied activator of TLR2 on both human and murine mast cells. In contrast to responses to LPS, activation of TLR2 in human mast cells by PGN results in degranulation and does not require priming with cytokines or soluble CD14 (109). Similar to LPS, PGN activates mast cells to release TNF, IL-5, and IL-13; however, unlike LPS, PGN and zymosan both induce mast cell release of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, and LTC_4 (116).

Mast cells also respond to viral-associated ligands that activate TLR. TLR3 recognizes double-stranded RNA (dsRNA) that exist as the genome of

dsRNA viruses and as replicative intermediates of single-stranded RNA (ssRNA) viruses (117). The synthetic dsRNA polyinosinic-polycytidylic acid (polyI:C) has been used extensively to study TLR3 activation. PolyI:C induces human mast cell production of IFNα and IFNβ, two cytokines important for activating numerous antiviral genes (104). W/W^v mice injected in the peritoneal cavity with polyI:C had reduced peritoneal recruitment of $CD8^+$ T-cells, revealing a role for mast cells in regulating $CD8^+$ T-cell migration through TLR3 activation (118). TLR7 responds to ssRNA sequences that occur during infection with RNA viruses and can be activated by the synthetic ligand imiquimod (119). Using W^{sh}/W^{sh} mice in a mast cell knock-in model, induction of early cutaneous inflammation and migration of Langerhans cells by imiquimod was delayed in mast cell-deficient mice but fully recovered in mast cell-reconstituted mice, demonstrating that mast cells contribute to early inflammatory responses induced by a TLR7 agonist (120). Unmethylated CpG motifs of bacterial DNA activate TLR9 and human mast cells have been shown to release TNF, IL-1β, and LT in response to CpG (104). Also, TLR3, 7, and 9 agonists are all capable of inducing release of TNF, IL-6, and chemokines such as CCL5 and CCL3 (MIP-1α) from murine fetal skin-derived cultured mast cells (FSMC) (121). Although some types of mast cells express TLR5, no studies have shown direct activation of this receptor in mast cells.

- ***Complement Receptors***

The complement pathways are activated during microbial infection or tissue injury to produce complement peptide fragments that activate receptors on

various innate immune cells. Mast cells express the complement receptors CR2-5, C3aR, and C5aR (9, 122). Both C3a and C5a are chemotactic for mast cells and are capable of activating mast cells to release histamine (123, 124). Also, C3a induces mast cell production of chemokines CCL2 (MCP-1) and CCL5 (125). When subjected to cecal ligation and puncture, a mast cell-dependent model of bacterial peritonitis, both C3- and C4-deficient mice exhibited higher mortality, revealing a potential for complement components to activate mast cells *in vivo* (126). In a recent study, zymosan-induced peritonitis was shown to involve mast cells through a C5aR-dependent mechanism using mast cell-deficient mice (127). Taken together, complement recognition provides another mechanism for mast cells to respond to pathogens.

- ***Fc Receptors Activated by Ig-Binding Proteins***

In addition to the high-affinity FcεRI, mast cells express other Fc receptors such as FcγRI, II, and III (9). The activation of Fc receptors through binding antigen-specific antibodies will be discussed in the section on mechanisms of mast cells in acquired immunity. With regards to innate immunity, Fc receptors on mast cells can be activated by Ig-binding proteins (superantigens) in an antigen-independent manner. Both *Staphylococcus aureus*-derived protein A and *Peptostreptococcus magnus*-derived protein L activate mast cells through binding IgG and/or IgE, resulting in the release of histamine, tryptase, and LTC_4

(128). HIV glycoprotein 120 (gp120) binds IgE to stimulate mast cell secretion of IL-4 and IL-13 (129).

- ***Other Innate Receptor Systems***

CD48 is a glycosylphosphatidylinositol-anchored protein that most likely acts as a coreceptor to detect mannose-binding proteins derived from pathogens, since CD48 by itself is unable to signal. Mast cells express CD48 and studies have shown the activation of mast cells by mannose binding proteins, such as FimH protein from *Escherichia coli* and Sm60 protein from *Schistosoma mansoni* (130, 131). Release of histamine from mast cells induced by *Mycobacterium tuberculosis* is also dependent on CD48 interaction (132). Another mast cell innate receptor is the FMLP receptor, capable of binding the gp41 peptides of HIV for activation (133). Meanwhile, dectin-1 mediates mast cell secretion of LTB_4 and LTC_4 when activated by the TLR2 ligand zymosan; however, dectin-1 does not mediate responses to another TLR2 ligand PGN (134). The studies were further extended to demonstrate that zymosan acts through dectin-1, but not TLR2, to induce mast cell generation of intracellular ROS (135).

ii. Mechanisms in Acquired Immunity

Mast cells are well-known to act as effector cells in Th2-mediated responses both in asthma and against helminth infections. In addition, the innate and acquired immune responses are linked by pathogen-activated mast cells that

promote the development of specific immunity by presenting antigens and influencing the responses of dendritic cells, T cells and B cells.

- ***Effector Cells***

In a host sensitized to certain helminths, mast cells are activated in a secondary infection by parasite-specific IgE that binds to cell surface FcεRI. As effector cells in the protective Th2 response, mast cells secrete chymase and many other mediators, which contribute to the inflammatory response and expulsion of helminths (136, 137). In a mouse mast cell knock-in study of helminth infection, mast cell production of IL-4 and TNF were required to efficiently expel the parasite (138). The Th2 cytokine IL-4 promotes parasite expulsion in the gut by inducing mucous hypersecretion, altering epithelial function and increasing gut motility (139). TNF could potentially contribute to the inflammatory response against helminths by recruiting immune cells through increasing vascular permeability and upregulating adhesion molecules on the vascular endothelium. The evidence for mast cells in specific helminth infections will be discussed below.

- ***Antigen Presentation***

Major histocompatibility complex (MHC) molecules on antigen presenting cells (APC) directly interact with T cell receptors (TCR) to initiate antigen-specific T cell expansion. Mast cells constitutively express MHC class I (MHCI)

and upregulate expression of MHC class II (MHCII) when stimulated with IFNγ, TNF, or LPS (140). Also, costimulatory molecules CD28, CD80, CD86, ICAM-1 and OX40 ligand (OX40L) that act on T cells, and CD40 ligand (CD40L) that acts on B cells are expressed on mast cells and in mast cell-derived exosomes (10, 140). CD80 and CD86 expression in mast cells can be upregulated by GM-CSF while CD28 and CD80 are upregulated by the TLR3 agonist polyI:C (118, 141). Interestingly, human tonsil mast cells that induce T cell proliferation through the OX40L-OX40 interaction also colocalize with T cells in human tonsils, while human lung mast cells do not express OX40L or colocalize with T cells (142). This provides evidence that mast cells may serve as APC in humans *in vivo*.

- ***Effects on Dendritic Cells, T Cells, and B Cells***

Dendritic cells (DC) are regarded as probably the most important APC due to their broad range and potency of antigen presentation. Resting immature DC in peripheral tissues have limited capacity to present antigens to T cells. Upon proper stimulation in the microenvironment, however, DC migrate to draining lymph nodes where they upregulate the expression of MHCII and costimulatory molecules. Various mast cell products enhance DC migration and functional maturation. DC migration is increased in response to TNF, IL-1, IL-16, IL-18, CCL5, and PG_{E2}, all mast cell secreted products (10). In addition, mast cell exosomes upregulate DC expression of MHCII and costimulatory molecules that correspond to a mature DC phenotype (143). Other mast cell mediators such as histamine, IL-17E, and thymic stromal lymphopoietin (TSLP) have also been

implicated as promoters of DC maturation (10). Both IgE and PGN stimulated mast cells facilitate the migration of DC to draining lymph nodes in mice, but migration of DC in response to LPS injection is not mast cell-dependent (144, 145). Effects of mast cells on DC in response to different pathogenic stimuli require further investigation.

Little direct evidence exists for the role of mast cells in mediating T cell responses in the context of microbial infections. However, mast cells have the capacity to influence T cell responses through the release of various chemotactic factors for T cells, upregulating adhesion molecules on the vascular endothelium for T cell recruitment, and releasing factors that affect T cell differentiation into Th1 or Th2 cells. When activated by IFNα, mast cells release CXCL10 (IP-10), while activation by SCF or TNF results in mast cell release of CCL5; both these chemokines are migratory for T cells (146, 147). Mast cell-derived TNF induces epithelial cell expression of the intracellular and vascular adhesion molecules, ICAM-1 and VCAM-1, and mediates T cell binding to epithelial cells (148). While mast cell histamine can polarize T cells to a Th1 phenotype through H1 receptors, another study has shown that histamine acts on H2 receptors of DC to induce T cell polarization towards a Th2 phenotype (57, 149). These contrasting results demonstrate potential differences in mast cell mediation of the Th1/Th2 balance depending on the cytokine milieu and cellular interactions present in the microenvironment. Also, a study demonstrated that mast cell were responsible for $CD8^+$ T cell recruitment to the peritoneal cavity after injection of mice with the TLR3 agonist polyI:C (118).

Most of the evidence for mast cell-B cell interactions involves the effect of mast cells on B cell isotype switching to IgE. Human lung mast cells express CD40L, which binds to CD40 on B cells to induce IgE synthesis in the presence of IL-4 (150). Also, B cells stimulated with LPS and IL-4 showed enhanced production of IgE and IgG1 when exposed to rat mast cell chymase (151). However, there is currently no evidence for the involvement of mast cells in mediating B cell antigen-specific antibody responses *in vivo* (10).

iii. Mast Cell Interaction with Specific Pathogens

In this section, evidence for direct interactions of mast cells against pathogenic organisms and viruses will be discussed. More emphasis will be placed on mast cell interactions with viruses since this PhD thesis focuses on effects of influenza A virus on mast cells.

- ***Helminth Infections***

Mast cells are involved in Th2 responses against certain helminths, parasitic worms that cause illness in humans and other mammals. Although mast cells are known to be activated by helminths and mast cell hyperplasia is observed in various helminth infections, the critical involvement of mast cells in protective immunity has only been demonstrated against a limited number of helminths.

Mast cells are important in the expulsion of the *Trichinella spiralis* nematode, as W/W^v mice show prolonged infection with the parasite compared to wildtype mice (152, 153). Also, reconstitution with intestinal mast cells by injection of spleen cells into W/W^v mice restores the *T. spiralis* expulsion rate to that of wildtype mice (154). Mice deficient in mouse mast cell chymase (mMCP-1) are unable to increase intestinal permeability and expel T. spiralis, demonstrating the importance of a mast cell protease as a mechanism of parasite expulsion (155). In a recent study, W/W^v mice reconstituted with $TNF^{-/-}$ or $IL\text{-}4^{-/-}$ bone marrow show less efficient *T. spiralis* expulsion and more intestinal pathology than with wildtype bone marrow, illustrating the importance of mast cell cytokines in protective immunity (138). Infection with *Nippostrongylus brasiliensis*, an intestinal nematode of rodents similar to human hookworms, also induces mast cell hyperplasia (156). However, in *N. brasiliensis* infection, the increased primary expulsion rate seen in W/W^v mice are not recovered by mast cell reconstitution and expulsion rates are similar upon secondary infection in W/W^v and wildtype mice, indicating that mast cells do not play an important role against this parasite (157).

- ***Fungal Infections***

Although mast cells are capable of responding to the yeast cell wall component zymosan, little evidence exists for mast cell immunity against fungal organisms *in vitro* or *in vivo*. Relatively high doses of the indoor fungus *Trichoderma viridae* are required to induce mast cell degranulation, but low doses

are capable of enhancing histamine secretion from mast cells activated by IgE/α-IgE (158). A study published this year (2009) shows that different *Aspergillus* species, especially *A. fumigatus*, induces IgE-independent mast cell degranulation (159). To the author's knowledge, there are currently no published studies demonstrating the importance of mast cells in fungal infections *in vivo*.

- ***Bacterial Infections***

In 1996, mast cells were shown for the first time to be protective against bacterial infections *in vivo,* both in a cecal ligation and puncture mouse model of septic peritonitis and in mice injected with fimbriated *E. coli* (160, 161). In both these studies, mast cell-deficient W/W^v mice were less efficient in clearing the bacteria with increased mortality rates, and reconstitution with mast cells restored the ability of the mice to clear the infection with enhanced survival. Mast cell-derived TNF is important in the bacterial peritonitis model (160). In the fimbriated *E. coli* infection model, TNF, LTC_4, and LTB_4 contribute to the ability of mast cells to recruit neutrophils and clear the infection (161, 162). Mast cells also play important roles against other bacterial infections such as *Klebsiella pneumoniae*, *Listeria monocytogenes,* and *Pseudomonas aeruginosa* through the activities of IL-6, $\alpha_2\beta_1$-integrins, and possibly IL-1α and β, respectively (163-166). In a pulmonary *K. pneumoniae* infection model, the administration of human tryptase-β1 increased neutrophil migration more than 100-fold, demonstrating the importance of a mast cell protease in host defense against bacteria (167).

- ***Viral Infections***

Although mast cells in viral infections are less well-characterized than in bacterial infections, more evidence for the role of mast cells against viral pathogens has recently emerged (Figure 1.3).

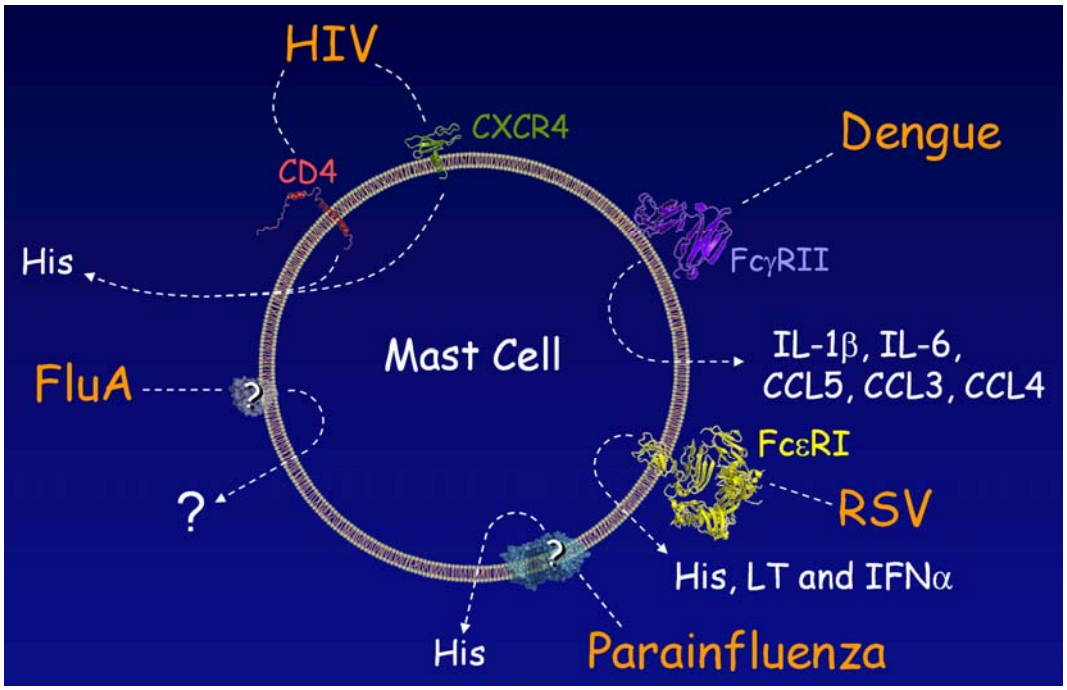

Figure 1.3: Mast cell responses to viral exposure (original figure). Viruses are shown with the receptor mediating the response when the molecule is known. The illustrated 3D structures for the mast cell receptors were obtained from the "Protein Data Bank" (www.pdb.org) and are an accurate representation for each receptor, except receptors annotated with question marks. Shown in the image are viruses reported to elicit release of mediators from mast cells, although the image is not inclusive of all viruses reported to affect mast cells. All viruses are shown indicating the specific response that they elicit in mast cells. HIV interacts with mast cells through the CD4 and CXCR4 receptors. The interaction stimulates the release of histamine (His). Dengue virus interacts with mast cells through the Fc-

gamma receptor II (FcγRII) and stimulates the release of the cytokines IL-6 and IL-1β, and the chemokines CCL5 and CCL3α/β. RSV-specific IgE activates FceRI and RSV has been shown to stimulate the release of His and leukotriene (LT). Parainfluenza act through unknown receptors to induce His release. Both RSV and influenza A virus (FluA) stimulate the release of IFNα. *The experiment showing release of IFNα from mast cells used a UV-inactivated FluA virus only.

a) Human immunodeficiency virus

Human immunodeficiency virus (HIV) infection can lead to the development of acquired immunodeficiency syndrome (AIDS), in which the patients are severely immunocompromised and susceptible to opportunistic infections. HIV infection is correlated with increased serum IgE levels in patients, which is indicative of worse prognosis (168). Also, AIDS patients have fewer intestinal MC_T in the mucosa (169). Later, it was shown that HIV infects mast cells in the peripheral blood of patients and HIV productively infects human mast cells *in vitro* (170, 171). HIV enters mast cells through the receptors CD4 and CXCR4, and exposure to HIV induces histamine release and reduces mast cell migratory activity towards chemokines (171). Evidence shows that mast cell progenitors are vulnerable to HIV infection, but as mast cells mature, they become increasingly resistant to infection (172). Placental mast cells from

pregnant women infected with HIV are a long-term reservoir for latent HIV, and these mast cells can be reactivated *ex vivo* to release infectious virus (173).

b) Dengue virus and Reovirus

In 1977, patients with dengue virus infection were found to have increased urinary histamine, suggesting a potential role for an important mast cell mediator in disease pathogenesis (174). More evidence showed that human mast cells are permissible to antibody-enhanced dengue virus infection and are activated to release the cytokines IL-1β and IL-6, as well as the chemokines CCL5, CCL3, and CCL4 (MIP-1β) (23, 175, 176). Infection of mast cells with antibody-enhanced dengue virus is dependent on the binding of virus to mast cell FcγRII (177). Dengue virus infection also induces caspase-dependent apoptosis of mast cells, but not of other Fcγ-expressing cell types (178). A recent paper also showed that mast cells can be infected by reovirus and upon infection, are capable of inducing NK cell chemotaxis (179).

c) Respiratory syncytial virus (RSV)

Although respiratory syncytial virus (RSV) is unable to infect mast cells, evidence suggests that mast cells respond to RSV infection (176). RSV is a major cause of lower respiratory illness in infants and young children, and RSV infection is associated with the development of asthma later on in life (180). Both RSV-specific IgE and histamine levels are increased in nasopharyngeal secretions

of infected infants(181). Mast cell density and levels of LT are increased in lungs of RSV-infected rats, implicating a role for mast cells in RSV infection (182). A study shows that reconstitution of wildtype mast cells into FcεRI$^{-/-}$ mice recovers their ability to develop AHR upon secondary infection with RSV, providing *in vivo* evidence for mast cells in development of asthma-like responses (183).

d) Parainfluenza virus

Human parainfluenza viruses (HPIV) are second only to RSV in causing lower respiratory tract infection (LRTI) in young children. Although mast cell-HPIV interactions have not been demonstrated, mast cells do respond to Sendai virus, a parainfluenza virus that affects rodents and pigs. Sendai virus induces histamine release from rat peritoneal mast cells (184). Bronchiolar mast cell numbers are increased in rats with Sendai virus-induced bronchiolitis, and mast cell numbers correlate with AHR and pulmonary neutrophil infiltration (185). Also, Sendai virus-induced increases in mast cell numbers are associated with prolonged viral replication, higher viral titres, and more persistent lymphocytic infiltration, implying a role for mast cells in viral clearance (186).

e) Influenza A virus

Influenza A virus (FluA) is an important respiratory pathogen that affects millions of people worldwide. There is little evidence for direct mast cell-FluA interactions and the focus of this PhD project is to investigate this knowledge gap.

Mast cell tryptase from pig lungs is capable of cleaving FluA hemagglutinin, thereby potentially enhancing viral infectivity (187). Also, cells from bronchoalveolar lavage (BAL) of normal, non-atopic individuals secrete histamine in response to FluA (188). One study in 2002 showed that FluA-antigen induces mast cell-dependent cutaneous anaphylaxis in mice mediated by FluA-specific antibodies; however, the conclusions from this study are in question since the purity of the gradient-purified FluA-antigen (cultivated from allantoic fluid of chicken eggs) is not specified and the allergic response could instead be attributed to allantoic contaminants (189). To date, the findings of this study have not been confirmed or extended by other studies. As the subject of this PhD thesis pertains to FluA, the clinical and biological aspects of FluA infection will be described in detail below.

2. Influenza Virus

Influenza is an acute respiratory illness caused by influenza virus infection (see review (190)). The threat of an influenza pandemic receives tremendous attention since previous pandemics have led to high levels of illness, death, and economic loss. Notably, the 1918 "Spanish flu" was estimated to be responsible for 50 million deaths worldwide. As of June 2009, the World Health Organization (WHO) officially announced the pandemic status of the H1N1 "swine flu" virus, indicating the widespread infection of a newly circulating influenza virus in humans with sustainable human-to-human transmission (191). However, seasonal epidemics that occur every year also result in substantial mortality and mobidity. Among the three species of influenza virus A, B, and C, influenza A virus (FluA) is associated with greater illness and mortality in human disease. Two subtypes of FluA, H1N1 and H3N2, are most important for the circulating seasonal influenza epidemics. The following background on influenza will focus on information regarding seasonal influenza and FluA, since this PhD thesis involves work on a non-pandemic strain of FluA of the H1N1 subtype.

A. Epidemiology of Influenza Virus Infection

The ability of influenza virus to cause recurrent seasonal epidemics and occassionally more threatening pandemics reflects the interplay between combinations of pathogen and host factors. The extent of antigenic variation of the virus, the relative virulence of the virus, and the degree of protective immunity of a population determine the transmissibility and impact of influenza infection.

i. Antigenic Properties

Influenza virus replication of its genome occurs through the activity of viral RNA polymerase. Antigenic changes or "antigenic drift" of influenza virus occurs as high rates of mutation arise from the faulty proofreading ability of the RNA polymerase (192). Accumulation of mutations, particularly changes in the surface proteins hemagglutinin and neuraminidase, results in new variants that are capable of evading humoral immunity, causing seasonal epidemic outbreaks (193). In contrast, the unique segmental character of influenza virus allows "antigenic shift" to occur when entire RNA segments of two influenza viruses recombine, resulting in a new virus with a mixture of surface and internal proteins (193). Pandemics occur as a result of antigenic shift where genetic reassortment between human and animal influenza strains produces a new virus subtype, which the population has little immunity against.

ii. Transmission

Transmission of seasonal influenza virus mainly occurs by the spread of respiratory droplets from person-to-person by sneezing and coughing. Influenza virus can also be transmitted by touching respiratory droplets on a surface and then touching the nose or mouth with the contaminated hand. The half life of influenza droplets ranges from 1-16 hours, with low humidity and low UV radiation as factors that promote longer survival (194). The incubation of influenza virus between exposure and the onset of symptoms is 1-4 days, and

healthy adults are contagious from 1 day pre-symptomatic to 5 days post-symptomatic illness (195).

iii. Seasonality

Influenza season occurs during the winter months in temperate climates and during the wet seasons in tropical areas (192). Influenza epidemics are explosive events that peak quickly and also resolve abruptly. In the United States, influenza season peaks between November and March (195). Although the mechanism behind the seasonality of influenza virus is unclear, increases in indoor crowding, enhanced survival of influenza virus particles at colder temperatures, and decreases in vitamin D levels in the winter are possible contributors (196-198).

iv. Mortality and Morbidity

According to the WHO, annual influenza epidemics account for upper respiratory tract infections (URTI) in 5 – 15% of the world population, resulting in up to 500,000 deaths every year (199). In the United States, influenza epidemics have both health and economic impacts, resulting in approximately 41,000 deaths and 44 million lost work days a year (200). Influenza is also the most medically-attended acute respiratory illness in the United States, leading to more than 300,000 annual hospitalizations (200). Although the elderly (> 65 years) make up the minority of people admitted to hospital for influenza, greater than 90% of influenza deaths occur in this age group (201). Increases in

influenza-associated mortality are attributed to an aging population, indicating the need to improve vaccination programs among the elderly as a primary prevention strategy (201).

B. Clinical Illness of Influenza Virus Infection

i. Signs and Symptoms

Influenza infections are usually self-limiting and last for approximately a week, although complications leading to more serious illness can occur. Patients typically present with a sudden onset of fever and chills, along with myalgia, malaise, sore throat, loss of appetite, and a non-productive cough (202). A high fever usually peaks within 24 hours of onset, declines by the third day, and resolves by the sixth day. Physical signs include a general appearance of unwellness, irritated watery eyes, reddened face, hot and moist skin, and a clear nasal discharge. Children may present with gastrointestinal symptoms such as nausea, vomiting, and diarrhea in addition to the other symptoms. In circumstances where infection occurs in immunocompromised patients or is caused by an especially virulent FluA strain, symptoms may last longer than usual and result in higher fatality rates (190). Human infection with the pandemic H1N1 "swine flu" virus generally presents with similar symptoms as seasonal influenza, however, a significant number of people have presented with diarrhea and vomiting (191).

ii. Complications

Complications from influenza infection usually occur in high-risk populations, including young children, the elderly, pregnant women, and patients with underlying medical conditions such as heart disease, asthma, AIDS, and diabetes (see review (203)). Pneumonia is the main complication of influenza that results in death. Pulmonary consolidation is a clinical sign of pneumonia that is confirmed by chest radiography. In influenza infections, secondary bacterial pneumonia caused by pathogens such as *Streptococcus pneumoniae, Staphylococcus aureus,* or *Haemophilus influenzae,* is more common than primary viral pneumonia caused by the virus itself. Other pulmonary complications arising from influenza include exacerbations of underlying conditions such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis.

Neurological complications of influenza also occur, especially in children, and include encephalopathy (Reye's syndrome), encephalomyelitis, aseptic meningitis, and Guillain-Barré syndrome. Symptoms from impairment of the central nervous system progress rapidly, often with fulminant or fatal outcomes. Other rare complications of influenza infection are myositis leading to potential renal failure and loss of ambulation, and exacerbation of underlying cardiac conditions such as congestive heart failure and ischemic heart disease.

Unlike seasonal influenza, elderly people infected with the new pandemic H1N1 virus are not at increased risk of complications, potentially due to natural immunity to the virus in older patients, apparently associated with a strain that

circulated in the 1950s (204). However, other risk groups such as young children, pregnant women, and people with underlying medical conditions are still at risk of more severe illness. Vaccines against this novel strain of FluA was distributed in the Fall of 2009 (191).

iii. Prevention and Treatment

Currently, the available methods for control of influenza are vaccines and antiviral drugs.

- ***Vaccines***

Vaccination is the most effective way of preventing influenza or the development of severe symptoms. WHO recommends annual vaccinations for the elderly, the disabled, people with chronic medical conditions, pregnant women, health care workers, and children between 6 months and 2 years (199). Two types of influenza vaccines are used globally: trivalent inactivated influenza vaccine (TIV) and live attenuated influenza vaccine (LAIV). Both types of vaccines are grown in eggs and are thus contraindicated in people with serious egg allergies. TIV consists of inactivated influenza particles of two FluA strains and one FluB strain, which vary annually depending on predictions based on global monitoring of circulating strains (202). When accurate predictions result in antigenic similarity between the vaccine and the circulating strains, TIV have a 70-90% efficacy in young healthy adults (205). TIV is injected intramuscularly and

approved for use in the United States and Canada for anyone ≥ 6 months of age, including those with medical conditions (206, 207).

In the United States 2008-2009 guidelines for influenza vaccination, LAIV is approved only for people 2 – 49 years of age without risks of medical complications (206). A study in adults showed similar efficacies between TIV (71%) and LAIV (85%), where the differences were not statistically significant (208). LAIV is a trivalent, cold-adapted, live attenuated preparation delivered intranasally in a spray form. Compared to TIV, LAIV is advantageous because it only replicates in the colder regions of the upper respiratory tract without replicating systemically, provides better cross-protection against drifted strains, and is easier to administer. However, the downside of LAIV is that it is slighty more expensive than TIV and requires refrigeration. Although LAIV has been shown to have greater efficacy in young children and appears to be safe for high risk populations, larger studies are needed before LAIV can be approved for these groups (209).

- ***Antiviral Drugs***

Antiviral drugs are recommended for prophylaxis in people who have not received vaccination in a community with an influenza outbreak, health care workers in contact with high risk patients, people with serious egg allergies, and immunocompromised patients (210). When administered within 2 days of onset, these compounds alleviate influenza symptoms and reduce the severity of illness with an efficacy of 70-90%. Two classes of antiviral drugs are currently used for

prophylaxis and treatment of influenza: M2 ion channel inhibitors and neuraminadase (NA) inhibitors. M2 ion channels are proton pumps important for FluA infectivity by acidifying the virus during the uncoating process. Amantadine and rimantadine are adamantane compounds that inhibit FluA replication by blocking the function of M2 channels, which are essential for virus uncoating. M2 channel inhibitors are not effective against FluB. Side effects of adamantanes include perturbations of the central nervous system (CNS) and gastrointestinal effects, with CNS side effects more common with amantadine. Adamantanes are currently not recommended for use in the United States due to widespread resistance of seasonal H3N2 viruses (211). The adamantanes retain activity against circulating H1N1 viruses and can be used in combination with neuraminidase inhibitors when H1N1 viruses predominate in the community (212).

Effective against both FluA and FluB, oseltamivir and zanamavir are NA inhibitors. Viral NA cleaves sialic acid residues to release virus particles from the cell surface allowing for viral spread to adjacent cells. In addition to treatment of influenza, NA inhibitors are also recommended for prophylaxis in high-risk patients (211). Side effects of orally administered oseltamivir include nausea and vomiting, and transient neuropsychiatric events of self-injury and delirium that occur primarily in children and adolescents (211). Frequency of resistance to oseltamivir (0.4 – 18%) is much lower than that for M2 inhibitors (30-80%) (213). Also, oseltamivir-resistant influenza virus strains remain susceptible to the effects of zanamavir. Zanamavir is delivered by inhalation, and common adverse events

include diarrhea, nausea, headaches, dizziness, and respiratory symptoms. Zanamavir is not recommended for individuals with underlying airway disease.

C. Pathophysiology

Pathology throughout the respiratory tract is observed in patients infected with influenza virus, with inflammation of the larynx, trachea, and bronchi. Respiratory epithelial cells are the primary target of FluA replication, producing large amounts of new virions. After a first cycle of replication, FluA infects other cells in the surrounding area, including neighbouring epithelial cells and alveolar macrophages. FluA infection causes epithelial cell death by both necrosis and apoptosis, resulting in severe desquamation of the epithelium and exposure of the basement membrane. Generally, influenza infection is restricted to the respiratory tract and does not result in viremia, although more pathogenic influenza strains have demonstrated extrapulmonary manifestations (214). Epithelial cells respond to FluA infection by producing cytokines such as TNF and IL-1β and chemokines such as CCL5 and CXCL8 to initiate an inflammatory response (215). Submucosal edema is evident during influenza infection, with an infiltration of macrophages, neutrophils, and T cells within 3 days after infection (216). Proinflammatory cytokines cause symptoms associated with influenza such as fever, headaches, and fatigue (217). Excessive inflammatory infiltrate and production of proinflammatory cytokines are associated with more severe pathology in influenza infection (216).

D. Virology of Influenza Virus

Unique aspects of FluA virology help explain the epidemiological success, clinical progression, and pathogenesis of the virus. This section will discuss the components that make up FluA and the FluA life cycle (see reviews (190, 218).

i. Classification

Human influenza viruses are enveloped, segmented, negative-sense RNA viruses that belong to the *Orthomyxoviridae* family. Of the three human influenza types (A-C), FluA is the most pathogenic. FluA is further classified into subtypes based on serological and genetic differences of the envelope proteins, hemagglutinin (HA) and neuraminidase (NA). The "H" and "N" subtypes are based on different HA and NA types, respectively, on the viral surface. There are 16 different "H" subtypes and 9 "N" subtypes, but only H1-3 and N1-2 are found on circulating strains of seasonal human influenza viruses. When reassortment occurs between human and animal strains, new strains with uncommon subtypes (H5N1, H9N2) capable of infecting humans can result in pandemics. Strains of human influenza virus are defined by their type, place of origin, strain number, year of isolation, and subtype (eg. A/Hong Kong/1/68(H3N2)).

ii. Structure

The structure of FluA is depicted in Figure 1.4. Influenza viruses are enveloped viruses that exist as spherical forms approximately 80-120 nm in

diameter, while filamentous forms also occur. FluA has 8 segments of negative-sense ssRNA, each encoding at least one protein. The lipid envelope is derived from host cell membrane. Inserted in the envelope are the viral glycoproteins hemagglutinin (HA) and neuraminidase (NA), which form spike-like structures. HA exists as an elongated homotrimer and is the most abundant surface protein of FluA, while NA forms a disc-shaped homotetramer. Another FluA integral membrane protein is matrix protein 2 (M2), which does not protrude to the extent of HA and NA. A protein layer made up of matrix protein 1 (M1) underlies the lipid envelope of FluA and forms a bridge between the envelope and the viral ribonucleoprotein (vRNP). Each vRNP consists of one segment of RNA associated with a polymerase complex and multiple copies of nucleoprotein (NP). The polymerase complex is made up of acidic polymerase protein (PA), and basic polymerase proteins (PB1 and PB2). There are two FluA proteins designated nonstructural proteins (NS1 and NS2), although only NS1 is truly nonstructural as NS2 is incorporated into viral particles in small amounts.

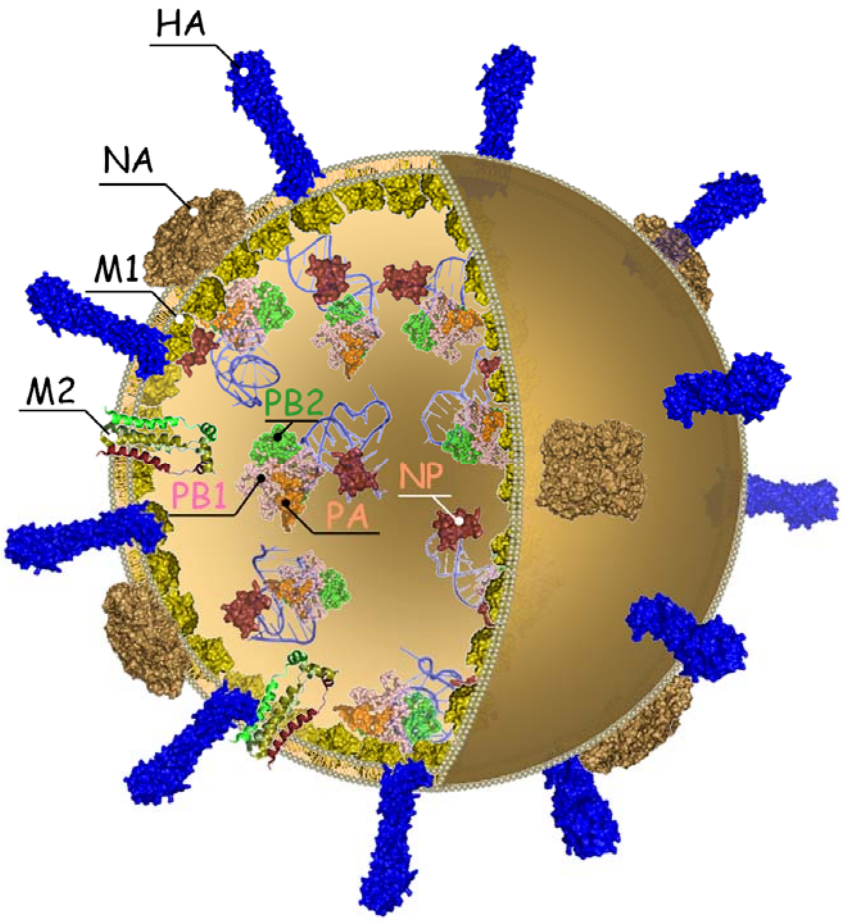

Figure 1.4: Diagrammatic representation of the influenza A (FluA) virus (original figure). All eight viral RNA segments are shown (blue ribbons). The RNA molecules are negative-sense single-stranded RNA (ssRNA) labeled polymerase B2 (PB2), polymerase B1 (PB1), polymerase A (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins (M encodes M1 and M2) and nonstructural proteins (NS encodes NS1 and NS2) according to the proteins they encode in descending order of molecular weight. The polymerase proteins PA, PB1 and PB2 form a complex as depicted. The viral ribonucleoprotein complexes (vRNP) consist of eight separate complexes of RNA, polymerase complex, and multiple copies of NP (only 1 copy shown). M1

lines the luminal surface of the virion. The envelope proteins HA, NA, and M2 are depicted.

iii. Properties of Viral Proteins

- ***Polymerase Proteins***

FluA polymerase subunits PB2, PB1, and PA are encoded by genomic segments 1, 2, and 3, respectively. The polymerase proteins together with NP make up the minimum required proteins necessary for FluA transcription and replication. The three polymerase subunits each contain a nuclear localization signal (NLS) for transport into the host cell nucleus. Also, PB2 is important for generating cap primers from host mRNA and bringing these cap structures to the viral RNA for initiation of transcription. A study of a H5N1 virus demonstrated that PB2 is a pathogenic determinant of FluA, since a PB2 mutation resulted in differences in virulence (219). PB1 is the catalytic subunit of the RNA polymerase and is required for the initiation and elongation of newly synthesized viral RNA. PB1 is also important for assembly of the three polymerase subunits into a complex. Although PA is essential for FluA RNA transcription and replication, the exact mechanisms of PA are unclear. PA has been shown to have endonuclease and proteolytic activities, but the functional significance of these activities is not understood (220, 221).

- ***Hemagglutinin and Neuraminidase***

Encoded by FluA segment 4, HA is the surface glycoprotein responsible for attachment of the virus to sialic acid residues on the surface of host cells. The specificity of the HA receptor-binding site is determined by the amino acid sequence at its globular head. The HA receptor-binding site of human FluA preferentially bind to α2,6-linked sialic acids, while that of avian FluA binds to α2,3-linked sialic acids (222). Cleavage of the HA precursor into two polypeptides, HA1 and HA2 linked by a disulphide bond, is essential for fusion of the viral envelope with the endosome as FluA enters the cytoplasm. Fusion activity is required for FluA infectivity as it allows the release of vRNP into the cytoplasm. HA also determines the pathogenicity of FluA, since host proteases are more efficient at cleaving HA precursors when multiple basic amino acids are present at the cleavage site, as occurs in virulent strains (223). Encoded by segment 6, NA is another FluA surface glycoprotein that acts as a sialidase by cleaving sialic acid residues, allowing the release of new virus particles from the host cell.

- ***Nucleoproteins***

NP is an essential protein for FluA transcription and replication encoded by gene segment 5. NP contains an RNA-binding domain and an NLS sequence, thereby allowing it to mediate transport of FluA RNA into the nucleus. Binding of NP to genomic RNA is also important in preventing early termination of

transcription (224). NP may promote switching of FluA RNA transcription to RNA replication.

- ***Matrix Proteins***

Two proteins, M1 and M2, are encoded by segment 7 of FluA. M1 is the most abundant FluA protein and is involved in assembly and disassembly of FluA particles (225). In the nucleus, M1 inhibits viral transcription and promotes export of vRNP into the cytoplasm. M1 also promotes FluA assembly and budding at the host cell surface. FluA integral membrane protein M2 acts as an ion channel that pumps protons from the endosome into virus particles (226). Thus, M2 acidifies the virus causing the dissociation of M1 from vRNP in the process of uncoating.

- ***Nonstructural Proteins***

Segment 8 also encodes two proteins: NS1 and NS2. As the only nonstructural protein of FluA, NS1 is non-essential for the virus life cycle, but functions as an important virulence factor. In the nucleus, NS1 is an RNA-binding protein that inhibits polyadenylation, splicing, and transport of host cellular mRNA (227, 228). On the other hand, NS1 enhances the translation of viral mRNA in the cytoplasm (229). NS1 counteracts the host antiviral response by inhibiting dsRNA protein kinase (PKR) and the effects of interferons (230, 231). Originally defined as a nonstructural protein, NS2 actually exists in FluA particles

associated with M1. Also termed nuclear export protein (NEP), NS2 contains a nuclear export signal (NES) and is important for nuclear export of vRNP (232).

iv. Replication Cycle

The replication cycle of FluA is depicted in Figure 1.5. FluA first binds to cell surface sialic acid using HA and enters cells by receptor-mediated endocytosis. Fusion of the viral and endosomal membranes requires the cleavage of HA by host proteases. M2 acidifies the virus particle, dissociates M1 from vRNP, and vRNP is released into the cytoplasm. Once imported into the nucleus, the vRNP undergoes primary viral transcription to synthesize messenger RNA (mRNA), and viral replication to produce full-length complementary RNA (cRNA). Both mRNA and cRNA are positive-sense ssRNA. cRNA is a template for generating copies of vRNA to be packaged into new virions. FluA mRNA is exported into the cytoplasm for translation into viral proteins (233). Secondary transcription of mRNA occurs from newly replicated negative-sense vRNA. Newly translated NP and polymerase proteins are imported into the nucleus and combine with replicated vRNA to assemble into vRNP. Next, vRNP are exported out of the nucleus. Translated FluA envelope proteins are targeted to the endoplasmic reticulum (ER) for proper folding and posttranslational modifications and subsequently to the trans-Golgi network for further processing. Both vRNP and envelope proteins are directed to the virion assembly site at the plasma membrane where budding of FluA particles occurs. Finally, NA cleaves attached sialic residues and new FluA virions are released from the host cell.

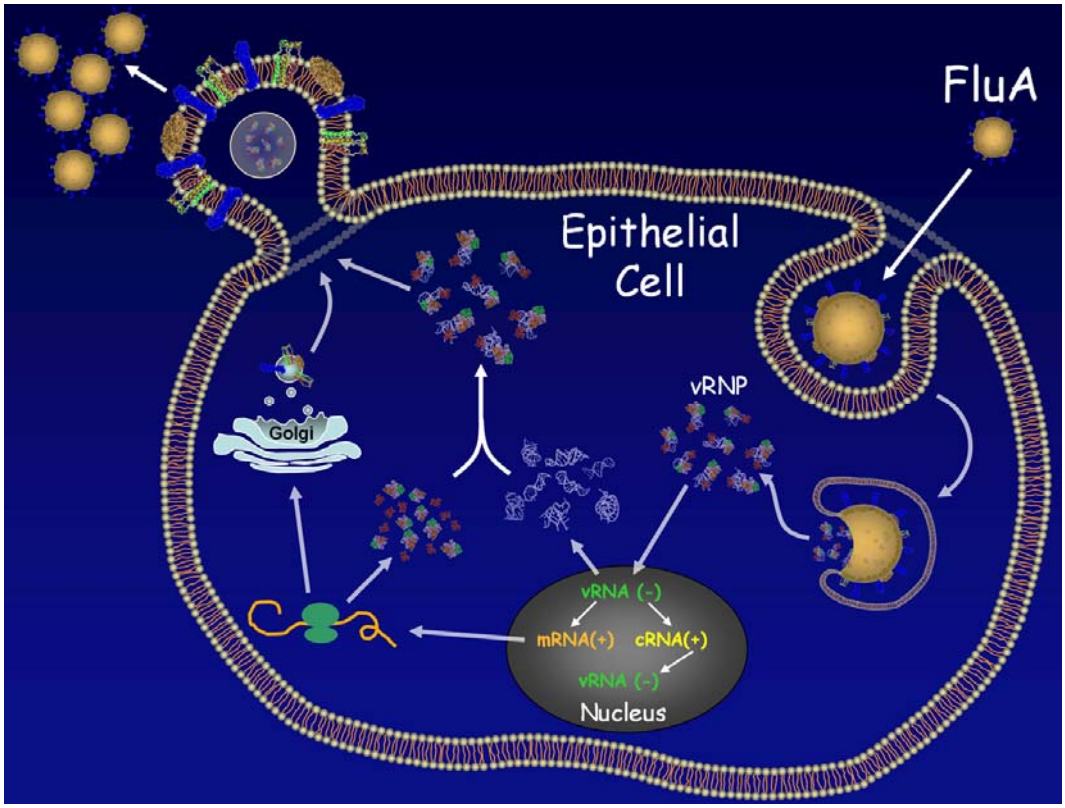

Figure 1.5: FluA replication cycle in lung epithelial cells (original figure). Internalized FluA releases its viral ribonucleoprotein complexes (vRNP) from the endosomal compartment. The negative-sense viral RNA (vRNA (-)) is transcribed into FluA mRNA (mRNA+) and the complementary RNA (cRNA+) from which more vRNA (-) is produced. Viral mRNA translates the viral proteins NA, HA, M2, NP and Ps which are assembled at the plasma membrane to form a new FluA particle.

3. Innate Immune Response to Influenza A Virus

Although FluA is capable of killing infected cells by both apoptotic and cytolytic mechanisms, the host cell also counteracts the viral infection to limit the spread of virus. Cells susceptible to FluA infection, such as epithelial cells and macrophages, have various methods of detecting FluA by recognition of viral components. Subsequently, cells respond to FluA by producing cytokines and chemokines to initiate inflammation and upregulating antiviral proteins to restrict viral spread. We will discuss both mechanisms of FluA detection as well as effector responses such as production of cytokines and antiviral proteins. However, in the experimental chapters of the thesis, we have chosen to focus on effector responses of mast cells.

A. Host Detection

Different pattern recognition receptors (PRR) recognize molecular patterns of FluA and activate various signaling pathways to result in production of IFN and other cytokines. Plasmacytoid DC and neutrophils recognize ssRNA of FluA through TLR7 (234, 235). In response to FluA, TLR7 acts through its adaptor molecule MyD88 to activate the nuclear translocation of interferon regulatory factor 7 (IRF7), resulting in type I IFN production (236). Interestingly, this study also showed that TLR7 signals through a phosphoinositol-3 kinase (PI3K)/p38 mitogen activated protein kinase (p38MAPK) pathway to induce antiviral genes in a signal transducer and activator of transcription 1 (STAT1)-

dependent and IFN-independent manner. The genome of FluA has complementary sequences at the 5' and 3' ends that form panhandle structures, which form base-pairings similar to dsRNA (237). This explains why FluA can be recognized by endosomal TLR3, which classically recognizes dsRNA (117).

Cytoplasmic PRR, such as the helicase proteins - retinoic acid inducible gene-I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) also detect different RNA moieties. RIG-I, but not MDA5, was shown to be critical for recognition of FluA infection (238). The natural ligand for RIG-I in detection of FluA is triphosphate-containing ssRNA forming panhandle structures (239). RIG-I and MDA5 respond to polyI:C sequences and viral dsRNA in a length-dependend manner, with RIG-I preferentially detecting short polyI:C sequences of ~ 300 bp while MDA5 recognizes long polyI:C sequences of > 1 kbp (240). In epithelial cells, FluA activates TLR3 through TIR domain-containing adaptor inducing IFN-beta (TRIF) whereas activation of RIG-I is mediated through the adaptor MAVS (241). TLR3 and RIG-I signaling result in a nuclear factor-κB (NF- κB)-dependent proinflammatory response while only RIG-I signaling results in a IRF3-dependent antiviral response (241). Macrophages, fibroblasts, and DC also activate RIG-I in response to FluA infection (238, 242, 243). FluA-infected $TLR3^{-/-}$ mice demonstrate higher survival rates than WT mice, despite higher viral titres, demonstrating a deleterious involvement of TLR3, potentially due to excessive proinflammatory cytokine production (244)

B. Inflammatory Response

Enhanced cytokine and chemokine production is a feature of human disease caused by FluA. Evidence suggests that the pathogenicity of FluA correlates with a "cytokine storm", characterized by a highly amplified and dysregulated production of cytokines and chemokines. Prominent in devastating pandemics, severe complications of FluA such as pulmonary edema and pneumonias are associated with the increases in levels of cytokines and chemokines.

i. Cytokine Production

Many cytokines contribute in the host inflammatory response to FluA. In FluA-infected patients, levels of TNF, IL-1β and IL-6 correlate with more severe symptoms (245). As the first line of defense against FluA, epithelial cells produce relatively small amounts of IL-1β, IL-6, and TNF. In contrast, FluA-infected monocytes/macrophages produce little newly synthesized virus, but produce large quantities of these proinflammatory cytokines (246). DC are also capable of producing cytokines in the innate response to FluA (247). TNF is involved in immunopathology in mice infected with FluA, as inhibition of TNF results in reduced lung pathology, decreased inflammatory infiltrates, and prolonged survival without changes in viral clearance (248). Mice lacking IL-1R, the receptor for IL-1β, also demonstrate decreased immunopathology; in contrast to TNF inhibition, however, IL-1R$^{-/-}$ mice have increased mortality and delayed viral clearance (249). Altogether, these results indicate that although proinflammatory

cytokines are involved in the immunopathology of FluA, some cytokines may have beneficial effects in protecting the host while others have detrimental effects.

ii. Chemokine Production

Clinically, the chemokines CCL2, CXCL9 (MIG) and CXCL10 are increased in patients infected with more pathogenic FluA strains associated with complications (250). *In vitro* studies show that FluA induces chemokine release from epithelial cells, monocytes/macrophages, neutrophils, and DC (235, 247, 251, 252). Both human alveolar and bronchial epithelial cells release more CXCL10 and CCL5 when exposed to H5N1 subtypes associated with more fatal disease when compared to H1N1 subtypes (253). Interestingly, mice lacking the chemokine receptor CCR5 (receptor for CCL3, CCL4 and CCL5) have higher mortality and increased pulmonary inflammation while mice lacking CCR2 (receptor for CCL2) have decreased mortality and less lung pathology due to defective macrophage recruitment into the lungs (254). As with the association of cytokine production and FluA infection, different chemokines seem to have positive or negative effects on the success of infection in the host.

C. Antiviral Response

Humans have evolved antiviral mechanisms to combat viruses such as FluA. Multiple pathways exist to regulate the antiviral response, providing cells with a greater capacity to resist viral infection. The type I IFNα and β are critical in the

early response of innate immune cells against FluA. Induction of a large number of IFN-stimulated genes results in upregulation of antiviral proteins with direct effects on the replication of FluA.

i. Type I Interferons

Cells infected with FluA produce the type I IFNα and β, major antiviral cytokines that function to restrict virus replication (255). Type 1 IFN are essential for the defense against viral infections by their ability to interfere with viral replication and promote an anti-viral state in host cells (256). They act by binding to the common IFNα receptor (IFNAR). A study showed that FluA replication occurs efficiently in fibroblasts derived from $IFNAR^{-/-}$ mice while wildtype cells were resistant to FluA infection, indicating the importance of type I interferons in antiviral defense against FluA (257). Binding of IFNα or β to IFNAR activates a JAK/STAT pathway to induce the assembly of ISG factor 3 (ISGF3). ISGF3 binds to IFN-stimulated response elements (ISRE) present in the promoter region of IFN-stimulated genes (ISG), resulting in transcription of hundreds of genes. Some ISGs have direct antiviral activities against FluA including PKR, RNaseL/2'5' oligoadenylate synthetase (OAS), and myxovirus resistance protein A (MxA) (258). ISG15 and viral stress inducible protein p56 (p56) are other ISRE-controlled genes that are upregulated by IRF3 and IRF7, and can be IFNα/β-dependent or independent (259).

ii. Proteins with Direct Antiviral Functions

Many host cellular proteins have antiviral activities that have direct effects on viral replication. The following section will discuss antiviral proteins that have been shown to be important against FluA.

- ***Myxovirus Resistance Protein A***

Mx1, the mouse homologue of human MxA, was first discovered in an inbred mouse strain (A2G) that demonstrated a high resistance to influenza infection (260). Type I IFN induce gene expression of MxA (261). The antiviral activity of MxA is demonstrated by MxA transfectants that confer resistance to cells normally susceptible to FluA (262). While mouse Mx1 is nuclear and inhibits FluA primary transcription, human MxA is cytoplasmic and has no effect on primary transcription (263). However, the exact molecular mechanism of MxA against FluA is not known. Mx proteins are high molecular weight GTPases that assemble into large multimers (258). MxA binds to vRNP through complex formation with NP protein and prevents import of vRNP into the nucleus, providing a potential explanation for the antiviral activity of MxA (264). Also, MxA associates with intracellular membranes, but the functional significance of this localization is not understood (265).

- ***Protein Kinase R***

PKR (also known as eukaryotic translation initiation factor 2α kinase 2 or eIF2AK2) is a protein kinase stimulated by type I IFN (266). Binding of FluA dsRNA to the N-terminal dsRNA binding domain of PKR activates the protein kinase domain to phosphorylate eukaryotic initiation factor 2α (eIF2α) (267). When eIF2α is phosphorylated, it acts as a competitive inhibitor of eIF2B, resulting in the inhibition of both cellular and viral protein translation. PKR-deficient mice are more susceptible to FluA infection, demonstrating the importance of PKR in antiviral defense against FluA (268). However, NS1 protein of FluA counteracts the effect of PKR as demonstrated by NS1 mutants that are unable to suppress the activation of PKR in infected cells, resulting in defective viral protein synthesis (269).

- ***RNaseL/2'5' Oligoadenylate Synthetase***

OAS is an enzyme induced by IFN in response to viral infections and contains a groove critical for dsRNA binding (270). There are three distinct forms of human OAS (OAS1-3) and they act by catalyzing the formation of a 2',5'-linked phosphodiester bond between ATP and 2',5'-linked oligomers of adenosine to produce short 2',5'-linked oligoadenylates (2-5A) with the general formula pppA(2'p5'A)n (270). These low molecular weight 2-5A specifically bind to and activate RNase L, an endonuclease that degrades cellular and viral

mRNAs, resulting in inhibition of translation (271). The OAS/RNase L pathway is activated by FluA, but its effects are opposed by viral NS1. Mutation of NS1 results in lower viral titres from infected cells, but siRNA knockdown of cellular RNase L significantly increases the replication of the NS1 mutant (272). This shows that the OAS/RNase L pathway is effective in inhibiting FluA replication in the absence of NS1.

- ***Interferon-stimulated gene 15***

Although both ISG15 and p56 are induced by IFN, they can also be induced by viral infections independently of IFN effects. FluA-infected cells deficient in IFNα/β are able to induce both ISG15 and p56 (259). ISG15 is an ubiquitin-like protein that in conjunction with its conjugating enzymes Ube1L and UbcH8, modifies protein functions by conjugating to cellular proteins (273). ISG15 conjugates to both IFN-induced antiviral proteins and constitutive cellular proteins (274). $ISG15^{-/-}$ mice are more susceptible to FluA infection, indicating ISG15 as an important antiviral protein (275). The link between the ability of ISG15 to modify proteins and its importance in resistance against FluA has not been established.

- ***Viral Stress-inducible Protein p56***

Viral stresses such as IFN, dsRNA, and viral infections induce the activity of p56 protein and its family members (p54, p58, p60) (276). P56 family members

have multiple tetratricopeptide (TPR) motifs as protein interaction domains. P56 binds to eIF3 and prevents eIF3 from stabilizing the eIF2•GTP•Met-tRNAi ternary complex, thus inhibiting translation (277). Although FluA induces transcription of the ISRE-controlled p56 gene, mature p56 mRNA is not produced, likely due to inhibtion of posttranscriptional mRNA processing by NS1 (259). Currently, the contribution of p56 antiviral activity on FluA replication is not clear. Evidence suggests that NS1 blocks posttranscriptional processing of cellular mRNA by sequestering proteins required for processing the 3' end of cellular pre-mRNA (278).

iii. Nitric Oxide

Nitric oxide (NO) is produced enzymatically by NO synthase (NOS) through conversion of the substrates L-arginine, NADPH, and oxygen into the products L-citrulline, NO, and NADP. Three isoforms of NOS exist: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). NO has both regulatory effects on physiological and disease processes as well as antimicrobial effects during pathogen infections.

In a study of human experimental influenza, individuals infected with FluA had higher oral exhaled NO levels compared to baseline (279). NOS2 is induced in epithelial cells infected with FluA through the detection of dsRNA by PKR and NF-κb signaling (280). Both NOS2 expression and NO production are induced in FluA-infected murine macrophages (281). Recently, a DC subset that produces TNF and iNOS (TipDC) was found to contribute to FluA clearance in

mice by promoting $CD8^+$ T-cell proliferation (282). However, it is important to understand how to balance the antimicrobial and inflammatory effects of NO, as excessive NO production can potentially lead to tissue damage and respiratory distress.

4. Mast Cell-Epithelial Cell Interaction

There is little existing evidence for direct interactions between mast cells and FluA. However, mast cells are in prime location and have the capacity to respond to FluA infection; not only are they expressed constitutively next to the epithelial lining in the respiratory tract, they also react to various mediators produced by epithelial cells during viral infections. Mast cells also have reciprocal effects on epithelial cells, and could potentially influence the progression of FluA infection of the epithelial barrier. This next section will discuss evidence for the interaction between mast cells and epithelial cells, and the response of mast cells to virus-induced epithelial cell products.

Epithelial cells are the target of FluA and line the entire respiratory tract including the nasal mucosa, trachea, bronchi, bronchioles, and alveoli. Mast cells exist along the entire respiratory tract situated both adjacent to and in direct contact with epithelial cells. Mast cells are found abundantly in the bronchial submucosa, pleura, peribronchial regions, and alveolar septa (283). Lung intraepithelial and lumenal mast cells also exist, which can have direct access to the external environment (284, 285).

A. Mast Cell-Epithelial Cell Co-Culture

Epithelial cells can both directly and indirectly influence mast cell function. Using transwell chambers, mast cell survival was increased when co-cultured with human airway epithelial cells, and this survival was further enhanced when mast

cells were cultured directly on epithelial cell monolayers (286). Co-culture with epithelial cells also induces phenotypic changes in primary human mast cells from the connective tissue-type MC_{TC} to the mucosal-type MC_T (286). Adhesion to epithelial cells modulates mast cell function as evidenced by diminished IgE-dependent mast cell degranulation when adhered to epithelial cells versus fibronectin (287). A recent study showed that while RSV-infected epithelial cells induce mast cell degranulation when placed in co-culture, neither direct RSV inoculation or culture medium from RSV-infected epithelial cells were able to induce mast cell degranulation (288). This study supports a role for direct mast cell-epithelial cell interaction in mast cell innate response to viruses.

When adhered in co-culture, mast cells inhibit epithelial cell release of secretory leukocyte protease inhibitor (SLPI), which inhibits endogenous proteases and has antimicrobial activity, demonstrating mast cell regulation of an epithelial cell innate response (289). Mast cells also affect epithelial cell function in co-culture models with other epithelial cell types; for example, mast cells increase paracellular permeability of colonocytes, and promoting proliferation and differentiation of keratinocytes similar to *in vivo* conditions (79, 290). Thus, there is evidence to suggest a reciprocal communication between mast cells and epithelial cells, and further exploration of this interaction is needed in the context of innate immunity against pathogens.

B. Response of Mast Cells to Virus-Induced Epithelial Cell Mediators

Part of the pulmonary innate immune system, epithelial cells are a prime source of signals during viral infections since they are the first to interact with pathogens and are often the target of viral invasion. Human lung epithelial cells express mRNA for all 10 known human TLRs, including viral TLR3, 7, and 9 (291). FluA infection of lung epithelial cells stimulates TLR3, resulting in production of type I interferons, CXCL8, and IL-6 (292). Activation of TLR7 and TLR9 by pathogenic products in epithelial cells also results in the release of these mediators (293, 294). Respiratory viruses also induce epithelial cell production of NO, which has multiple antiviral properties (295).

Mast cell functions are regulated by soluble mediators released by epithelial cells. Virus-associated stimuli released from infected epithelial cells that have effects on mast cells include type I interferons, TLR ligands released during viral replication, and NO. Although produced by many different cell types, IFNα/β are first produced by epithelial cells since they are the first site of contact with respiratory viruses (296). Treatment of mast cells with IFNα and/or β results in inhibition of TNF and histamine release, increase in TGF-β production resulting in decreased co-stimulatory activity on T-cells, and decreased adhesion to extracellular matrix proteins (297-299). TLR ligands have many effects on mast cell function, which was discussed in section 1 on mast cell mechanisms in innate immunity (Page 19-22). NO has inhibitory effects on the release of mast cell mediators, including histamine, proteases, leukotrienes, cytokines, and

chemokines (300). However, the role of IFN and NO on the regulation of mast cell response to viruses needs further study. Also, TSLP production in epithelial cells is activated by viruses such as RSV and HIV, and TSLP has been shown to stimulate Th2 cytokine production by mast cells (301-303). Investigations of other epithelial cell products important for mast cell function, such as SCF and IL-33, in the context of viral infections would provide greater insight into the role of mast cell-epithelial cell interactions in response to viruses (304, 305).

C. Effect of Mast Cell Mediators on Epithelial Cell Function

Once mast cells have been alerted to the invading pathogen, they can exert effects, in part, back onto the epithelium to encourage host defense. Tryptase is a mast cell-specific mediator that has effects on epithelial cells relevant to innate immunity. Tryptase induces release of PGE_2, MCP-1, and CXCL8 from epithelial cells (306-308). Mast cell tryptase activates protease-activated receptors (PAR), which are expressed on various cell types including epithelial cells (309). There are four forms of PAR, with PAR-2 having both pro and anti-inflammatory effects in the airways (310). Activation of PAR-2 on epithelial cells can be protective in the airways by suppressing LPS-mediated leukocyte recruitment (311). Conversely, PAR-2 activation induces MMP9, GM-CSF, and proinflammatory cytokine release from epithelial cells (312-314). Recently, PAR-2 was found to be important for FluA replication in epithelial cells and protective against lung inflammation and death in FluA-infected mice (315). However, whether mast cell tryptase contributes to the effect of PAR-2 against FluA was not determined.

Mast cell chymase induces mucin production from human bronchial epithelial cells (316). Also, chymase induces the release of latent TGF-β from the extracellular matrix of epithelial cells (317). Mast cell histamine upregulates TLR3 expression in human airway epithelial cells, suggesting a role for histamine in promoting innate responses to respiratory viral infections (318). Histamine also contributes to AHR in guinea pigs infected with parainfluenza virus (319). Taken together, these studies demonstrate the ability of mast cell-derived products to alter epithelial cell functions that may be relevant in innate responses to viruses. However, the exact role of these mast cell products in defense of epithelial cells against viral infections requires elucidation.

5. Synthesis and Conceptual Model

Mast cells are secretory cells that contribute to the innate immune response against pathogens by releasing various mediators including a repertoire of cytokines and chemokines (Fig. 1.6). Heterogeneous as mast cells are, each mast cell subtype can demonstrate fine-tuned responses that differ depending on the specific stimuli and microenvironment present. There is evidence that mast cells are infected by certain viruses and respond to viral exposure by increasing proliferation and releasing cytokines, chemokines, and proteases. Whether these mast cell responses promote viral replication and pathology or protect against viral infection in each situation is uncertain.

Our aim is to study the innate responses of mast cells to FluA, which has not been investigated. We will use the human mast cell lines HMC-1 and LAD2, and primary human cultured mast cells (HCMC) derived from peripheral blood progenitors. Human mast cells will be treated with intact or UV-inactivated FluA of the strain A/Puerto Rico/8/1934(H1N1) as well as the virus-associated stimuli IFNα and/or β and viral TLR ligands. These stimuli will be used to compare responses of mast cells to replication-competent FluA versus different environmental cues present during FluA infection. The human bronchial epithelial cell line Calu-3 and the standard canine cell line Madine-Darby Canine Kidney cells (MDCK) used for growing FluA in culture will be used as comparative cell types for studying FluA infection. We will characterize FluA replication cycle in human mast cells, measure cytokine release from mast cells exposed to FluA, and study mast cell expression of antiviral genes induced by FluA. Both single-

mediator assays and multiplex cytokine arrays will be used to assess the mast cell mediator profile in response to the different viral stimuli. Antiviral proteins known to be important during FluA infection (MxA, PKR, ISG15, p56, NOS) will be studied in mast cells.

We anticipate that FluA will productively infect human mast cells, with FluA transcription, translation, and release of progeny virus occurring in mast cells. Since mast cells are activated upon exposure to other pathogenic stimuli, we predict that the release of pro-inflammatory cytokines such as IL-6, chemokines such as CXCL8, and immunomodulatory cytokines such as TGF-β will increase from mast cells in response to FluA, IFNα and/or β, and viral TLR ligands. However, the levels of secretion are expected to vary depending on the specific stimuli, demonstrating the tightly controlled responses of mast cells. FluA infection could also modulate the levels of cytokines induced, since the virus has mechanisms to combat cellular defenses to enhance its own survival. Mast cells are important in protection against various pathogens, thus, we predict that FluA infection will induce mast cells to express antiviral genes in an attempt to suppress FluA replication.

Finally, we will assess the effect of mast cells on FluA infection of epithelial cells using a transwell co-culture system. FluA infection needs to be limited at the epithelial barrier since epithelial cells are the primary target for FluA replication. It is reasonable to postulate that mast cell-epithelial cell interactions occur during FluA infection. We predict that addition of mast cells in

co-culture will be protective against FluA infection of epithelial cells, with decreased viral production from epithelial cells.

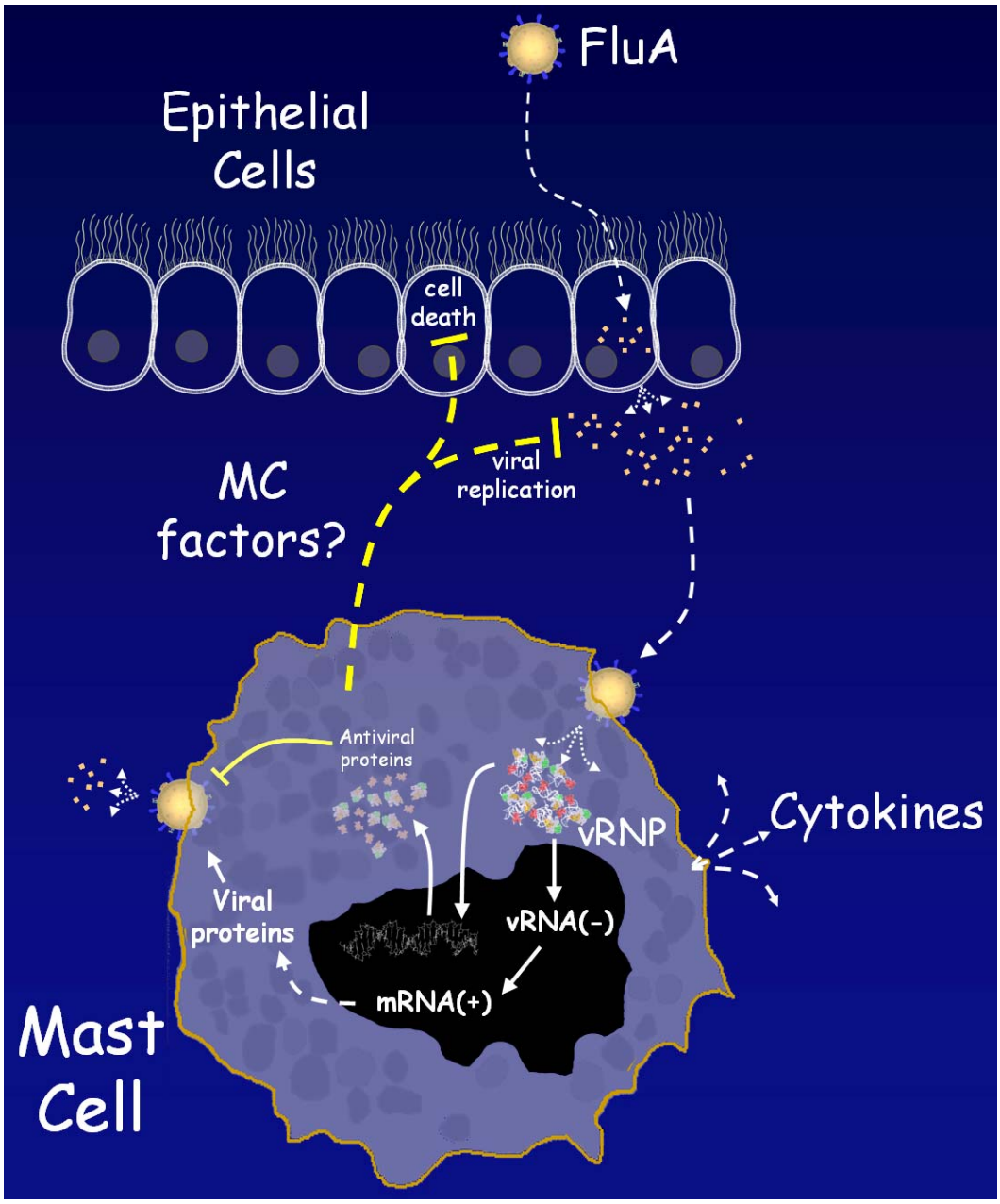

Figure 1.6: Conceptual model (original figure). In our conceptual model, FluA first replicates in epithelial cells to produce progeny virus, which could infect mast cells in the proximity. I hypothesize that FluA enters mast cells where vRNA

is transcribed into mRNA and replicated into cRNA. Viral proteins are translated and viral components are packaged and released as infectious FluA. FluA infection induces mast cell expression of antiviral genes for MxA, PKR, ISG15, and eNOS. FluA-induced IFNα,β released from epithelial cells would decrease the ability of FluA to infect nearby mast cells. Mast cells exposed to FluA release cytokines such as IL-6 and TGF-β. IFNα,β and TLR ligands produced from FluA-infected epithelial cells also induce cytokine release from mast cells. Mast cell interaction with epithelial cells results in decreased viral replication in and decreased cell death of epithelial cells.

6. Hypothesis

Mast cells are productively infected by FluA and respond to FluA infection by upregulating the expression of antiviral genes. Both FluA and other viral-associated stimuli induce mast cells to release mediators important in innate immunity. As mast cells are capable of initiating an innate immune response against FluA, mast cells can be protective against FluA infection of epithelial cells.

7. Objectives

1. To characterize the FluA replication cycle in human mast cells.

2. To compare FluA replication in human mast cells versus human epithelial cells.

3. To study the expression of antiviral genes in FluA-infected human mast cells.

4. To measure cytokine release from human mast cells treated with virus-associated stimuli: intact and UV-inactivated FluA, IFNα and β, and TLR ligands.

5. To determine the effect of mast cells on FluA infection of epithelial cells in a human mast cell-epithelial cell co-culture system.

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Chapter II: Influenza A Virus Replication and Infectivity in Human Mast Cells

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2. Introduction

Influenza virus causes a febrile respiratory disease in humans that ranges from self-limiting infection to primary viral pneumonia that may result in death (1). Of the three influenza virus types A, B, and C, influenza A virus (FluA) causes the majority of human disease (2). Although FluA can be associated with pandemics, yearly ongoing seasonal influenza results in significant morbidity and mortality, with an estimated half a million deaths worldwide every year (3).

An improved understanding of how the immune system defends against FluA is critical in the development of preventative and therapeutic strategies for influenza. Research on innate immunity against FluA has focused on the inflammatory and antiviral response of epithelial cells, the primary target of FluA replication (4). However, the complexity of immune responses against pathogens is being unraveled and involves the interplay of many cell types in the host. It is important to distinguish the roles of different immune cell types in the response against FluA to better appreciate the dynamic interactions within the immune system and elucidate critical players in host defense.

Mast cells are emerging as an important cell type in innate immunity with beneficial roles in host defense (5). Situated in vascularized tissues and abundant in mucosal surfaces, mast cells are in prime locations to encounter microbes and alert the immune system (6). Evidence first began to build for mast cell responses to bacterial and fungal infections, and more recently to responses against viruses (7). For example, dengue virus is capable of infecting mast cells and induces release of cytokines such as IL-1β and IL-6, as well as the chemokines CCL5, CCL3α, and CCL3β (8, 9). HIV-infected mast cells have also been shown to harbor HIV, keeping the virus dormant until cells are reactivated to release infectious virus (10).

Not only are mast cells capable of responding to viruses, they also respond to surrounding signals released from other cells during an infection. Based on their close association with epithelial cells at many sites in the body, it is not surprising mast cells and epithelial cells interact; they directly influence each other by cell-cell contact and indirectly by exchanging signals in the form of soluble mediators. For example, when epithelial cells and mast cells adhere, there is a decrease in mast cell degranulation (11). On the other hand, mast cells decrease epithelial cell release of secretory leukocyte protease inhibitor (SLPI) (12), a protein with antiviral activity against HIV (13). Since FluA induces a myriad of changes in epithelial cells, including antiviral, inflammatory, and apoptotic responses, these changes are likely to affect mast cell function. In turn, mast cells exposed to FluA could modulate the response of epithelial cells to the

initial infection. Understanding how mast cells respond to FluA could provide insight into signals present in the epithelial environment during an infection.

Currently, there is little evidence that human mast cells respond to FluA. However, FluA has been associated with increased histamine levels in the lungs of patients, implicating a potential interaction between FluA and mast cells (14). Another study showed that human mast cells can respond to UV-inactivated FluA by producing interferon (IFN)-α (15). To understand the response of human mast cells to FluA, it is essential to determine whether or not FluA infects mast cells and how FluA infection proceeds. During viral replication, FluA mRNA transcription is the first process we studied that requires host components. As the definition of infection varies, we consider that mast cells are infected once there is evidence that FluA is using host machinery i.e. mRNA transcription has occurred. In the current study, we have shown that FluA can productively infect mast cells through the process of viral entry, RNA replication and transcription, protein synthesis, but release a near absence of infectious progeny FluA particles. The low amounts of FluA released from mast cells suggest that FluA may induce anti-viral inhibitory mechanisms in mast cells.

3. Materials and Methods

Cell culture

All cells were cultured in a humidified incubator with 5 % CO_2 at 37^{o}C. Mycoplasma testing was performed once a month using the "MycoAlert® Mycoplasma Detection Kit" (Lonza Rockland, Inc., Rockland, ME, USA) to

ensure cell cultures were free from mycoplasma contamination. Briefly, 50 µl of cell culture supernatant was mixed with 50 µl of MycoAlert® reagent for 5 min and measured for 1st luminescence in a luminometer. Then, 50 µl of MycoAlert® substrate was added for 10 min and measured for 2nd luminescence. Samples are considered negative according to manufacturer's protocol if the ratio of 1st/2nd luminescence is < 1.0. Positive samples were immediately discarded from the incubator with subsequent testing of other cultures in that incubator. Incubators were periodically cleaned with detergent and 30% ethanol.

The immature human mast cell line HMC-1 (a kind gift from J.H. Butterfield, Mayo Clinic, Rochester, MN, USA) was cultured in Iscove's Modified Dulbecco's Medium (Invitrogen Canada Inc., Burlington, ON, Canada) supplemented with 5% fetal bovine serum (Invitrogen Canada Inc.), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. HMC-1 were passaged three times a week and were seeded at 1.0 x 10^5 cells/ml in 10ml of media in 75 cm^2 flasks (BD Falcon, BD Canada, Oakville, ON, Canada) at every passage. The cell concentration at the end of each passage reached 0.8 to 1.0 x 10^6 cells/ml. The more mature human mast cell line Laboratory of Allergic Diseases 2 (LAD2) (a generous gift from Drs. A.S. Kirshenbaum and D.D. Metcalfe, National Institutes of Health, Bethesda, MD, USA), was cultured in Stempro-34 SFM complete medium (Invitrogen Canada Inc.) supplemented with 100 ng/ml recombinant human stem cell factor (rhSCF, Peprotech Inc., Rocky Hill, NJ, USA) and 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. LAD2 were hemidepleted weekly i.e. half the volume of cell culture would be

removed, then cells were pelleted and resuspended in fresh media, and the newly resuspended cells would be added back to its original flask. Equal volumes of fresh media were added to the culture when LAD2 concentration exceeded 1.0 x 10^6 cells/ml. When LAD2 concentration was maintained between 1.0 to 5.0 x 10^5cells/ml the cell concentration approximately doubled weekly.

Primary human cultured mast cells (HCMC) were developed from $CD34^+$ peripheral blood mononuclear cells (PBMC) as described previously (16). Briefly, 100 ml of blood was drawn from healthy human donors in 10 ml heparinized Vacutainer™ tubes (BD Canada, Oakville, ON, Canada) and layered on Histopaque (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). $CD34^+$ progenitors were selected from the PBMC fraction using the "Human CD34 positive selection kit" (StemCell Technologies, Vancouver, BC, Canada). $CD34^+$ cells were cultured in Stempro-34 SFM complete medium supplemented with 100 ng/ml rhSCF and 100ng/ml rhIL-6 for 8 weeks, with 30 ng/ml rhIL-3 for the first week only. Isolated $CD34^+$ cells were seeded at 5.0 x 10^4 cells/ml. The volume of Stempro media was doubled every 3.5 d until the final volume reached 20 ml. When the volume in the flask reached 20 ml, bi-weekly hemidepletions were performed where half the old media would be replaced by fresh media while maintaining the total volume at 20 ml (see LAD2 hemidepletion above). At 8 weeks, the entire volume of old media was replaced by fresh media prior to use in experiments. Also at 8 weeks, > 99% of the cultured cells were confirmed as mast cells by tryptase and/or chymase staining (see below).

The human lung carcinoma cell line Calu-3 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured with MEM + Earle's salts (Invitrogen Canada Inc.) and L-glutamine supplemented with 10% fetal bovine serum, 10mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Calu-3 were passaged three times a week and were seeded at 1.0 x 10^5 cells/ml in 12 ml of media in 75 cm^2 flasks. The cell concentration at the end of each passage reached 0.6 to 0.8 x 10^6 cells/ml.

Tryptase/Chymase Immunohistochemical Staining

Intracytoplasmic staining was performed with modifications from methods described previously (16, 17). Cytospins of cells (5 x10^4/slide) were fixed in methanol/0.6% H_2O_2 for 30 min at room temperature (RT) to inhibit endogenous peroxidase activity and rinsed once with dH_2O. All incubations were performed in a humidified chamber. For chymase staining, 50μl of mouse anti-human mast cell chymase biotin-conjugated monoclonal antibody (MAB1254B, mouse $IgG_{1\kappa}$, Chemicon, Temecula, CA, USA) diluted to 3μg/ml in "DakoCytomation Antibody Diluent with Background Reducing Components" (S3022, Dako North Amercia, Inc., Carpinteria, CA, USA) was added to the slide and incubated overnight at 4°C. Slides were rinsed with 1x dH_2O, 3x TTBS (0.1%Tween in tris-buffered saline), then 1x dH_2O. Two drops of 1μg/ml horseradish peroxidase streptavidin (SA-5704, Vector Laboratories (Canada), Inc., Burlington, ON, Canada) was added to slides and incubated for 1 h at RT. Slides

were rinsed as above. One hundred μl of NovaRED peroxidase substrate (SK-4800, Vector Laboratories (Canada), Inc.) were added and incubated at RT for 2 to 10 min until staining developed. The reaction was stopped by immersing slides in dH_2O.

Tryptase staining was performed similarly as for chymase staining with the following differences: we used mouse anti-human mast cell tryptase alkaline phosphatase-conjugated monoclonal antibody (MAB1222A, mouse IgG_1, Chemicon) diluted to 1.5 μg/ml. Vector® Blue alkaline phosphatase substrate (SK-5300, Vector Laboratories (Canada), Inc.) was used in the staining reaction. Slides were permanently mounted with Fluoromount-G (0100-01, Southern Biotech, Birmingham, AL, USA).

Influenza Virus and Ultraviolet (UV)-Inactivation

Influenza A virus (FluA) of the A/PR/8/34 strain (H1N1) grown in allantoic fluid of duck eggs was obtained as a kind contribution from Dr. K.P. Kane (University of Alberta, Edmonton, AB, Canada). Stock FluA hemagglutination titer was 16,000 HAU/ml. We also determined the $TCID_{50}$/ml (see below for method) for the stock virus, and calculated that 20 HAU/ml = 1 x 10^4 $TCID_{50}$/ml. Stock FluA was diluted 50x with PBS prior to UV-inactivation,. UV-inactivation of virus was performed using a UV lamp (ENF-280C, Spectroline, Westbury, NY, USA) at 254 nm of wavelength, placed 20 cm from the sample in sterile conditions. Effective inactivation was confirmed by positive

hemagglutination indicating intact viral envelope proteins and negative hemadsorption indicating no infectivity (see below for assay details).

Virus expousure

HMC-1, LAD2, and HCMC were plated at 1.0 x 10^6 cells/ml in 1 ml of media, and Calu-3 was plated at 1.5 x 10^5 cells/ml in 5.0 ml of media in 6-well tissue culture plates. Mast cells were rested for 1 h at 37^{o}C prior to FluA treatment. Calu-3 were incubated at 37^{o}C overnight prior to FluA treatment to allow monolayer formation. 1 ml of FluA was added to the cells at various concentrations for 1 h to allow for virus adsorption onto the cell surface. After 1 h of FluA adsorption, cells were washed with 3 ml of PBS, 3 times. No FluA was detected by hemagglutination assay (see below) in the PBS of the 3rd wash. Cells were incubated in the original volume of fresh media for various time periods.

Cell Viability

Cell number was counted using a BRIGHT-LINE^{TM} hemacytometer (Hausser Scientific, Horsham, PA, USA). Cell viability was calculated as the percentage of live cells as determined by trypan-blue (0.4%) exclusion.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) for Specific Species of RNA

Total RNA was isolated from cell pellets using a RNeasy Plus Mini Kit (Qiagen Inc., Mississauga, ON, Canada). From 0.1μg of RNA, cDNA was

synthesized using the Superscript® III First-Strand Synthesis System (Invitrogen Canada Inc.). Oligo-dT was used to transcribe all messenger RNA (mRNA). We performed PCR for the mRNA of FluA polymerase B1 (PB1, forward primer: 5'-AAC GAT GGA GGT TGT TCA GC-3', reverse primer: 5'-AAA CCC CCT TAT TTG CAT CC-3', 427 bp product), non-structural protein 1 (NS1, forward primer: 5'-CTT CGC CGA GAT CAG AAA TC-3', reverse primer: 5'-GTG GAG GTC TCC CAT TCT CA-3', 535 bp product), and hemagglutinin (HA, forward primer: 5'-GGA GAA GGA GGG CTC ATA CC-3', reverse primer: 5'-CCT GAC GTA TTT TGG GCA CT-3', 469 bp product). The cDNA products for mRNA, vRNA, and cRNA of hemagglutinin was confirmed by sequencing. β-actin forward primer was 5′-GGCATC CTC ACC CTG AAG TA-3′ and reverse primer was 5′-AGG GCA TAC CCC TCG TAG AT-3′, yielding a 326 bp product. Gene specificities were ensured using the BLAST sequencing program at GenBank. PCR amplification of FluA genes was performed using recombinant Taq DNA polymerase (Sigma-Aldrich Canada Ltd.), in cycles of 45 sec at 94^{o}C, 45 sec at 58^{o}C, and 1 min at 72^{o}C with a final stage of 10 min at 72^{o}C. The same conditions were used for amplification of β-actin, except with an annealing temperature of 55^{o}C. We optimized the cycle number for PB1, NS1, HA, and β-actin to be 32, 32, 28, and 28 cycles, respectively, to be within the exponential phase of amplification. PCR products were visualized under UV light after separation on a 1.5% agarose gel containing ethidium bromide.

The HA forward primer listed above was used as a gene-specific primer to transcribe negative-sense viral genomic RNA (vRNA), and a complementary

RNA (cRNA)-specific reverse primer (5'-AGT AGA AAC AAG GGT GTT-3') was designed to transcribe the positive-sense cRNA of the FluA HA gene. Subsequently, PCR was performed as described above. DNA sequencing of the mRNA, vRNA, and cRNA of HA was performed by the DNA Core Services Lab (University of Alberta, Edmonton, AB, Canada) and products were tested as > 99% homologous using the BLAST sequencing program. Thus, we could distinguish the three different species of RNA using this method.

Western Blot

Proteins from the flow-through of the RNeasy Spin Column were precipitated using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad Laboratories, Hercules, CA, USA) and reconstituted with resuspension buffer (Tris-Cl/Urea/SDS) to a final protein concentration of 1 μg/ml using the BCA (bicinchoninic acid) protein assay (Promega Corp., Madison, WI, USA). Samples were loaded onto a 10% SDS-polyacrylamide gel and proteins were separated using electrophoresis. Proteins were blotted onto PVDF membranes using semi-dry transfer technique. FluA proteins were detected using an anti-serum to FluA of the A/PR/8/34 strain from an immunized rabbit (generous gift from Dr. Earl Brown, University of Ottawa, Ottawa, ON, Canada). Isotype controls were performed using normal rabbit serum (also from Dr. Earl Brown). β-actin was detected using a mouse monoclonal antibody (AC-15) against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). IRDye®680-conjugated polyclonal goat anti-mouse IgG and IRDye®800CW- conjugated anti-rabbit IgG were used with the Odysey® Infrared

Imaging System for immunodetection of proteins (Li-cor Biosciences, Lincoln, NE, USA).

Hemagglutination Assay

A hemagglutination assay was used to quantify total FluA particles in a sample by the ability to bind red blood cells (RBC) (18). A 50 µl supernatant sample was placed in a round-bottom 96-well microtitre plate (BD Biosciences, Mississauga, ON, Canada) and serially diluted 2-fold along the long axis of the plate using PBS/0.1% BSA. An equal volume of 0.5% human RBC solution from phlebotomy of healthy donors was mixed with the diluted samples and allowed to incubate for 2-3 h at room temperature. Positive hemagglutination was verified with the presence of lattice formation, while negative hemagglutination was verified by a dot formation of RBC at the bottom of the well. The lowest dilution at which hemagglutination was present was determined as the hemagglutination unit (HAU) of that sample. Viral titres were expressed in HAU/ml where volume is calculated based on the 1 ml of FluA added during adsorption of virus.

Hemadsorption Assay

The hemadsorption assay detects the presence of infectious FluA particles in a sample (19). We performed the hemadsorption assay with the following modifications: Calu-3 is capable of binding RBC after infection with FluA, and was used as the substrate for infectious FluA detection. 48-well plates containing Calu-3 monolayers at > 80% confluency were treated with 125 µl samples placed

in each well for 1 h to allow FluA to bind to the cell surface, then washed 3x with 0.5 ml PBS. After 5 day (d) incubation at 37^{o}C, media was removed, 2 ml of 0.5% RBC suspension was added to cover the wells, and plates were placed at 4^{o}C for 30 min. Cell monolayers were washed gently 2x with 0.5 ml PBS. After removing the last PBS wash, plates were observed with an inverted microscope to detect positive hemadsorption as evidenced by RBC binding in rosette or plaque formations. No hemadsorption was observed with sham-treated wells.

Determination of 50% Tissue Culture Infectious Dose ($TCID_{50}$)/ml

$TCID_{50}$/ml was determined by the dilution at which 50% of FluA-infected wells show hemadsorption. Calu-3 monolayers at > 80% confluency were treated with 125 μl of serial 10-fold dilutions of each sample in 8 identical wells. Plates were incubated for 5 d at 37^{o}C and assayed for hemadsorption. $TCID_{50}$/ml was calculated from the data using the Reed-Muench method (20).

Immunofluorescence Staining and Confocal Microscopy

Cytospins of cells (3.3 x10^{4}/slide) were fixed and permeabilized with 3% paraformaldehyde/0.5% Triton X-100 for 5 min on ice. Washes consisted of 3x wash with PBS for 5 min. Cells were fixed with 3% paraformaldehyde for 20 min on ice and washed. Cells were blocked with 0.5% gelatin in PBS for 30 min at RT and rinsed 1x with PBS. 100 μl/slide of rabbit anti-hemagglutinin polyclonal IgG antibody (ab46968, Abcam Inc., Cambridge, MA, USA) or rabbit IgG antibody at 1 μg/ml was added to the cells and incubated overnight at 4^{o}C in a humidified

chamber. Cells were washed and 150 µl/slide of goat anti-rabbit IgG Alexa Fluor 488 antibody (A-11034, Molecular Probes®, Invitrogen Canada Inc.) at 2.5 µg/ml was added to the cells as the secondary antibody. DAPI (D1206, Invitrogen Canada Inc.) at 1.7 µg/ml was also added to the cells for nucleic acid staining. Secondary antibody and DAPI was incubated with the cells for 30 min at RT and cells were washed. Cells were rinsed 1x with dH_2O and slides were permanently mounted with Fluoromount-G. Cells were visualized using a confocal microscope (Fluoview™ FV 1000, Olympus America Inc., Center Valley, PA, USA).

Data and Statistical Analysis

Densitometric analysis was performed using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA). The average background densitometric value was calculated from three independent locations on the image. The area of the circle used to capture the densitometric value was optimized for each image to enclose the thickness of the largest band. One single measurement was taken per band for each image. The average background was subtracted from each value. The graphs were created using GraphPad Prism 3.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis was performed using GraphPad Instat 3.05 (GraphPad Software, Inc.). The student's paired t-test when comparing two sets of values and one-way analysis of variance (ANOVA) was used when comparing greater than two sets of values. A p-value of < 0.05 was considered significant.

4. Results

FluA treatment affects viability of LAD2

To analyze the response of mast cells to FluA treatment, we exposed mast cells to FluA *in vitro*. We investigated whether FluA treatment affects the viability and cell number of human mast cells in three individual experiments (except *n*=1 for HCMC at 6 d and 8 d). The cell number and viability of LAD2 followed similar trends in response to 0 – 100 HAU/ml of FluA treatment (Fig. 2.1A). Viability was significantly lower ($p < 0.0001$) with FluA treatment at 100 HAU/ml (58.1 ± 2.5 %) when compared with 0 HAU/ml treatment (90.0 ± 4.1 %) (Fig 2.1A.) Cell number was also significantly lower ($p = 0.037$) with FluA treatment at 100 HAU/ml (65.4 ± 9.7 %) when compared to 0 HAU/ml treatment (set at 100 %). Viability was determined for LAD2, HCMC, and Calu-3 from 0 to 8 d post-sham or post-FluA treatment (Fig. 2.1B-D). LAD2 demonstrated significantly lower viability at 4, 5, and 8 d post-FluA treatment (20 HAU/ml) compared to sham (Fig. 2.1C). Viability between sham and FluA treatments in Calu-3 and HCMC were not significantly different (Fig. 2.1B, D).

Transcription and RNA replication of FluA takes place in human mast cells.

We investigated whether transcription and replication of the different FluA RNA species occurs in two mast cell lines LAD2 (Fig. 2.2A-D, *n*=3) and HMC-1 (Fig. 2.2E-F, *n*=2). Genomic FluA RNA was identified by the presence of viral vRNA, FluA transcription was identified by the presence of viral mRNA, and FluA RNA replication was identified by the presence of viral cRNA. RT-PCR for

specific species of RNA for the hemagglutinin (HA) gene of FluA was used to evaluate expression of vRNA, mRNA, and cRNA by exposing mast cells to 0.01 to 100 HAU/ml of FluA. RT-PCR of stock FluA showed presence of genomic vRNA, but mRNA and cRNA were not detected (data not shown), confirming that the detected mRNA and cRNA in infected cells were newly synthesized. Representative gels for LAD2 and HMC-1 samples are shown in Fig. 2.2A and 2.2E, respectively. Study of the different RNA types in the sham treatments did not generate a signal above background. For each particular RNA type, the highest densitometric value in the experiment was used as 100% and all other values were compared to this value. LAD2 expression of FluA vRNA was not significantly different between the 100 HAU/ml dose compared to any of the lower doses; the lowest dose tested was 0.01 HAU/ml (Fig. 2.2B). LAD2 expression of mRNA did not differ between the two highest doses (100 and 10 HAU/ml), but doses 1 HAU/ml and lower generated significantly less cRNA than the 100 HAU/ml dose (Fig. 2.2C). LAD2 expression of FluA cRNA followed a dose response, where 100 HAU/ml of FluA generated the highest RNA levels, with smaller doses generating significantly less mRNA products (Fig. 2.2D). The difference between the dose responses of mRNA production compared with vRNA production may reflect that mRNA transcription and vRNA production from cRNA are regulated by different mechanisms, as suggested by previous studies (21). FluA-treated HMC-1 showed a dose response trend, with lower doses generating less vRNA, mRNA, and cRNA (Fig. 2.2G). These dose response experiments showed that the two highest FluA doses generated similar amounts of

vRNA and mRNA, while cRNA was significantly higher with 100 HAU/ml. Thus, we chose to use 20 HAU/ml of FluA, a dose between the highest two doses, for subsequent time course comparison studies.

To compare the amount and duration of mRNA transcribed between epithelial cells and mast cells, we performed a time course analysis of FluA mRNA expression in human mast cells and lung epithelial cells. Transcripts for HA, a FluA envelope protein, and polymerase B1, a FluA polymerase protein, were analyzed. We used LAD2 and HMC-1 mast cell lines along with primary human cultured mast cells (HCMC) from peripheral blood $CD34^+$ progenitors. Our positive control for FluA infection was Calu-3, a human lung epithelial cell line known to be permissive to FluA infection (22). There was no detectable signal for FluA in the sham treatments for all cell types (Fig. 2.3). Of note, the 0 h time point indicates the beginning of the post-FluA treatment period, after 1 h of FluA adsorption and subsequent washes. In both Calu-3 and mast cells, the time course of mRNA production followed similar trends. HA and PB1 mRNA were detectable within 6 h post-FluA treatment, peaking at 1 d (except for HCMC, which peaked at 6 hr), and remained detectable at 5 d (Fig. 2.3). The peak signal intensity for HA and PB1 as measured by the FluA mRNA to β-actin mRNA ratio was similar for Calu-3 and LAD2, ranging from approximately 1.0 to 1.3 (Fig. 2.2B-C, G-H). In HMC-1, the peak intensity for HA was lower, at 0.5 (Fig. 2.3E). HCMC showed higher peak intensity for HA mRNA at 2.4 compared with PB1 at 1.4 (Fig. 2.3J-K). Overall, human mast cells appear to produce relatively similar

amounts of FluA mRNA as compared to Calu-3 epithelial cells and over comparable time courses as Calu-3.

FluA protein synthesis takes place in human mast cells

As PCR is an extremely sensitive technique, the possibility exists that contamination of mast cell cultures with other cell types, such as monocytes, is responsible for the detected FluA RNA in Fig. 2.2. In this next set of experiments, we show that both LAD2 and HCMC express FluA proteins with comparable or higher signal than that of Calu-3 when loaded at the same total protein concentration. This signal is unlikely to be attributed to a 1% contamination in culture, thus providing support that it is indeed mast cells that are infected by FluA. Since LAD2 and HCMC expressed FluA mRNA in a similar fashion as Calu-3 (Fig. 2.3), we investigated these cells for FluA protein expression in comparison with Calu-3. Western blot analysis was used to determine when FluA proteins were expressed. The FluA proteins recognized by an anti-FluA serum (gift from Dr. E. Brown, U of Ottawa) are: polymerase complex proteins (Ps) consisting of PB1, PB2, and PA; HA; NP; M1; and NS1 (confirmed by correspondence with Dr. E. Brown) (23). None of these proteins were detected in cells from sham or UV-inactivated FluA treatments (Fig. 2.4).

All FluA proteins recognized by the anti-FluA serum were detected in Calu-3, LAD2, and HCMC, with the exception of Ps in Calu-3 (Fig. 2.4A-C). The expected molecular weight (MW) for Ps is above 150 kDa and was detected in LAD2 and HCMC from 2 to 6 d post-FluA treatment (Fig. 2.4B-C). HA (70 kDa)

could be detected at 6 h in Calu-3, LAD2, and HCMC and ended by 4 d post-FluA treatment in Calu-3 and 6 d post-FluA treatment in LAD2 and HCMC (Fig. 2.4A-C). Expression levels of HA was sustained from 2 to 6 d post-FluA treatment in LAD2 and HCMC, but in Calu-3, started to diminish at 4 d post-FluA treatment. NP at 58 kDa was detected in all three cell types from 6 h to 6 d post-FluA treatment (Fig. 2.4). M1 at 27 kDa and NS1 at 26 kDa have similar gel mobilities. Interestingly, only one band corresponding to this MW was expressed in Calu-3, while two bands (M1 and NS1) were expressed in LAD2 and HCMC at 6 h to 6 d post-treatment with FluA (Fig. 2.4). Non-specific bands at approximately 40 kDa were detected in all treatments including sham (Fig. 2.4).

Release of FluA particles from Calu-3 compared with MDCK

To determine that the human lung carcinoma cell line Calu-3 could be used for production of FluA particles, we compared the release of FluA from Calu-3 with that from MDCK, a standard canine cell line for growing FluA (24). Total FluA particle release was determined by the hemagglutination assay. Treatment with 0 HAU/ml showed no detectable FluA release from Calu-3 or MDCK (Fig. 2.5). We evaluated the FluA dose at which release of FluA progeny virus was detected in the supernatant of MDCK and Calu-3 at 3 to 6 d post-FluA treatment (Fig. 2.5). FluA particle release was detected from MDCK at 3 d at 0.2 HAU/ml, on day 4 at 0.002 HAU/ml, on day 5 at 0.02 HAU/ml, and on day 6 at 0.002 HAU/ml of FluA treatment (Fig. 2.5A-D). FluA particle release was detected from Calu-3 day 3 to 6 at 0.02 HAU/ml of FluA treatment (Fig. 2.5E-H).

Release of FluA particles from human mast cells

Next, we investigated whether FluA undergoes productive infection in human mast cells by measuring the amount of FluA particles released into FluA-treated mast cell supernatants. We compared the release of FluA from mast cells and epithelial cells using the hemagglutination assay. Hemagglutination is only detectable in the presence of FluA. Release of FluA particles from Calu-3, LAD2, HMC-1, HCMC was measured from 0 to 6 d post-FluA treatment at 20 or 100 HAU/ml. No release of FluA particles was detected from 0 d treatments or UV-inactivated FluA treatments at 100 HAU/ml with any of the cell types (Fig. 2.6). At 20 HAU/ml of FluA treatment, Calu-3 release of FluA particles was detected at 1 d with significant release at 3, 4, and 5 d (2533.5 ± 910.0 HAU/ml, $p = 0.039$ for 5 d), when compared with 0 d (Fig. 2.6A). LAD2 and HCMC did not release significant amounts of FluA with 20 HAU/ml of FluA treatment (Fig. 2.6C, E). HMC-1 showed no release of FluA at 20 HAU/ml dose of FluA treatment (Fig. 2.6G), consistent with its low amount of transcribed FluA mRNA (Fig. 2.3C-D).

We increased the dose of FluA treatment to see if we could detect higher levels of FluA particle release from LAD2 and HCMC. By increasing the dose of FluA to 100 HAU/ml, we were able to detect release of FluA particles from Calu-3, LAD2, and HCMC at 2, 4, and 6 d (Fig. 2.6B, D, F). At 6 d, Calu-3 released 3200 HAU/ml, LAD2 released 160 HAU/ml, and HCMC released 320 HAU/ml of FluA (n=1). In the same experiment, treatment of all cell types with 100 HAU/ml of UV-FluA did not produce virus (Fig. 2.6B, D, F).

Release of infectious FluA particles from Calu-3 compared with MDCK

To determine the sensitivity of Calu-3 compared with MDCK to detect infectious FluA release in the hemadsorption assay, we compared the FluA dose which resulted in detectable FluA infectivity by hemadsorption in MDCK and Calu-3 at 4, 5, and 6 d post-FluA treatment. Infectious FluA, as determined by positive hemadsorption, was detected in MDCK at 0.002 HAU/ml of FluA at 4, 5, and 6 d following exposure in 2, 2, and 3 experiments out of 5, respectively (Table 2.1, upper). In 1 experiment, infectious FluA was detected in MDCK treated with 0.0002 HAU/ml of FluA at 5 d (Table 2.1, upper). Similarly, Calu-3 showed positive hemadsorption when treated with 0.002 HAU/ml of FluA at 5 and 6 d in 2 and 2 experiments out of 5, respectively (Table 2.1, lower). At 4 d, infectious FluA was detectable in Calu-3 treated with 0.02 HAU/ml of FluA in 4 experiments out of 5 (Table 2.1, lower).

Release of infectious FluA particles from human mast cells

We tested whether human mast cells release infectious FluA particles by measuring the infectivity of supernatants from FluA-treated HMC-1 and LAD2 in comparison to Calu-3. FluA-treated supernatants from HMC-1, LAD2 and Calu-3 were tested for infectious virus by detecting positive hemadsorption on Calu-3 monolayers. Calu-3 released infectious FluA when treated with 0.02 to 20 HAU/ml of FluA (3 out of 3 experiments for each dose) and with 0.002 HAU/ml in 2 out of 3 experiments (Table 2.2, upper). LAD2 released infectious FluA when treated with 100 HAU/ml in 6 out of 6 experiments and with 20 HAU/ml of FluA

in 1 out of 6 experiments (Table 2.2, lower). HMC-1 treated with doses of FluA up to 100 HAU/ml released no detectable infectious FluA (Table 2.2, lower). These results show that release of infectious FluA from mast cells was only detectable at > 4 log higher doses of initial FluA infection compared with the FluA dose used in epithelial cells (Table 2.2).

Next, we compared the time course of infectious FluA release from human mast cells with that of Calu-3. At 20 HAU/ml of FluA treatment, Calu-3 released infectious FluA in 2 out of 3 experiments at 6 hr, and in 3 out of 3 experiments for each time point from 1 to 5 d (Table 2.3, upper). At the same dose, LAD2 released infectious FluA at 6 h (2 out of 3 experiments) and at 2 d (1 out of 6 experiments) (Table 2.3, upper). No detectable infectious FluA was released by HMC-1 or HCMC at any time points when treated with 20 HAU/ml of FluA (Table 2.3, upper). However, by increasing the dose of FluA to 100 HAU/ml, we were able to detect release of infectious FluA from LAD2 at 6 h to 2 d, and from HCMC at 6 h and at 4 d (Table 2.3, lower). At this dose, Calu-3 released infectious FluA from 6 h to 6 d (Table 2.3, lower).

To quantify the titer of infectious FluA released, we determined the log $TCID_{50}$/ml value of Calu-3, LAD2, and HCMC supernatants from 0 to 6 d post-treatment with 100 HAU/ml of FluA. The sensitivity of this method was 0.5 log $TCID_{50}$/ml. The infectious titre of Calu-3 supernatants was detectable 2 to 6 d of FluA treatment, ranging from 3.5 log $TCID_{50}$/ml at 2 d to 2.9 log $TCID_{50}$/ml at 6 d (Fig. 2.7). The infectious titres of both LAD2 and HCMC supernatants were below the detectable limit of this assay. The $TCID_{50}$ experiments revealed that

although some of the FluA-infected mast cell supernatants had detectable infectious virus in the hemadsorption assay (Table 2.3), less than 50% of Calu-3 wells in tissue-culture would show positive hemadsorption.

Confocal microscopy of HA expression in HCMC and Calu-3

Although FluA transcription and protein synthesis were similar in human epithelial cells and mast cells, Calu-3 released at least 100-fold more progeny FluA particles than all human mast cells tested (Fig. 2.6-7). This suggested that FluA viral replication was restricted at a post-translational step in mast cells. Thus, we used confocal microscopy to visualize any differences in the cellular distribution of a FluA protein, HA, in Calu-3 compared with HCMC. We used 2 d post-FluA treatment since this is the time of maximal FluA protein expression (Fig. 2.4). DAPI staining was used to visualize the nucleus, while a FITC secondary antibody was used to show HA signal. Calu-3 showed diffuse cytoplasmic staining and plasma membrane distribution of HA (Fig. 2.8). In contrast, HCMC showed diffuse nuclear HA staining and several punctate dots of HA staining in the cytoplasm in 2 independent experiments (Fig. 2.8). Approximately 30 – 50% of visualized HCMC and nearly 100% of Calu-3 were positive for HA staining, although this was not quantified using software (data not shown). Although it is well-known that mast cell granules autofluoresce with FITC staining, we would expect the entire population of HCMC to show positive staining if the signal was due to autofluorescence only (25). These confocal images show that viral HA did not localize to the plasma membrane in mast cells,

suggesting that transport of HA to the cell surface is defective or blocked by a mast cell antiviral mechanism.

5. Discussion

We found that FluA is capable of infecting human mast cell lines as well as primary mast cells cultured from human peripheral blood progenitors. While epithelial cells generated greater numbers of progeny FluA than the initial infecting dose of FluA, infected mast cells did not produce significant amounts of virus. FluA infection proceeded through RNA transcription in mast cells in a time course similar to that in epithelial cells (Fig. 2.3). Indeed, FluA mRNA transcription followed similar trends between LAD2 and Calu-3 at corresponding time points (Fig. 2.3). FluA proteins were detectable starting at 6 h post-FluA treatment in both mast cells and Calu-3 (Fig.4). However, expression of FluA proteins was no longer detectable in Calu-3 at 4 d post-FluA treatment, whereas LAD2 and HCMC expressed FluA protein for up to 6 d (Fig. 2.4).

In contrast to RNA and protein synthesis, mast cells did not release significant amounts of FluA particles as determined by hemagglutination. Also, infectious FluA particles were only detected from mast cell supernatants at high doses of initial FluA infection compared with epithelial cells. This near absence in the release of FluA particles by mast cells could explain the higher levels of FluA protein expression inside mast cells as a result of an accumulation of viral proteins. While release of infectious FluA from Calu-3 was detected with 0.002 HAU/ml of FluA (Table 2.1), release of infectious FluA from mast cells was rarely detected at

20 HAU/ml (Table 2.3), a 4 log higher dose. Only by increasing the FluA dose to 100 HAU/ml were we able to observed infectious FluA particle release from LAD2 and HCMC (Table 2.3). From our observations, it appears that FluA is capable of producing sufficient amounts of viral RNA and proteins in mast cells. However, we speculate that at a post-translational phase, there is either a defect in the machinery of mast cells necessary for completion of the FluA life cycle or that mast cells have antiviral mechanisms to prevent the completion of the FluA life cycle. Immunofluorescence staining for HA of FluA and imaging with confocal microscopy showed that HA protein expression was restricted to the nucleus and cytoplasm of mast cells with no localization at the plasma membrane (Fig. 2.8). This provides evidence that although FluA proteins are produced in mast cells, the proteins are unable to be transported to the proper location for assembly of new viruses. Further studies are needed to investigate the means by which FluA release is reduced in mast cells compared with epithelial cells. Possibly, mast cells produce antiviral proteins that directly inhibit interactions between various FluA proteins that are necessary for viral assembly. Thus, future studies may reveal antiviral mechanisms unique to mast cells that could be harnessed for antiviral drug development.

Our study is the first to show that human mast cells are susceptible to infection by FluA to produce infectious virus. We measured the log $TCID_{50}$/ml of our stock FluA and determined that 100 HAU/ml, the dose at which mast cells produce infectious FluA, is equivalent to 4.5 log $TCID_{50}$/ml. This FluA dose is comparable to peak median FluA titres found in nasopharyngeal washes of

influenza patients (4.8 log $TCID_{50}$/ml) (26) and in human experimental influenza (4.2 log $TCID_{50}$/ml) (27). We are also the first to show that FluA transcription and protein synthesis takes place in mast cells. Additionally, our direct comparison of FluA infection in mast cells and epithelial cells is valuable for understanding the varied responses of different cell types to FluA.

Our results complement previous studies looking at virus infection of mast cells. King et al. demonstrated that dengue virus is capable of infecting a human mast cell/basophil line to produce infectious virus, and that virus production can be enhanced using a dengue-specific antibody (8). Another study showed that patients with acquired immunodeficiency syndrome (AIDS) have HIV-infected mast cells, and that mast cells cultured from uninfected patients are capable of being infected with HIV (28). Interestingly, mast cells infected with reovirus were shown to mediate chemotaxis of NK cells, providing evidence that mast cells may contribute to the host response against viral stimuli (29). As another example, respiratory syncytial virus (RSV) does not infect mast cells, but instead, activates mast cell degranulation through virus-specific IgE (30). However, the previous researchers did not explore the life cycle of these viruses in mast cells and how viral replication differs in mast cells compared to other cell types.

So far, mast cells have been overlooked as a potential player in FluA infection. However, the results of this study indicate possible new roles for mast cells. It would be interesting to examine whether mast cells are infected in influenza patients, and investigate the location of mast cell-FluA interactions in the human lung. Secondly, except for one study demonstrating that mast cells

produce interferon-α when exposed to different viruses (15), the role of mast cell innate antiviral mechanisms in response to viruses has not yet been explored. Potentially, mast cells have distinct antiviral mechanisms that determine the susceptibility of mast cells to different viruses. There is substantial evidence that mast cells respond to viral stimuli by releasing various cytokines and chemokines (31), and it would be worthwhile to test whether the production and secretion of these mediators are modulated by FluA infection.

Also, Shirato et al. showed that mast cell degranulation is induced when co-cultured with RSV-infected epithelial cells (32), suggesting an important role for epithelial-mast cell interactions in the pathogenesis of viral infections. As mentioned before, mast cells and epithelial cells exist in proximity in the lungs. There is evidence of communication between these two cell types in literature; for example, co-culture models show that mast cells increase epithelial cell proliferation while epithelial cells promote mast cell survival (33, 34). By studying epithelial-mast cell interactions in the context of FluA infection, we could discover that mast cells are protective against FluA infection of epithelial cells. Alternatively, we could reveal that FluA-infected epithelial cells send signals to activate mast cell defense mechanisms against viral invasion.

In conclusion, our results show that human mast cells are infected by FluA *in vitro* and undergo FluA mRNA transcription and protein synthesis. FluA-infected mast cells release infectious virus, albeit at a much lower level than epithelial cells. The mechanisms by which mast cells are less permissive to a complete FluA replication cycle need to be clarified. Our research on the

characterization of FluA infection in mast cells contributes to the evolving evidence for the involvement of mast cells in innate immunity against viruses.

6. Figures

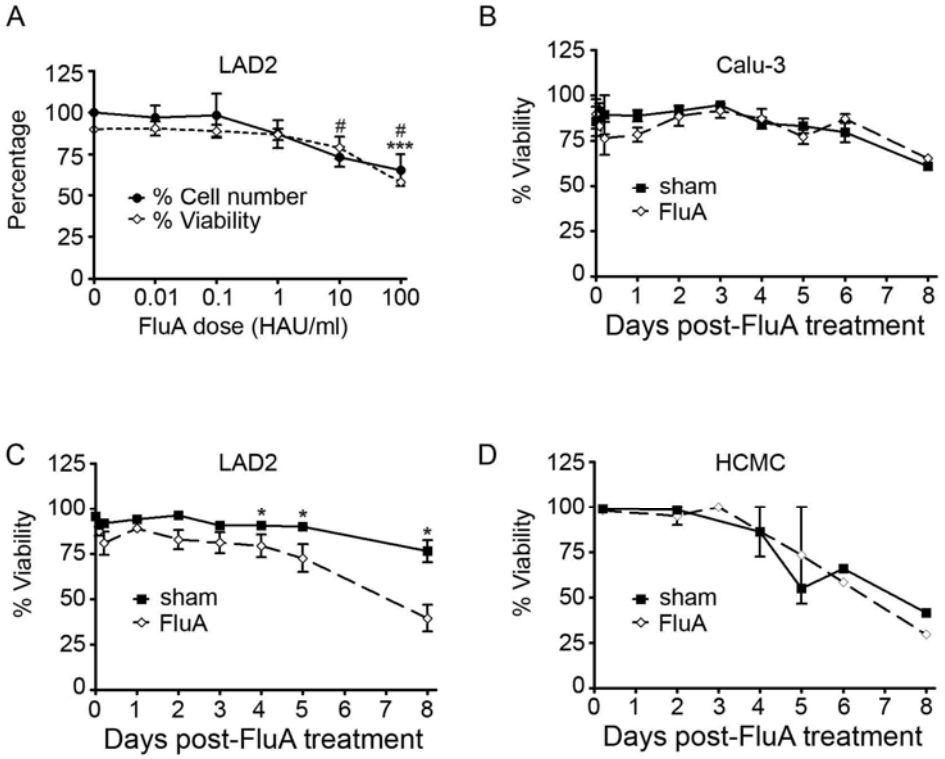
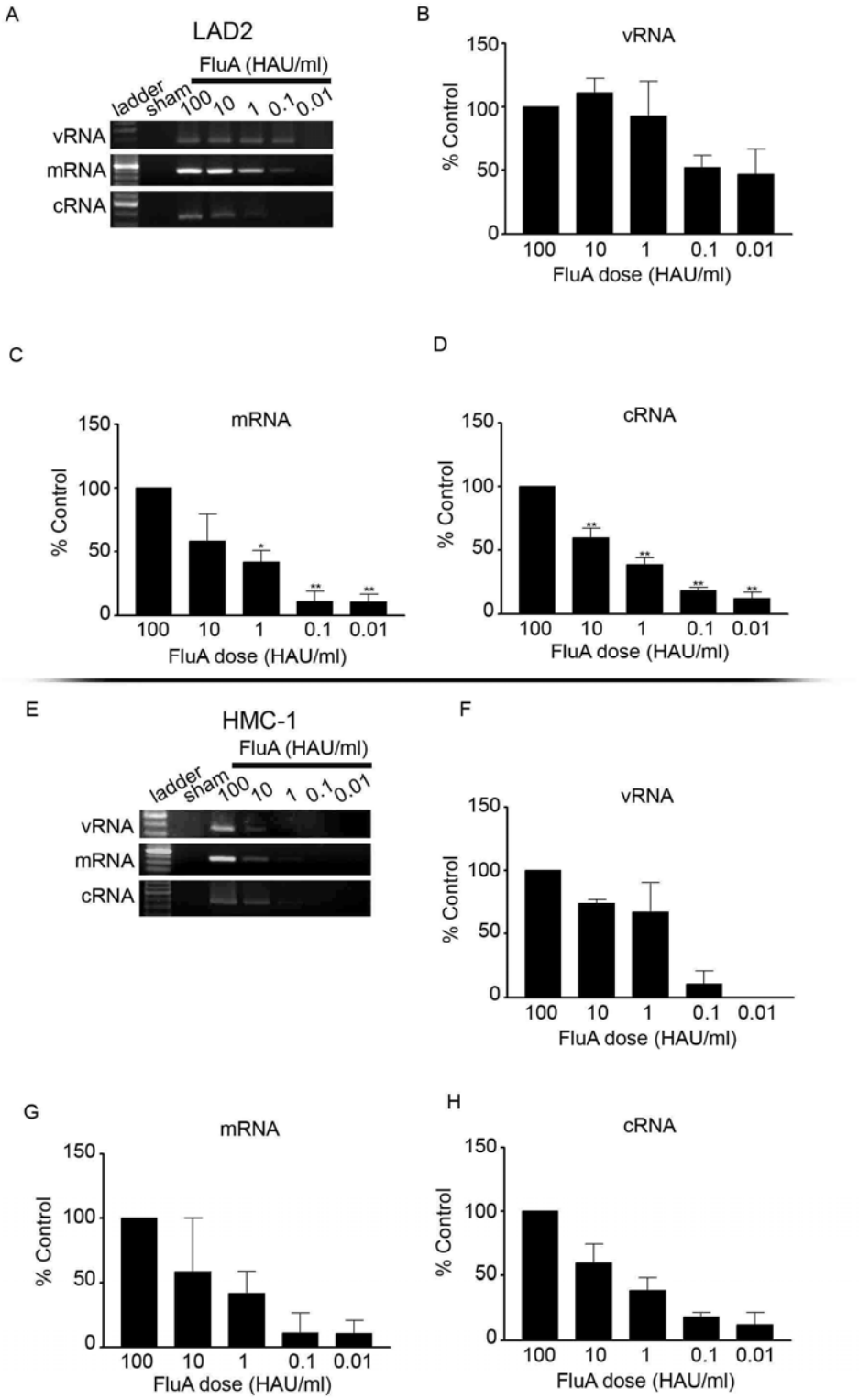


Figure 2.1: Cell number and viability of human mast cells and lung epithelial cells treated with FluA. Sham treatment (0 HAU/ml) consisted of media only. Cell number was calculated as percentage of sham (% sham) where sham was set at 100%. Viability was determined as percentage of live cells (% Viability). Note that SCF was added in the fresh media at 0 days post-FluA treatment. (A) Cell

number and viability of the human mast cell line LAD2. Cells were sham-treated or FluA-treated from 0.01 to 100 HAU/ml for 2 d. (B,C,D) Viability of the human lung epithelial cell line Calu-3, LAD2, and primary human cultured mast cells (HCMC), respectively, was determined at 0 to 8 d post-treatment with sham or FluA at 20 HAU/ml. Statistical significance for cell number is represented as # $p < 0.5$ in panel A; statistical significance for cell number is represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, when compared to sham treatments in panels A-D. ($n = 3$).


A
LAD2
FluA (HAU/ml)
ladder
sham
100
10
1
0.1
0.01
vRNA
mRNA
cRNA
B
vRNA
% Control
150
100
50
0
100 10 1 0.1 0.01
FluA dose (HAU/ml)
C
mRNA
% Control
150
100
50
0
*
**
**
100 10 1 0.1 0.01
FluA dose (HAU/ml)
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cRNA
% Control
150
100
50
0
**
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**
**
100 10 1 0.1 0.01
FluA dose (HAU/ml)
E
HMC-1
FluA (HAU/ml)
ladder
sham
100
10
1
0.1
0.01
vRNA
mRNA
cRNA
F
vRNA
% Control
150
100
50
0
100 10 1 0.1 0.01
FluA dose (HAU/ml)
G
mRNA
% Control
150
100
50
0
100 10 1 0.1 0.01
FluA dose (HAU/ml)
H
cRNA
% Control
150
100
50
0
100 10 1 0.1 0.01
FluA dose (HAU/ml)

Figure 2.2: Transcription and RNA replication of FluA in mast cells. PCR analysis of the viral RNA species: viral genomic RNA (vRNA), messenger RNA (mRNA) and complementary RNA (cRNA) of the FluA hemagglutinin gene, was conducted. FluA doses from 0.01 to 100 HAU/ml were used. Sham treatment consisted of media only. The average signal for sham treatment was equal to background signal and therefore was not plotted. All samples were taken at 2 d post-treatment. (A) Dose response to FluA in the human mast cell line LAD2 (n=3). (B-D) Densitometric analysis of (A). (E) Dose response to FluA in the human mast cell line HMC-1 (n=2). (F-H) Densitometric analysis of (E). Densitometric data was plotted by calculating the percentage of sham (% sham). Statistical significance is represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, when compared to sham treatments.

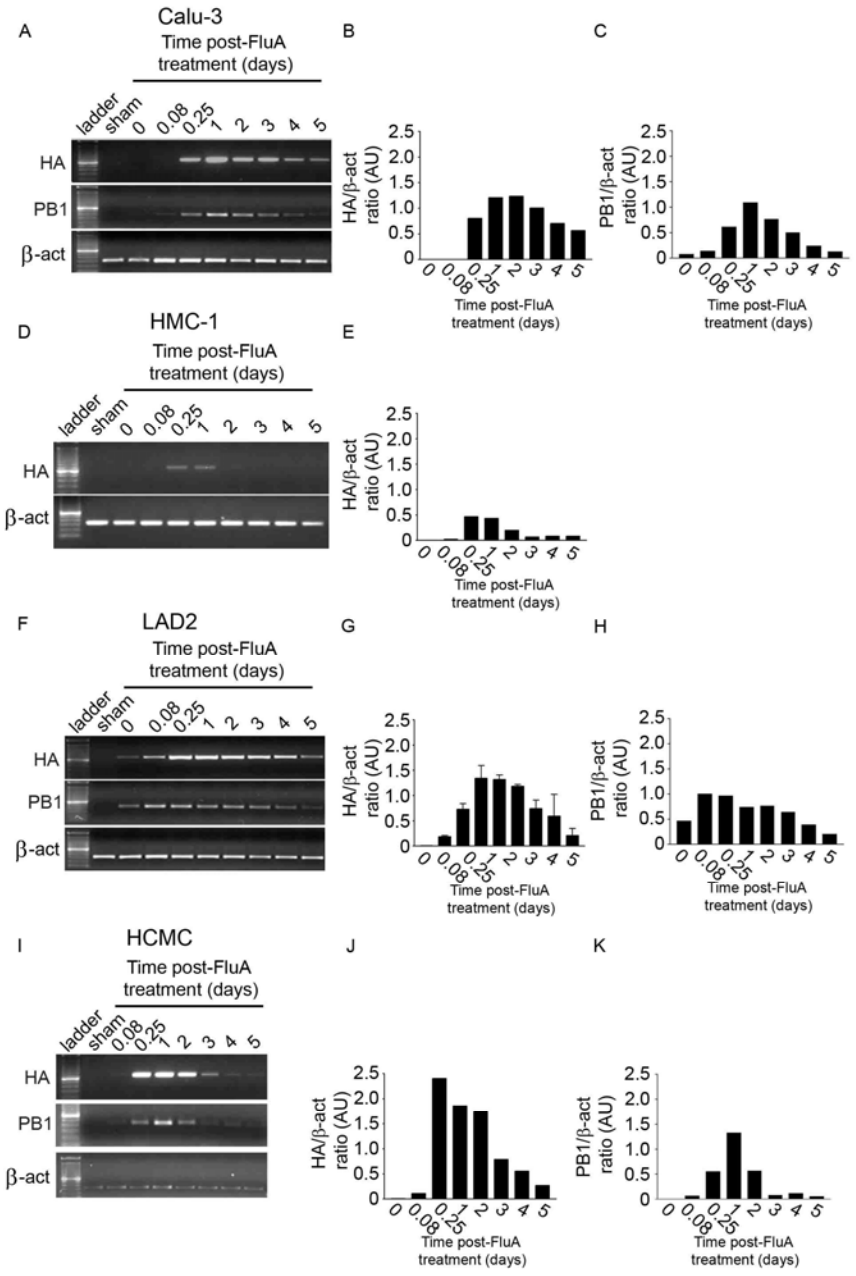

Figure 2.3: Time course comparison of FluA mRNA transcription in human mast cells and lung epithelial cells. PCR analysis of FluA hemagglutinin (HA) and polymerase B1 (PB1) mRNA expression. Cells were treated with FluA for 1 h to allow for adsorption of virus, media was removed then subsequently washed 3

times, and replaced with fresh media. 0 h time point indicates the time at which fresh media was added back to the cells. FluA dose used was 20 HAU/ml. Sham indicates treatment with media only at 2 d. Gels representing time course expression of FluA mRNA from 0 h (0h) to 5 d post-FluA treatment in (A) the human lung epithelial cell line Calu-3, the human mast cell lines (D) HMC-1 and (F) LAD2, and (I) primary human cultured mast cells (HCMC). Densitometric analysis of HA expression was performed for (B) Calu-3, (E) HMC-1, (G) LAD2, and (J) HCMC. Similarly, densitometric analysis of PB1 expression was performed for (C) Calu-3, (H) LAD2, and (K) HCMC. Densitometric data was plotted by calculating the FluA mRNA (HA or PB1) to β-actin (β-act) ratios expressed in arbitrary units (AU). All panels indicate n=1, except panel G which represents n=3 experiments.

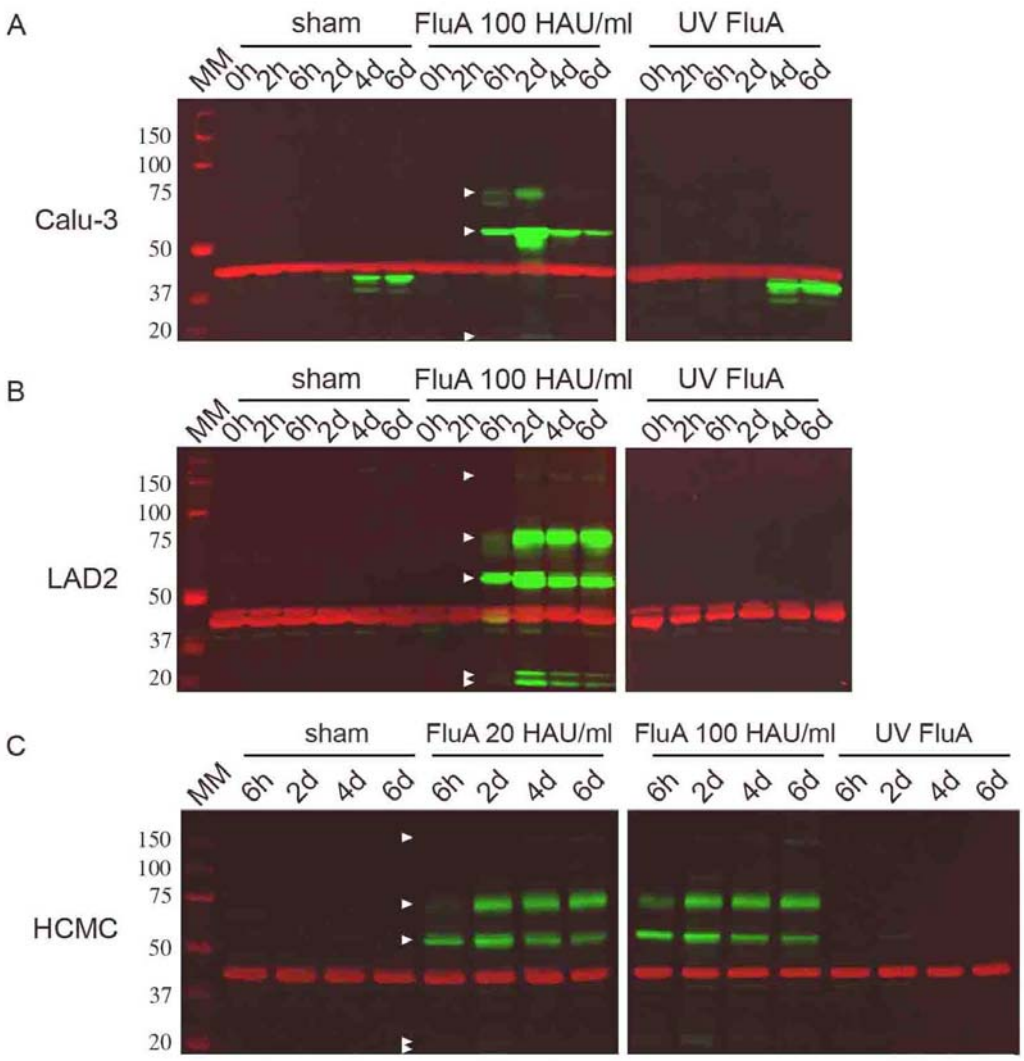

Figure 2.4: Time course comparison of FluA protein synthesis in human mast cells and lung epithelial cells. Western blot analysis of translated FluA proteins. UV-inactivated FluA (UV FluA) dose used was 100 HAU/ml. Sham treatment consisted of media only. FluA proteins appear in the green channel and β-actin appears in the red channel. FluA proteins detected were Ps (polymerase proteins) at >150 kDa, HA (hemagglutinin) at 70 kDa, NP (nucleoprotein) at 58 kDa, M1 (matrix protein 1) at 27 kDa, and NS1 (non-structural protein 1) at 26 kDa. M1 and NS1 cannot be distinguished because of their similar molecular weights.

Arrowheads indicate HA, NP, and M1 and/or NS1 from top to bottom. Time course expression of FluA proteins from 0 h (0h) to 6 d post-FluA treatment in (A) the human lung epithelial cell line Calu-3, (B) the human mast cell line LAD2, and (C) primary human cultured mast cells (HCMC).

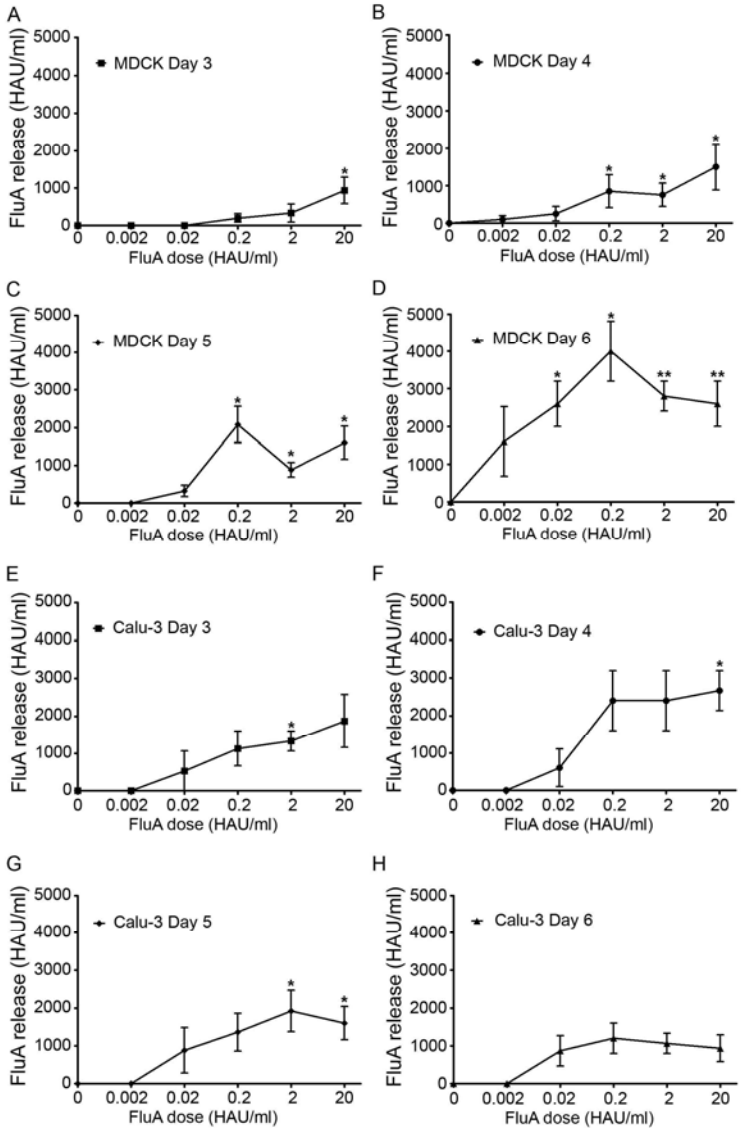

Figure 2.5: Release of FluA particles from epithelial cells. Hemagglutination assay of supernatants from epithelial cells after FluA treatment from 0 to 20 HAU/ml. (A-D) Release of FluA particles from the canine epithelial cell line MDCK at 3 to 6 d post-FluA treatment. (E-H) Release of FluA particles from the human lung epithelial cell line Calu-3 at 3 to 6 d post-FluA treatment. Statistical significance is represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, when compared to 0 HAU/ml treatments. ($n = 3 - 5$).

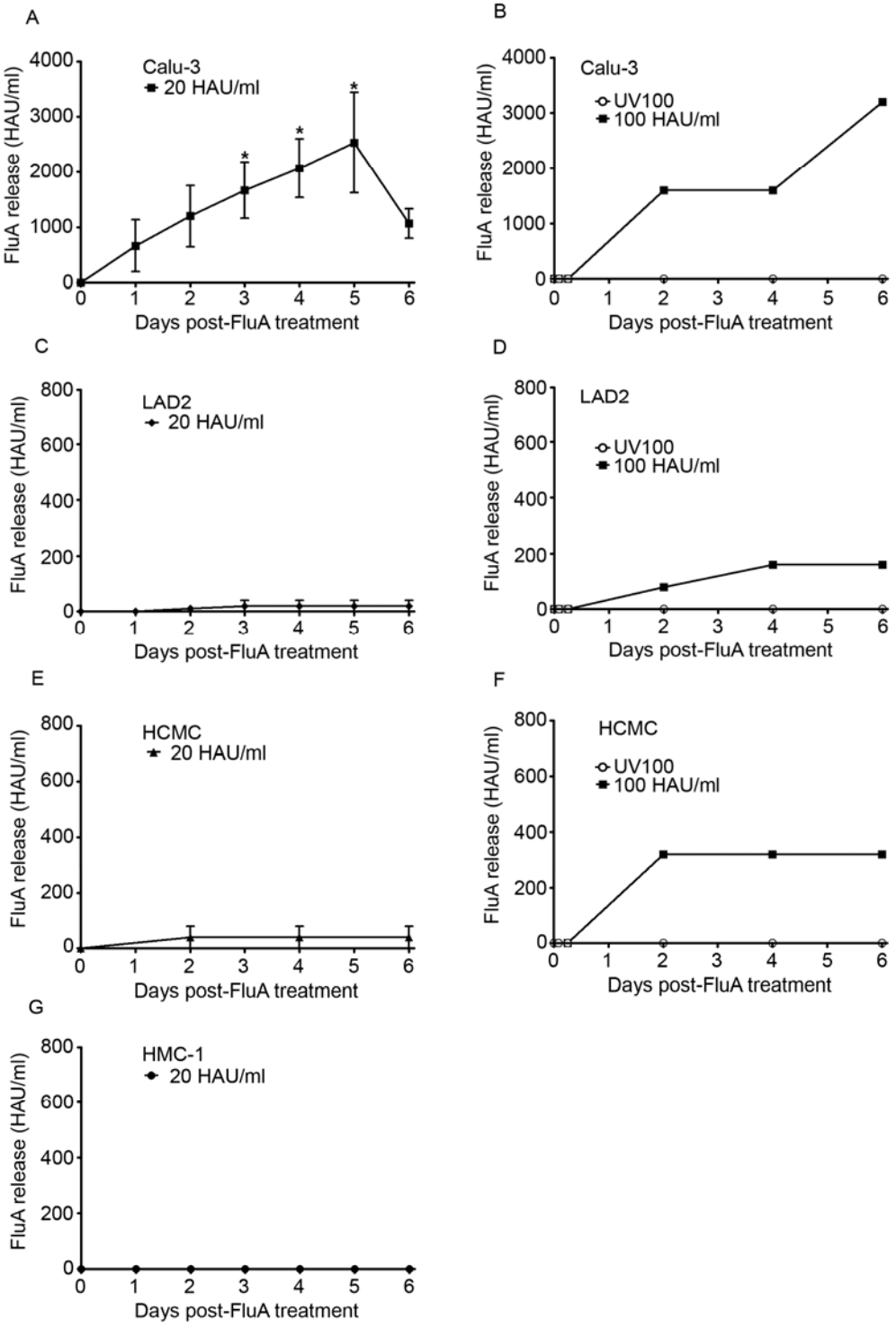

Figure 2.6: Time course comparison of FluA particle release from human mast cells and lung epithelial cells. Hemagglutination assay of supernatants from

epithelial cells and mast cells from 0 to 6 d post-FluA treatment. Release of FluA particles from (A) the human lung epithelial cell line Calu-3, (C) the human mast cell line LAD2, (E) primary human cultured mast cells (HCMC), and (G) the human mast cell line HMC-1, using 20 HAU/ml FluA treatment. Release of FluA particles from (B) Calu-3, (D) LAD2, and (F) HCMC, using 100 HAU/ml of FluA or UV-inactivated FluA (UV100) treatment. Statistical significance is represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, when compared to 0 d treatments for panels A, C, E, and G ($n = 3 – 6$). Statistics were not performed for panels B, D, and F ($n = 1$).

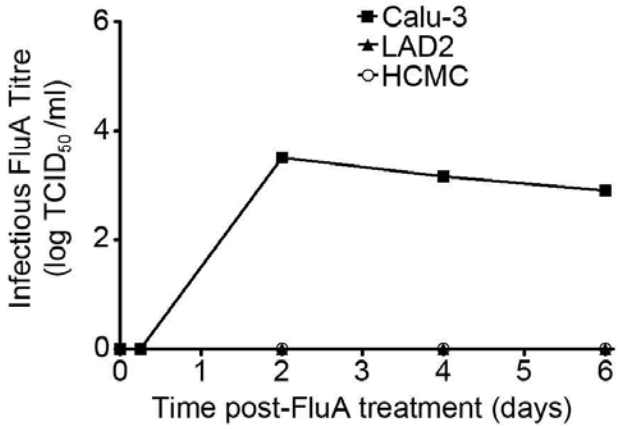

Figure 2.7: Time course comparison of infectious FluA titer from human mast cells and lung epithelial cells. $TCID_{50}$/ml of supernatants was determined using hemadsorption assay of FluA-treated supernatants on human lung epithelial cell (Calu-3) monolayers. The human lung epithelial cell line Calu-3, human mast cell line LAD2, and primary human cultured mast cells (HCMC) were treated with FluA at 100 HAU/ml. Samples were taken at 0 to 6 d post-FluA treatment. Statistics were not performed (n = 1).

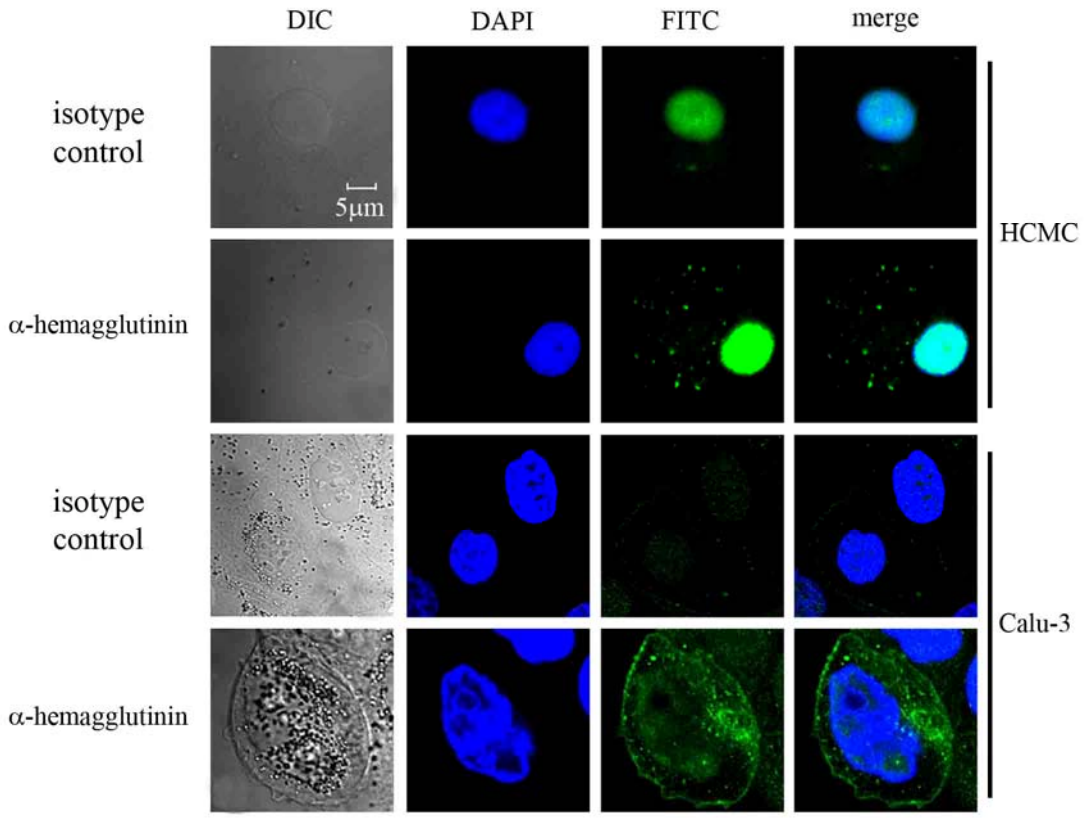

Figure 2.8: Confocal microscopy of hemagglutinin in Calu-3 and HCMC. Cells were visualized under DIC, DAPI nuclear staining, FITC α-hemagglutinin (HA), and merge of DAPI / FITC. Isotype controls are shown for both cell types. Cells were treated with 20 HAU/ml of FluA and harvested at 2 d. Images for HCMC are representative of $n = 2$ experiments, while $n = 1$ was performed for Calu-3.

7. Tables

FluA doses (HAU/ml) treatment of MDCK							
Days	20	2	0.2	0.02	0.002	0.0002	sham
4	$(+)^5$	$(+)^5$	$(+)^4(-)^1$	$(+)^4(-)^1$	$(+)^2(-)^3$	$(-)^5$	$(-)^5$
5	$(+)^5$	$(+)^5$	$(+)^5$	$(+)^5$	$(+)^2(-)^3$	$(+)^1(-)^4$	$(-)^5$
6	$(+)^5$	$(+)^4(-)^1$	$(+)^4(-)^1$	$(+)^4(-)^1$	$(+)^3(-)^2$	$(-)^5$	$(-)^5$
FluA doses (HAU/ml) treatment of Calu-3							
4	$(+)^5$	$(+)^4(-)^1$	$(+)^4(-)^1$	$(+)^4(-)^1$	$(-)^5$	$(-)^5$	$(-)^5$
5	$(+)^5$	$(+)^5$	$(+)^4(-)^1$	$(+)^4(-)^1$	$(+)^2(-)^3$	$(-)^5$	$(-)^5$
6	$(+)^5$	$(+)^5$	$(+)^4(-)^1$	$(+)^4(-)^1$	$(+)^2(-)^3$	$(-)^5$	$(-)^5$

Table 2.1: Infectivity of FluA in epithelial cells. Hemadsorption assay of epithelial cell monolayers after FluA treatment. FluA doses of 0.0002 to 20 HAU/ml were used. Samples were taken at 4 to 6 d post-FluA treatment. Positive hemadsorption is indicated by (+); negative hemadsorption is indicated by (-). Superscript numbers indicate *n*-values for each outcome (total of 5 experiments). Upper table represents hemadsorption on the Madine-Darby canine epithelial cell line (MDCK) and lower table represents hemadsorption on the human epithelial cell line Calu-3.

Infected supernatants on Calu-3 monolayers							
FluA doses (HAU/ml)							
Supernatants	20	2	0.2	0.02	0.002	0.0002	sham
Calu-3	$(+)^3$	$(+)^3$	$(+)^3$	$(+)^3$	$(+)^2$ $(-)^1$	$(-)^3$	$(-)^3$
FluA doses (HAU/ml)							
Supernatants	100	20	10	1	0.1	0.01	Sham
HMC-1	$(-)^3$	$(-)^3$	$(-)^3$	$(-)^3$	$(-)^3$	$(-)^3$	$(-)^3$
LAD2	$(+)^6$	$(+)^1$ $(-)^5$	$(-)^5$	$(-)^5$	$(-)^5$	$(-)^5$	$(-)^5$

Table 2.2: Release of infectious FluA particles from human epithelial cells and mast cells. Hemadsorption assay on human lung epithelial cell (Calu-3) monolayers after treatment with supernatants from FluA-infected epithelial cells and mast cells. FluA doses of 0.0002 to 100 HAU/ml were used. Samples were taken at 2 d post-treatment with supernatants from infected Calu-3, HMC-1, and LAD2. Positive hemadsorption is indicated by (+); lack of hemadsorption is indicated by (-). Superscript numbers indicate *n*-values for each outcome (from a total of 3 to 6 experiments). Upper table represents supernatants from infected human epithelial cell line Calu-3. Lower table represents supernatants from infected human mast cell lines HMC-1 and LAD2.

Infected supernatants on Calu-3 monolayers							
Time post-FluA treatment (20 HAU/ml)							
Supernatants	6 h	1 d	2 d	3 d	4 d	5 d	sham
Calu-3	$(+)^2$ $(-)^1$	$(+)^3$	$(+)^6$	$(+)^5$	$(+)^5$	$(+)^6$	$(-)^6$
HMC-1	$(-)^3$	$(-)^3$	$(-)^3$	$(-)^3$	$(-)^3$	$(-)^3$	$(-)^3$
LAD2	$(+)^2$ $(-)^1$	$(-)^4$	$(+)^1$ $(-)^5$	$(-)^4$	$(-)^4$	$(-)^6$	$(-)^6$
HCMC	$(-)^2$	$(-)^1$	$(-)^2$	$(-)^1$	$(-)^2$	$(-)^2$	$(-)^1$

Time post-FluA treatment (100 HAU/ml)					
Supernatants	6 h	2 d	4 d	6 d	UV FluA
Calu-3	$(+)^1$	$(+)^1$	$(+)^1$	$(+)^1$	$(-)^1$
LAD2	$(+)^1$	$(+)^6$	$(-)^2$	$(-)^2$	$(-)^1$
HCMC	$(+)^1$	$(-)^1$	$(+)^1$	$(-)^1$	$(-)^1$

Table 2.3: Time course comparison of infectious FluA particle release from human epithelial cells and mast cells. Hemadsorption assay of human epithelial cell (Calu-3) monolayers after addition of supernatants from FluA-treated epithelial cells and mast cells. The human lung epithelial cell line Calu-3, human mast cell lines HMC-1 and LAD2, and primary human cultured mast cells (HCMC) were treated with FluA. Samples were taken at 6 hours (6 h) and 1 to 5 d post-FluA treatment. Positive hemadsorption is indicated by (+); lack of hemadsorption is indicated by (-). Superscript numbers indicate *n*-values for each outcome (total of 1 to 6 experiments). Upper table represents supernatants from cells treated with 20 HAU/ml of FluA. Lower table represents supernatants from cells treated with 100 HAU/ml of FluA. Sham indicates post-treatment with media only at 2 days. UV FluA indicates post-treatment with UV-inactivated FluA at 100 HAU/ml at 2 days.

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Chapter III: Human Mast Cells Release Immunomodulatory and Proinflammatory Mediators in Response to Viral-Associated Stimuli and Influenza A Virus

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2. Introduction

Mast cells play diverse roles in the immune system, acting as sentinel and effector cells in both health and disease. Although mast cells are most often appreciated for their ability to promote allergic responses, they are also important cell types in innate immunity against various pathogens (1). Over the last 15 years, mast cells have been shown to secrete pro-inflammatory mediators in response to pathogenic stimuli and to be protective against bacterial infections, partly by secreting cytokines such as TNF (1, 2). However, the evidence for involvement of mast cells in viral infections is limited.

Since the discovery of toll-like receptors (TLR) as important pattern recognition receptors (PRR) for pathogen-associated molecular patterns (PAMP), much attention has focused on the responses of innate immune cells to various ligands that specifically target different TLR (TLR1-10 in humans) (3). For example, lipopolysaccharide (LPS) found in gram-negative bacteria activates

TLR4, whereas double-stranded (ds) RNA, or analogues such as polyinosinic:polycytidylic acid (polyI:C), activates TLR3 (4, 5). Interestingly, dsRNA and polyI:C are also known to activate cytosolic PRR such as RIG-I and MDA5. RIG-I is activated by its natural ligand - 5'ppp-containing ssRNA with panhandle structures, but can also be activated by short length polyI:C; in contrast, MDA5 recognizes long forms of dsRNA and polyI:C (6). To our knowledge, the expression of RIG-I and MDA5 in mast cells has not yet been studied.

Studies have shown that mast cells selectively produce different mediators in response to activators of specific TLR. One study showed that bacterial PGN and yeast zymosan, which are thought to activate the TLR2/TLR6 complex, induce mast cells to release cysteinyl leukotrienes (cysLT); in contrast, bacterial lipoproteins thought to activate the TLR2/TLR1 complex do not induce release of cysLT, but activate mast cells to release mediators stored in granules (7). As for viral stimuli, polyI:C acts through TLR3 to induce the production of IFNα and IFNβ, by various immune cells, including dendritic cells, epithelial cells and mast cells (5, 8, 9).

Type I interferons (IFN), IFNα and IFNβ, when released from cells in response to viral exposure, induce numerous antiviral genes to protect host cells from viral invasion, replication and spread (10). Released from both epithelial cells and mast cells, IFN may have effects on mast cells in an autocrine or paracrine manner. Previous studies have shown that IFNα has regulatory effects on IgE-mediated mast cell responses as well as mast cell-dependent T cell

activation (11, 12). However, few studies have investigated whether IFN stimulation alone can induce mast cell mediator secretion.

In addition to activating TLR and inducing type I IFN production, pathogens contain many additional signals that contribute to the activation of cells and their phenotypic response. Hence, it is important to study the responses of mast cells to intact viruses in comparison to TLR ligands and IFN. In addition to their responses to TLR agonists, mast cells release inflammatory mediators in response to intact pathogens such as fungi, bacteria, and viruses; some examples of these inflammatory cytokines and chemokines include TNF, IL-1β, IL-6, CCL5, CXCL8, and cysLT (13-18). However, other mediators released by mast cells, such as the immunomodulatory cytokine TGF-β, have not been studied in the context of mast cells in innate immune responses (19). In the respiratory tract, mast cells are abundant at mucosal surfaces near epithelial cells, which are the primary target for influenza A virus (FluA) infection. However, there is little evidence that FluA has direct effects on mast cells, other than one *in vitro* study showing that UV-inactivated FluA induces IFNα secretion (9). Based on the strategic location of mast cells in the lungs and the ability of mast cells to respond to pathogenic stimuli by releasing a spectrum of mediators, mast cells are likely to respond to FluA exposure.

Thus, we investigated the response of human mast cells to different viral-associated stimuli, namely polyI:C, type I IFN, and intact FluA virus. Instead of focusing solely on chemokines and pro-inflammatory cytokines as previous studies have done, we tested the effects of these viral-associated stimuli on mast

cell secretion of growth factors and immunomodulatory cytokines, such as VEGF and TGF-β, as well as chemokines and pro-inflammatory cytokines. TGF-β can be detrimental or beneficial in viral infections, depending on the viral model studied (20, 21). Thus, we wanted to study whether FluA exposure would lead to changes in TGF-β secretion. Also, no studies to our knowledge have looked at the response of human mast cells to intact FluA virus. We hypothesized that while the profile of secreted mediators from mast cells may follow a similar trend in response to all viral-associated stimuli, mast cells will have components of their responses that are unique to each stimulus.

3. Materials and Methods

Reagents

TLR agonists used include: TLR4 agonist lipopolysaccharide (LPS) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), TLR3 agonist polyinosinic:polycytidylic acid or polyI:C (4 – 8 kbp, InvivoGen, San Diego, CA, USA), TLR7 agonist loxoribine (InvivoGen), and TLR9 agonist CpG DNA (InvivoGen). IFNα and IFNβ were purchased from Sigma-Aldrich Canada Ltd and were endotoxin tested and had a certified purity ≥ 95%.

Cell culture

Cell culture was performed as described previously in Chapter 2 for the immature human mast cell line HMC-1 (a kind gift from J.H. Butterfield, Mayo Clinic, Rochester, MN, USA), the more mature human mast cell line Laboratory

of Allergic Diseases 2 (LAD2) (a generous gift from Drs. A.S. Kirshenbaum and D.D. Metcalfe, National Institutes of Health, Bethesda, MD, USA), and primary human cultured mast cells (HCMC) developed from $CD34^+$ peripheral blood mononuclear cells (PBMC) (22).

Influenza Virus and Ultraviolet (UV)-Inactivation

As described in more detail in Chapter 2, influenza A virus (FluA) was of the A/PR/8/34 strain (H1N1) (kind contribution from Dr. K.P. Kane, University of Alberta, Edmonton, AB, Canada) and UV-inactivation was performed using a UV lamp (ENF-280C, Spectroline, Westbury, NY, USA) at 254 nm. Hemagglutination units (HAU) for the stock FluA virus was determined using the hemagglutination assay as described in Chapter 2 (23).

Virus infection

All mast cells were plated at 1.0 x 10^6 cells/ml in 1 ml of media in 6-well tissue culture plates and rested for 1 hour (h) at 37^{o}C prior to experimental treatments. Cells were pelleted and supernatants were obtained for use in ELISA assays.

For the studies using HMC-1, TLR agonists, type I IFN, or media only (sham) were added to the cell suspensions for 24 h. For the multiplex membrane array studies using LAD2, polyI:C, UV-FluA, FluA, or media only (sham) were added to the cell suspensions for 48 hr. In treatment groups using IFNα and β, cells were pre-treated with IFNα and β 24 h prior to other treatments. For the time

course experiments with FluA where ELISA for CCL5, IFNγ, and GM-CSF were performed by Dr. John Gordon's laboratory (University of Saskatchewan), 1 ml of FluA or UV-FluA was added to LAD2 and HCMC for 1 h to allow for virus adsorption onto the cell surface. After 1 h of FluA adsorption, cells were washed with 3 ml of PBS, 3 times, then subsequently incubated in the original volume of fresh media for the time periods indicated.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA were performed (according to manufacturer's protocol) on supernatants from HMC-1 treated with TLR agonists or type I IFN (in duplicate wells) for human TGF-β1 (DY240, R&D Systems, Inc., Minneapolis, MN, USA), VEGF (DY293B, R&D Systems, Inc.), TNF (555212, BD Biosciences, Mississauga, ON, Canada), IL-6 (555220, BD Biosciences), and CXCL8 (555244, BD Biosciences). The TGF-β ELISA measured total TGF-β1 (latent and activated) as supernatant samples were acid-activated prior to assay. TGF-β ELISA for time course experiments using supernatants from LAD2 and HCMC (in duplicate wells) were performed by Dr. John Gordon's laboratory (University of Saskatchewan, Saskatoon, SK, Canada).

Multiplex membrane-based cytokine array

The "Human MultiAnalyte Profiling Base Kit A" (R&D Systems, Inc.) was used to simultaneously determine the concentrations of 36 cytokines (listed in manufacturer's product insert) in a single supernatant sample. This assay was

performed in duplicate for each supernatant sample. The films generated from the technique were scanned at 16 bit and 1600 dpi. Images were saved in TIFF format and imported into Odyssey Infrared Imaging System v1.2.15 software. A circle was drawn around the largest spot and the size of that circle was used to measure all spots. Background measurements were taken from two blank areas of the film and the average subtracted from all other readings. Values were then plotted in arbitrary units (AU).

4. Results

HMC-1 secretes TGF-β, IL-6, and CXCL8 in response to polyI:C

Previous studies have shown that human mast cells secrete pro-inflammatory cytokines and chemokines in response to TLR stimulation (9, 24). We investigated the effects of TLR agonists on mast cell secretion of TGF-β and VEGF, in comparison to the inflammatory cytokine IL-6 and the chemokine CXCL8 from the stem cell factor (SCF)-independent human mast cell line HMC-1 (Fig. 3.1A-D). Using ELISA, we measured the mediators in HMC-1 supernatants after 24 hour (h) treatment with various TLR agonists. Both activated and latent TGF-β1 (one isoform of TGF-β) were measured by ELISA. Dose response experiments (n = 3, except n = 1 for CpG DNA) were performed on HMC-1 for release of TGF-β to determine the doses of TLR agonists that were used for the experiments performed in Fig. 3.1 (Fig. 3.2A-D). We chose lipopolysaccharide (LPS) at 100 ng/ml since this is the lowest dose that showed

significant release of TGF-β from HMC-1 (Fig. 3.2A); previous studies used 1 μg/ml of LPS treatment to measure mast cell responses (25, 26). Similarly, we chose to use 10 μg/ml of polyinosinic:polycytidylic acid (polyI:C) since this dose showed significant TGF-β release and is the same dose used in previous mast cell literature (Fig. 3.2B) (9, 24). We used a long form of polyI:C (4 - 8 kbp), which can activate TLR3 and MDA5. Also, previous studies have administered polyI:C intracellularly by transfection to mimic intracellular dsRNA present during viral infections (27). However, we chose to administer polyI:C externally, consistent with our conceptual model that mast cells are exposed to dsRNA released by FluA-infected epithelial cells. Subsequently, mast cells would be activated by dsRNA through cell surface TLR3, or by activation of endosomal or cytoplasmic receptors after dsRNA uptake (9). Loxoribine (Loxo) did not induce significant release of TGF-β from HMC-1 (Fig. 3.2C); we used 100 μM of Loxo in subsequent experiments for release of other cytokines since this is the dose used in previous studies (28). For CpG DNA (CpG), we only performed $n = 1$ dose response experiment, which showed a increasing trend for TGF-β release at 500 nM and 1 μM doses, comparable to the 1 μM dose used in a previous study (24).

The TLR3 agonist polyI:C significantly induced HMC-1 secretion of TGF-β (298.0 ± 30.8 pg/ml, $p<0.01$) compared with sham (108.3 ± 13.9 pg/ml), IL-6 (142.8 ± 20.2 pg/ml, $p<0.05$) compared with sham (51.4 ± 10.3 pg/ml), and CXCL8 (110.3 ± 16.8 pg/ml, $p<0.05$) compared with sham (60.0 ± 7.6 pg/ml) (Fig. 3.1). HMC-1 also secreted significantly more TGF-β in response to LPS (235.0 ± 21.7 pg/ml, $p<0.05$). Loxo and CpG did not increase HMC-1 secretion of

any mediators tested compared to sham levels. PMA, an activator of the PKC-dependent pathway for mast cell exocytosis, was used as a positive control for TGF-β secretion (641.4 ± 49.9 pg/ml, $p < 0.01$) (Fig. 3.1A). VEGF secretion was not induced by any of the TLR agonists or PMA, only by CaI (Fig. 3.1B). The VEGF ELISA used detected three splice variants of human VEGF-A: $VEGF_{165}$, $VEGF_{121}$, and $VEGF_{165b}$ and showed no cross-reactivity with human VEGF-C, human VEGF-D, mouse $VEGF_{120}$, mouse $VEGF_{164}$, or rat $VEGF_{164}$ according to manufacturer's protocol. These results show that HMC-1 secrete both immunomodulatory and pro-inflammatory cytokines in response to polyI:C, which activates the viral-associated TLR3.

HMC-1 secretes TGF-β and IL-6 in response to IFNα and IFNβ

PolyI:C acting through TLR3, or the cytosolic receptors RIG-I or MDA5, can induce the production of type I interferons, IFNα and IFNβ, which bind the common IFN-α receptor (IFNAR) to initiate signaling cascades that activate numerous antiviral genes, including antiviral proteins such as myxovirus resistance protein A (MxA) and protein kinase R (PKR), to protect host cells from viral invasion (5). We investigated whether 24 h treatment with these IFNs can affect mast cell secretion of mediators (Fig. 3.3). A time course experiment was performed (Fig. 3.4) and we determined that 24 h was the best time point to measure increases in TGF-β secretion above spontaneous release in response to IFN. HMC-1 secretion of TGF-β was significantly induced by both IFNα (91.7 ± 22.1 pg/ml, $p<0.01$) and IFNβ (74.6 ± 14.0 pg/ml, $p<0.05$) compared to sham

(22.9 ± 8.3 pg/ml) (Fig. 3.3A). The combined effects of IFNαβ on TGF-β secretion were not significantly different than either substance alone. IFNα, but not IFNβ, induced significantly more IL-6 secretion (68.0 ± 7.7 pg/ml, $p<0.05$) than sham treatment (41.4 ± 5.6 pg/ml) (Fig. 3.3B). No synergistic effect of IFNαβ was shown on IL-6 secretion, since it did not differ from the effect of IFNα alone. None of the IFN treatments affected HMC-1 secretion of CXCL8 (Fig. 3.3C). Although the purchased IFN were endotoxin tested and were certified to have purities of ≥ 95%, the possibility of mast cell cytokine secretion due to LPS contamination still exists. However, 5% contamination would be expected to have a lower concentration of LPS than the 100 ng/ml that we tested. LPS at 100 ng/ml only induced significant secretion of TGF-β whereas IFNα induced significant TGF-β and IL-6, providing evidence that the effects of IFN on mast cell secretion is not solely due to effects of LPS contamination. Thus, HMC-1 can be activated to secrete TGF-β and IL-6 in response to type I IFN.

PolyI:C and FluA differentially affect LAD2 secretion of cytokines in pilot study

Since polyI:C mimics dsRNA produced by viruses during infection, we investigated whether mast cells secrete mediators in response to UV-inactivated influenza virus (UV FluA) or intact FluA. We used the SCF-dependent mast cell line LAD2 after 48 h treatment for these experiments. This time of treatment was used since both FluA mRNA and protein are detected at this time in FluA-treated LAD2 (Chapter 2 – Fig. 3.2 & 3.3). We used a multiplex membrane-based

cytokine array (R&D systems) in an $n = 1$ exploratory pilot study to screen for cytokines secreted from LAD2 in response to polyI:C, UV FluA, or FluA treatment with or without the addition of IFNαβ (Fig. 3.5). A total of 36 cytokines were tested with the "Human Cytokine Panel A" (R&D systems). The sensitivities for cytokines ranged from 0.47 - 9.94 pg/ml for this multiplex array kit. After membranes were exposed, the membranes were scanned using the Li-cor Odyssey machine and signals were quantified using the Odyssey software as described in Materials and Methods. IL-6 and CXCL8, two cytokines that we measured in previous ELISA experiments using HMC-1, were undetectable from LAD2 using this assay (data not shown). Interestingly, the secretion of IFNγ, CD40 ligand, CCL5, and PAI-1 that showed increases in signal intensity with either UV-FluA or FluA treatment compared to sham were not increased by polyI:C treatment (Fig. 3.5A-D). polyI:C treatment abolished the constitutive secretion, as shown in sham treatment, of PAI-1, IL-16, GM-CSF, and CCL-1 (I-309) to undetectable levels (Fig. 3.5D, F-H). The addition of IFNαβ to polyI:C treatment was able to partially recover the secretion of PAI-1, IL-16, and GM-CSF (Fig. 3.5D, F & G). There was little observable difference in IL-13 secretion with polyI:C or FluA treatment (Fig. 3.5E). polyI:C did not inhibit all cytokines analyzed as IL-1ra and MIF showed similar levels in all treatment groups from this multiplex array (Fig. 3.6A & B).

Similar to polyI:C, these preliminary results showed that FluA treatment decreased LAD2 secretion of GM-CSF and CCL1 to nearly undetectable levels (Fig. 3.5G & H). In contrast to polyI:C, FluA treatment resulted in detectable

LAD2 secretion of IFNγ and CCL5 compared to undetectable levels in sham (Fig. 3.5A & C). The secretion of IFNγ, PAI-1, GM-CSF, and CCL1 followed similar trends in response to either intact FluA or the replication-independent UV FluA (Fig. 3.5A, D, G & H). However, there were also differences in LAD2 secretion of cytokines in response to UV FluA vs. FluA. For example, CCL5 was not induced by UV FluA in contrast to FluA (Fig. 3.5C); on the other hand, UV FluA induced CD40 ligand secretion while FluA did not (Fig. 3.5B). Generally, pre-treatment with IFNαβ did not have much effect on FluA-induced changes in cytokine levels. The exception is CD40 ligand, which could be induced with IFNαβ pretreatment prior to FluA; although IFNαβ treatment alone was not performed, IFNαβ pretreatment prior to polyI:C had no effect on CD40 ligand secretion (Fig. 3.5B). The results from the multiplex cytokine array supports the idea that cytokine secretion from mast cells is finely-tuned to the specific stimuli present during viral infections, and likely represents the outcome of counteractive host and pathogen forces. However, since this multiplex array was used as a preliminary screening tool, subsequent ELISA assays were performed to validate this data.

Time course of mast cell cytokine secretion in response to FluA

To follow up on the multiplex array results, we tested LAD2 supernatants from time course experiments (n = 3) exposed to FluA using ELISA for CCL5, IFNγ, and GM-CSF (Dr. John Gordon's laboratory, University of Saskatchewan). LAD2 secretion of CCL5 was significantly increased by FluA at 2 days (d) (50.0

± 17.5 pg/ml, $p<0.05$) compared to sham (3.9 ± 1.9 pg/ml), and at 4 d (38.3 ± 12.8 pg/ml, $p<0.05$) compared to sham (4.8 ± 2.1 pg/ml) (Fig. 3.7A). Although the multiplex array showed that IFNγ levels were increased at 2 d of FluA treatment, the ELISA data showed no significant increase at 2 d when compared to sham (Fig. 3.7B). Interestingly, IFNγ levels were significantly decreased at 8 d of FluA treatment (17.5 ± 3.0 pg/ml, $p<0.05$) compared to sham (39.4 ± 7.2 pg/ml) (Fig. 3.7B). Levels of GM-CSF were unchanged between FluA and sham treatments (Fig. 3.7C). We also tested the time course of TGF-β and CXCL8 secretion by LAD2 using ELISA (Dr. John Gordon's laboratory, University of Saskatchewan) following the results of HMC-1 secretion. Levels of both these cytokines were not significantly affected by FluA (Fig. 3.7D & E). These results show that FluA has effects on mast cell cytokine secretion, with a significant increase in CCL5 secretion and an apparent decrease in IFNγ secretion at 8 d post-exposure.

Also, we tested whether the effects of FluA on chemokine secretion were dependent on viral replication by treating both LAD2 and the primary human cultured mast cells (HCMC) with UV FluA compared to intact FluA ($n = 1$). We have preliminary results showing that HCMC secretes more CCL5 in response to 6 h - 4 d of FluA treatment, but not in response to UV FluA at the same dose (Fig. 3.8A). Preliminary results from LAD2 supernatants also show increased secretion of CCL5 from 2 – 6 d of FluA, but not UV FluA treatment (Fig. 3.8B). This suggests that a replication-competent FluA is required to induce mast cell secretion of CCL5.

5. Discussion

Although a role for mast cells in innate immunity against bacteria is now well-established, the importance of mast cells in viral infections is less well understood. Our experiments show that human mast cell lines respond to various viral-associated stimuli, including synthetic mimics of viral replication, type I IFN, and FluA by changing their profile of secreted mediators. Within 48 hours, HMC-1 release TGF-β, IL-6, and CXCL8 in response to polyI:C and IFNα, and LAD2 release CCL5 in response to FluA (Fig. 3.1, 3.3 & 3.7). In the preliminary multikine array study, IL-6 and CXCL8 were not detected from LAD2 in response to polyI:C (data not shown), in contrast to our HMC-1 data (Fig.1). Although this discrepancy could be due to differences in the secreted cytokine profile between the two cell lines, these results are more likely attributed to the higher detection limits of the multikine assay for IL-6 and CXCL8, as we have data from our lab that LAD2 secrete both IL-6 and CXCL8 in response to IgE/anti-IgE when measured by ELISA but was also undetectable in the multikine array (unpublished). FluA is also capable of inhibiting IFNγ secretion by LAD2 at a later time point (8 d). PolyI:C has been shown in published studies to activate mast cell secretion of IFNα, pro-inflammatory cytokines, and chemokines; however, the results vary greatly between human and murine mast cells, and between different murine mast cell subsets (9, 24). In one study, HCMC were activated by polyI:C to secrete IFNα and leukotrienes, but not IL-1β, TNF, GM-CSF, or IL-5 (9). Our results from the multiplex cytokine array and ELISA using LAD2 confirm that polyI:C does not induce mast cell secretion of the latter

cytokines (Fig. 3.5 & 3.7, and data not shown). Type I IFN are known to have regulatory effects on IgE-induced mast cell secretion, by decreasing TNF production while increasing IL-10 and TGF-β production (11, 29). However, these studies did not determine the effects of IFN on mast cell secretion in the absence of IgE stimulation.

With the TLR agonist and type I IFN experiments, we specifically explored whether mast cells can release TGF-β and VEGF, factors that do not have a classic inflammatory role, in response to pathogen-associated stimuli. Both TGF-β and VEGF have pleiotropic effects on immune cells, but have not been studied extensively in the context of viral infections (30-33). We were interested in the effects of TLR activation and IFN treatment on mast cell secretion of these mediators. Our results showed that polyI:C induces release of the immunomodulatory cytokine TGF-β, in addition to IL-6 and CXCL8 (n = 6 to 10 for polyI:C treatment, Fig. 3.1A, C, D). None of the TLR agonists had any effects on mast cell release of VEGF. We also showed that IFNα and IFNβ activate mast cells to release TGF-β and IL-6 (n = 5 to 7, Fig. 3.3A-B). TGF-β has been reported to inhibit viral replication of hepatitis C virus and human T-cell leukemia virus, while other studies showed that TGF-β enhances rhinovirus infection and respiratory syncytial virus replication (20, 21, 30, 34). Several studies have shown that IgE-crosslinking induces mast cell production of TGF-β, which contributes to tissue remodeling by increasing fibroblast proliferation and collagen deposition (35-37). Interestingly, TGF-β has been shown to have autocrine effects on mast cells, by inhibiting the release of histamine and TNF induced by IgE (19). To our

knowledge, our results are the first to show that mast cells release TGF-β in response to polyI:C and IFN alone, suggesting a role for TGF-β in mast cell responses against viral infections. Further studies will help elucidate whether TGF-β promotes or modulates mast cell innate responses in the context of viral infections.

In addition to autocrine effects, mast cell-derived TGF-β could have effects on surrounding epithelial cells, which are the primary target of most respiratory viral infections. TGF-β has the ability to induce lung epithelial cells to transition into mesenchymal cells, which promote fibrosis through deposition of extracellular matrix proteins (38). In the context of viral infections, treatment of lung epithelial cells with TGF-β has been shown to enhance RSV replication (30). Potential mast cell-epithelial interactions during FluA infection and involvement of mast cell-derived cytokines such as TGF-β will be pursued in future studies.

Even though many studies use polyI:C as a surrogate for viral stimulation, our results demonstrate that cytokine secretion by mast cells differs in response to polyI:C compared to FluA. A study using HCMC showed that IFNα secretion was induced by both polyI:C and UV FluA (9). In a preliminary multiplex array, we observed that FluA induced the release of IFNγ and CCL5 while polyI:C had no effect on these cytokines (Fig. 3.5). IFNγ acts both directly to inhibit viral replication and indirectly by its immunostimulatory effects on macrophages, T cells, and NK cells (39). However, subsequent ELISA results showed that IFNγ is inhibited by FluA treatment at 8 d (n = 3 for Fig. 3.7), potentially as a result of FluA counteraction of antiviral immunity. Our results suggest that mast cells

detect FluA signals in addition to a dsRNA intermediate to initiate an immune response against FluA.

CCL5 was consistently elevated in response to FluA treatment in both LAD2 and HCMC and in both the multiplex cytokine array and ELISA experiments (n = 1 for Fig. 3.5, n = 3 for Fig. 3.7). In our ELISA studies, a replication competent FluA was capable of inducing release of CCL5 from mast cells (n = 3 for Fig. 3.7), whereas UV FluA had no effect (n = 1 for Fig. 3.5 & Fig. 3.8). Although only n = 1 study, our results showing that HCMC secreted CCL5 at 30 min after exposure to FluA at 100 HAU/ml suggest that CCL5 may be stored in mast cell granules to be released within minutes. CCL5 is important both for T cell recruitment and activation (40). FluA-infected human patients have increased levels of CCL5 in the bloodstream (41). Also, CCL5-deficient mice infected with FluA showed delayed viral clearance and increased airway inflammation with decreased survival compared with sham mice (42). Thus, secretion of CCL5 by mast cells in response to FluA may contribute to immune protection against the virus. Another interesting preliminary finding was that treatment with both polyI:C and FluA inhibited the release of CCL1 (Fig. 3.5), a chemokine with chemotactic activity for monocytes and certain T cell subsets. This supports a study where CCL1 production by mast cells is inhibited by the viral-associated cytokine IFNγ, although the relevance of this inhibition is unclear (43). The multiplex data also show novel results for PAI-1, a serine protease inhibitor linked to the development of atherosclerosis and asthma (44, 45). Induction of PAI-1 by FluA treatment (Fig. 3.5) suggests a role for this molecule

in innate responses, which may or may not be specific to mast cells. Another interesting molecule is CD40 ligand, which was found to be elevated in supernatants of mast cells exposed to UV-FluA or FluA+IFNαβ. Mast cells have been shown to express membrane bound and soluble forms of CD40 ligand also exists (46, 47). Interestingly, decreased levels of soluble CD40 ligand was found to be a negative prognostic factor for patients with influenza virus-associated encephalopathy (48). Whether CD40 ligand contributes to the pathogenesis of influenza virus infection is yet to be determined. It is important to follow up on the results from the multiplex cytokine array to confirm the data and further elucidate the specific mechanisms by which viral-associated stimuli regulates mast cell secretion.

A study using mast cell-deficient mice showed that mast cells activated through TLR3 by polyI:C promote $CD8^+$ T cell recruitment into the peritoneal cavity (49). It would be interesting to assess the effect of mast cells on FluA infection using mast cell-deficient mice. As $CD8^+$ T cells are important for viral clearance during FluA infection, mast cells may be contributing to viral elimination by attracting T cells to the site of infection (50). Based on this rationale, we would predict that FluA infection of mast cell-deficient mice would result in enhanced FluA replication and airway inflammation along with increased mortality.

We have presented evidence that cytokine secretion by mast cells is activated by factors that are present in the environmental milieu during FluA infection, including type I IFN, dsRNA, and intact FluA particles. We showed

that mast cell responses differ depending on the specific viral stimuli used, suggesting complex interactions between host and viral factors that culminate in particular outcomes. Our results provide evidence that mast cells play an important role in innate immunity against viruses. With future studies, we may discover unique mast cell mechanisms that will enhance our understanding of host defenses.

6. Figures

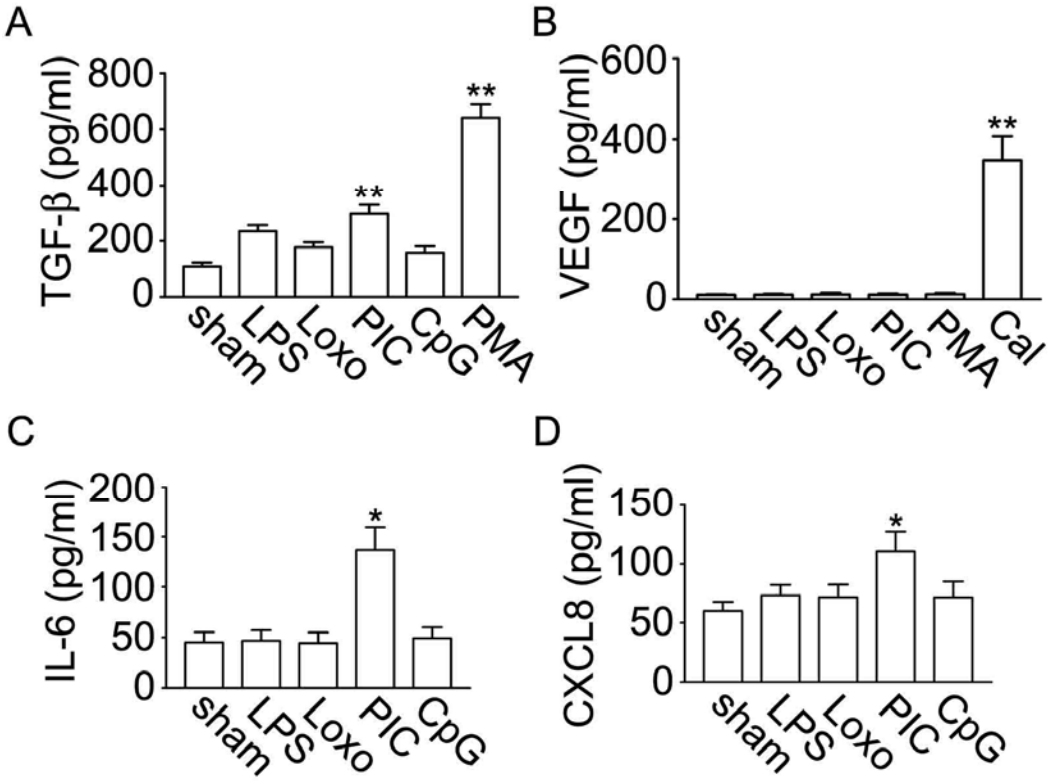

Figure 3.1: Human mast cell line HMC-1 secretes mediators in response to TLR agonists. ELISA were performed on HMC-1 supernatants to specifically quantify

the release of (A) TGF-β (n = 3 – 10), (B) VEGF (n = 6), (C) IL-6 (n = 8), and (D) CXCL8 (n = 3 - 6). Supernatants were collected from HMC-1 after 24 hour treatment with media only (sham) or TLR agonists. Agonists used were for TLR4 – lipopolysaccharide (LPS), TLR7 - loxoribine (Loxo), TLR3 - polyinosinic:polycytidylic acid (polyI:C) and TLR9 – CpG DNA (CpG). Phorbol myristate acetate (PMA) at 20 nM and calcium ionophore (CaI) at 1μM were used as positive controls for mast cell secretion (51, 52). Concentrations used for TLR agonists were determined from dose response experiments (see Fig. 3.2): 100 ng/ml LPS, 10 μg/ml polyI:C, 100 μM Loxo, and 1 μM CpG. Statistical significance as determined by Dunnett post-ANOVA test is represented as *$p < 0.05$; **$p < 0.01$, when compared to sham treatments.

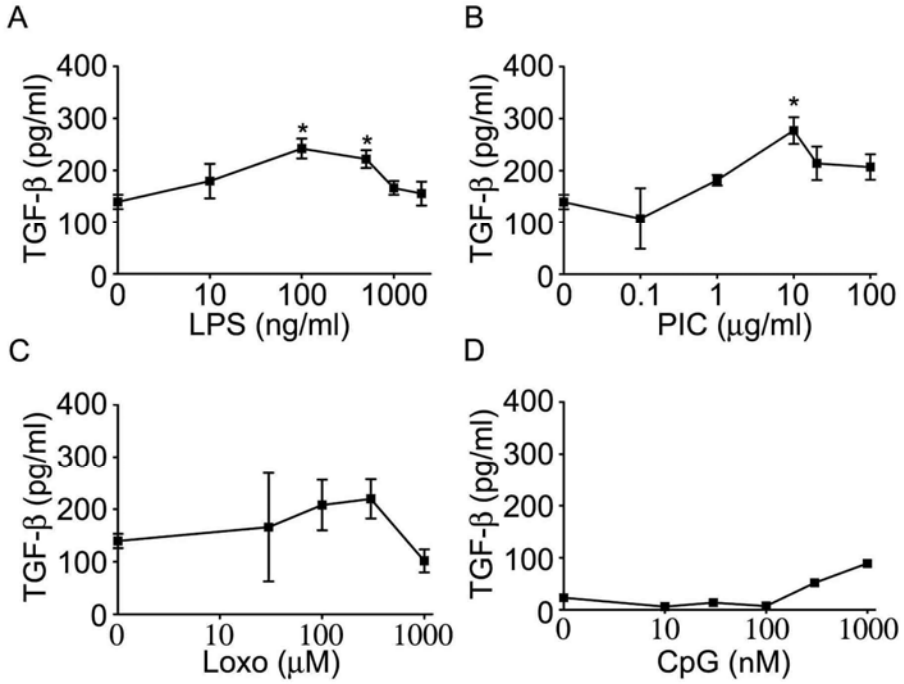

Figure 3.2: Dose response of TGF-β secretion in response to TLR agonists in HMC-1. ELISA for TGF-β were performed on HMC-1 supernatants collected after 24 h treatment with media only (concentration of zero) or increasing concentrations of TLR agonists (n = 3, except n = 1 for CpG). Dose response of TGF-β secretion to (A) LPS from 10 to 2000 ng/ml, (B) polyI:C (PIC) from 0.1 to 100 μg/ml, (C) Loxo from 30 to 1000 μM, and (D) CpG from 10 to 1000 nM. Statistical significance as determined by student's t-test is represented as *$p < 0.05$; **$p < 0.01$, when compared to sham treatments.

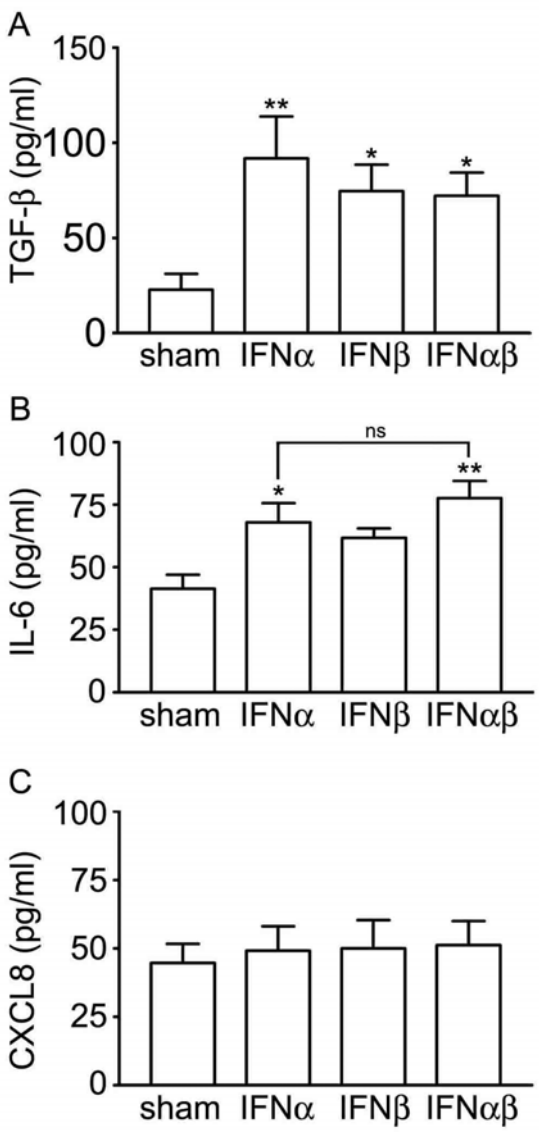

Figure 3.3: HMC-1 secretes mediators in response to type I interferons (IFN). ELISA were performed on HMC-1 supernatants to specifically quantify the release of (A) TGF-β (*n* = 7), (B) IL-6 (*n* = 5) and (C) CXCL8 (IL-8) (*n* = 7). Supernatants were collected from HMC-1 after 24 h treatment with sham or type I IFN – IFNα, IFNβ, or both (IFNαβ). IFN concentrations of 1 ng/ml were used. Statistical significance as determined by Dunnett post-ANOVA test is represented as *$p < 0.05$; **$p < 0.01$, when compared to sham treatments.

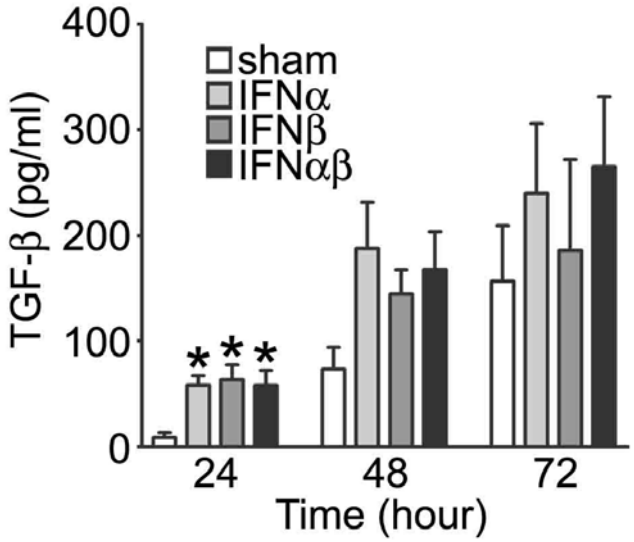

Figure 3.4: Time course comparison of TGF-β secretion in response to type I IFN in HMC-1. Supernatants were collected from HMC-1 after 24, 48 or 72 h treatment with sham, IFNα, IFNβ, or IFNαβ (n = 4). IFN concentrations of 1 ng/ml were used. ELISA for TGF-β were performed on HMC-1 supernatants. Statistical significance as determined by Dunnett post-ANOVA test is represented as *$p < 0.05$ for each time point when compared to sham treatments.

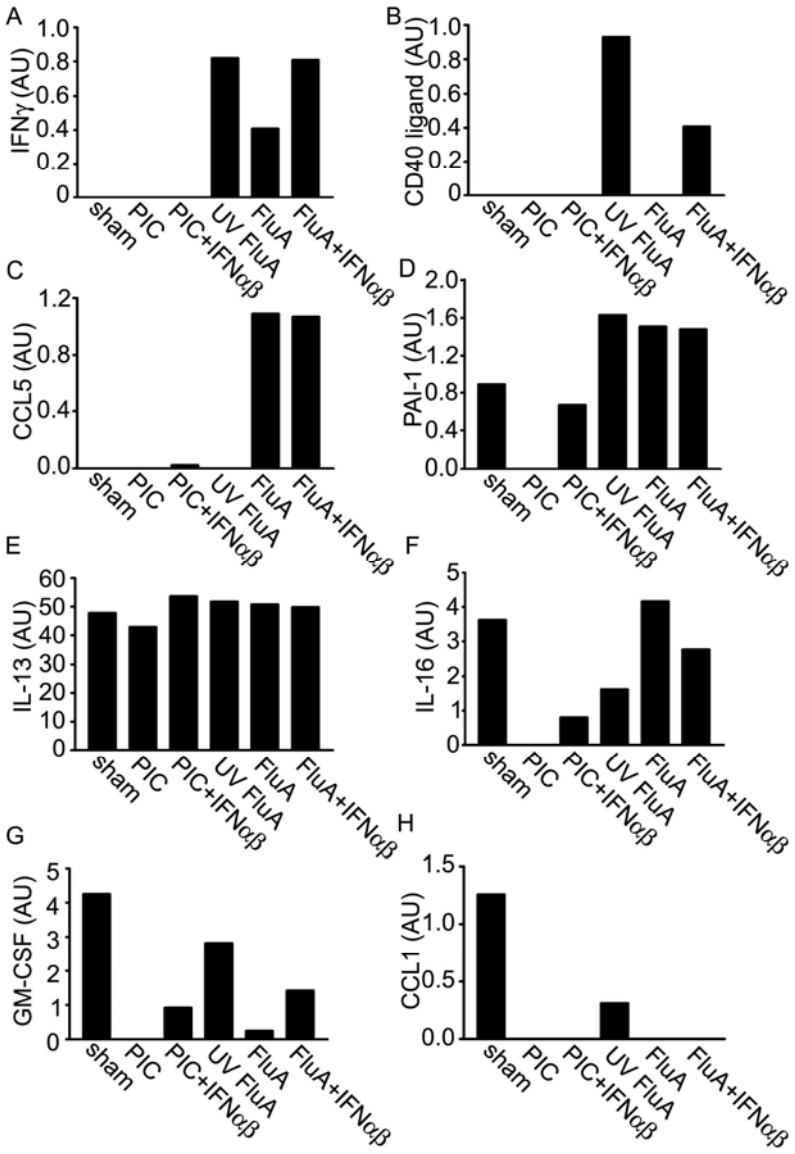

Figure 3.5: Changes in secretion of mediators from the human mast cell line LAD2 in response to viral-associated stimuli. Supernatants were collected from LAD2 after 48 h treatment with sham, polyI:C (PIC), polyI:C (PIC) + IFNαβ, UV-inactivated influenza virus (UV FluA), FluA, or FluA + IFNαβ. Concentrations used were 10 μg/ml polyI:C, 1 ng/ml IFNαβ, 20 hemagglutination units (HAU)/ml UV FluA, and 20 HAU/ml FluA. A multiplex membrane-based cytokine array (R & D systems) was used as a screening tool in a preliminary

experiment (n = 1) to detect changes in levels of 36 different mediators including cytokines, chemokines, and acute phase proteins from a single supernatant sample (in duplicate). Cytokine levels are expressed in arbitrary units (AU) of signal intensity (average densitometry of duplicate signals) as described in Materials and Methods. In response to the viral-associated stimuli, changes were detectable in LAD2 secretion of (A) IFNγ, (B) CD40 ligand, (C) CCL5 (RANTES), (D) plasminogen activator inhibitor-1 (PAI-1), (E) IL-13, (F) IL-16, (G) granulocyte macrophage colony-stimulating factor (GM-CSF), and (H) CCL-1 (I-309).

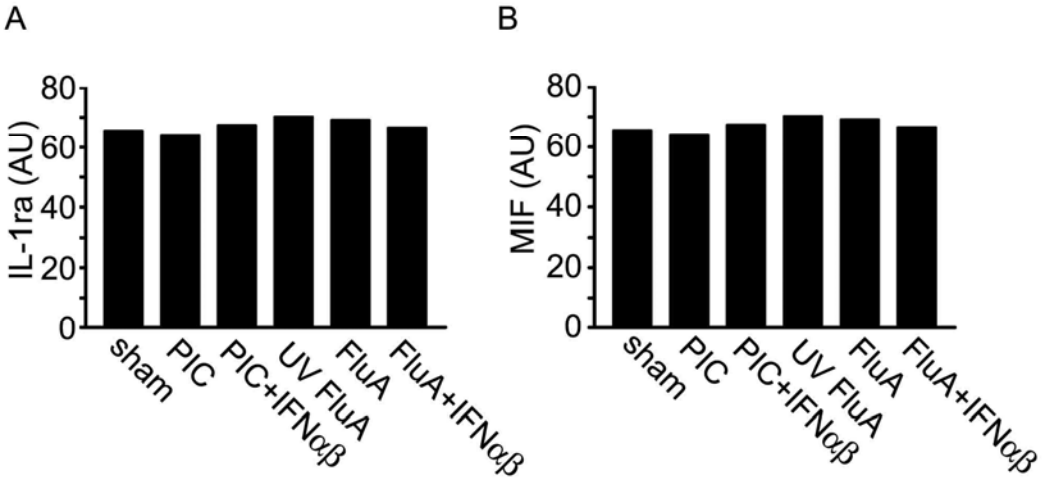

Figure 3.6: Changes in secretion of mediators from the human mast cell line LAD2 in response to viral-associated stimuli. Supernatants were collected from LAD2 after 48 hour treatment with sham, polyI:C (PIC), polyI:C (PIC) + IFNαβ, UV-inactivated influenza virus (UV FluA), FluA, or FluA + IFNαβ. Concentrations used were 10 μg/ml polyI:C, 1 ng/ml IFNαβ, 20 hemagglutination units (HAU)/ml UV FluA, and 20 HAU/ml FluA. A multiplex membrane-based

cytokine array (R & D systems) was used as a screening tool (n = 1) to detect changes in levels of 36 different mediators including cytokines, chemokines, and acute phase proteins from a single supernatant sample (in duplicate). Cytokine levels are expressed in arbitrary units (AU) of signal intensity (average densitometry of duplicate signals) as described in Materials and Methods. LAD2 secreted similar levels of (A) IL-1ra and (B) MIF in all treatment groups including sham.

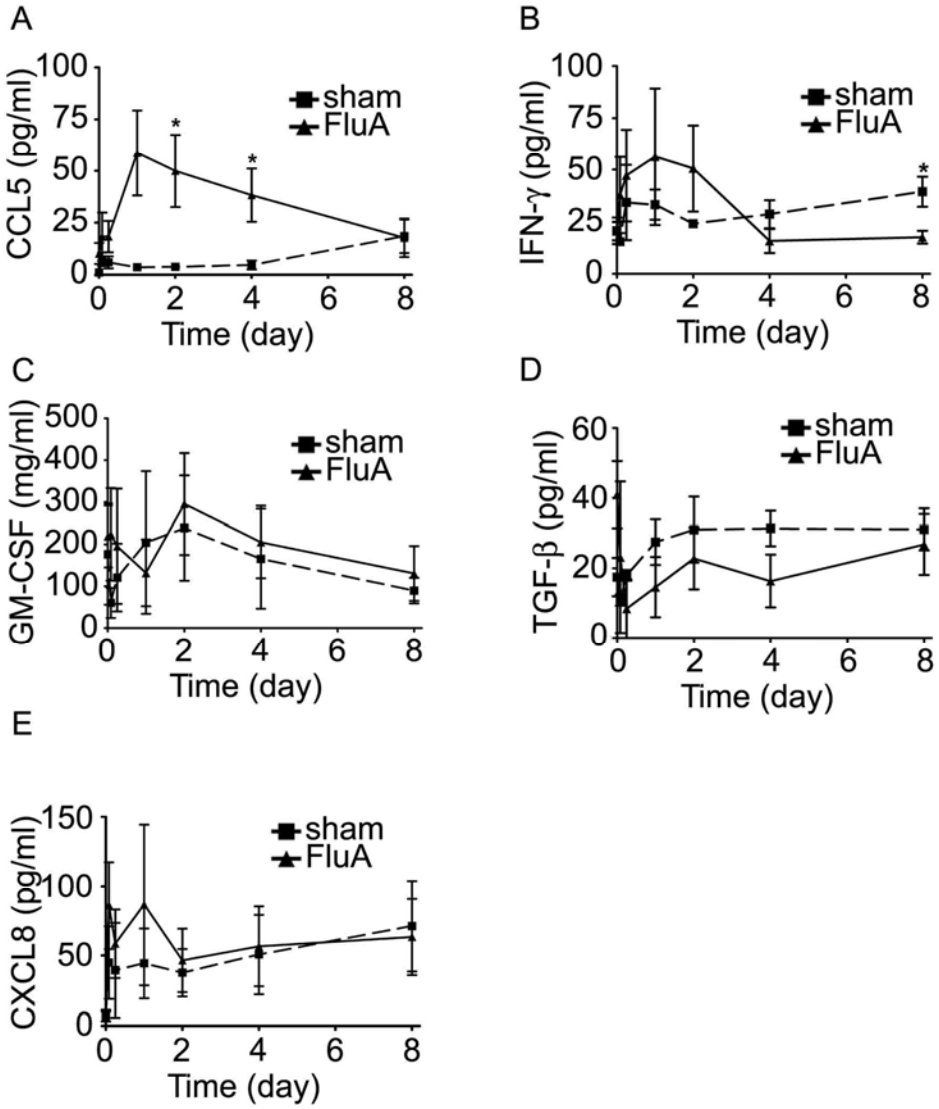

Figure 3.7: Time course comparison of mediator secretion from LAD2 in response to FluA. Supernatants were collected from LAD2 after 0 to 8 d treatment with sham or 20 HAU/ml FluA. ELISA on LAD2 supernatants were performed by Dr. John Gordon's laboratory for (A) CCL5 (RANTES), (B) IFNγ, (C) GM-CSF, (D) TGF-β, and (E) CXCL8 (IL-8) (n = 3). Statistical significance as determined

by student's t-test is represented as *p < 0.05 when compared to sham treatments for each time point.

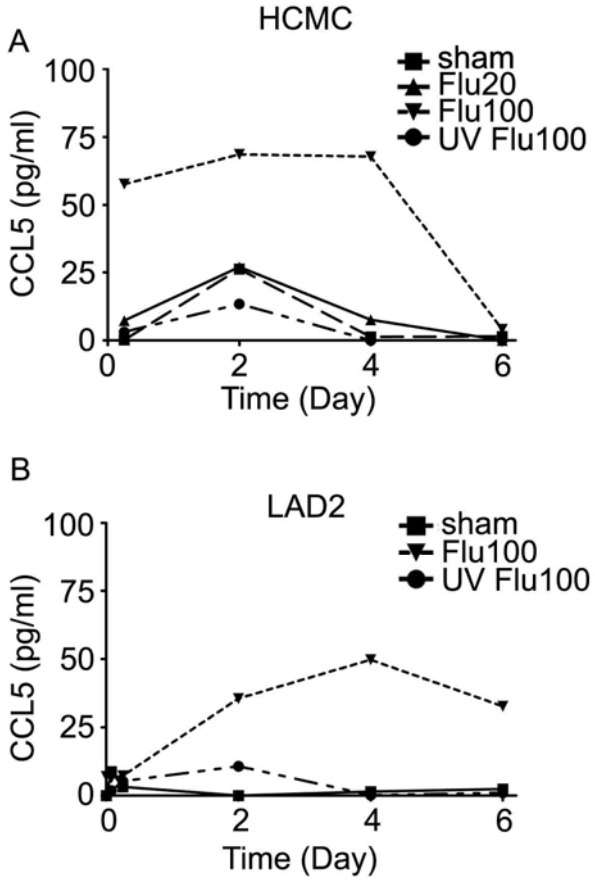

Figure 3.8: Time course comparison of CCL5 secretion from primary human-cultured mast cells (HCMC) and LAD2 in response to FluA and UV-FluA. Supernatants were collected from HCMC and LAD2 after 0 h to 6 d treatment with sham, 20 HAU/ml UV FluA, 20 HAU/ml FluA, or 100 HAU/ml FluA (n = 1). ELISA on (A) HCMC supernatants or (B) LAD2 supernatants were performed by Dr. John Gordon's laboratory for CCL5.

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Chapter IV: Human Mast Cells Express Antiviral Proteins in Response to Influenza A Virus and Inhibit Viral Replication in Human Lung Epithelial Cells

1. Authors

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2. Introduction

Many mechanisms exist in humans to restrict viral infections. For decades, mast cells have been proposed as sentinel cells at mucosal surfaces such as the lungs, thereby defending the host against pathogens (1). Mast cell-deficient mice are more susceptible to bacterial infection, providing an example of the protective role of mast cells against pathogens. However, relatively little is known about the role of mast cells against viruses, although there is evidence that mast cell hyperplasia is associated with viral infections (2). Several studies have demonstrated that antibody-enhanced dengue virus infection activates the release of cytokines and chemokines from mast cells, as well as induces apoptosis of human mast cells (3-5). In addition, activation of viral toll-like receptors (TLR) such as TLR3 and TLR7 induce mast cells to release various pro-inflammatory cytokines as well as IFNα (6, 7). TLR3 activation by the synthetic dsRNA

polyI:C also inhibits mast cell adhesion and promotes mast cell-mediated recruitment of CD8+ T lymphocytes (8, 9). Because of their large repertoire of mediators and wide distribution in the body, it is not surprising that mast cells have an important role in innate immunity (10).

Influenza A virus (FluA) is a highly pathogenic respiratory virus that causes significant morbidity and mortality in seasonal epidemics worldwide (11, 12). FluA primarily replicates in lung epithelial cells. During infection, FluA triggers an immune response that results in inflammation in affected patients. Currently, the role of mast cells in FluA infection has not been studied. Based on the abundance of mast cells in the lungs near the mucosal surface, they are in prime location to interact with FluA that enters the respiratory tract. Our investigations show that FluA infects human mast cells and produces viral mRNA and proteins, but releases little infectious virions (Chapter 2). Thus, mast cells may have antiviral mechanisms that restrict FluA infection.

Antiviral proteins are involved in protection against FluA infection in animal models and in lung epithelial cells. FluA has been shown to be detected by TLR3, TLR7, and the cytoplasmic RNA sensor RIG-I, which subsequently activates signaling pathways to induce cytokine and antiviral responses (13-15). Type I interferons (IFN$\alpha\beta$) are major antiviral cytokines that upregulate IFN-stimulated genes (ISG) in virus-infected cells to restrict virus replication (16). Some ISG have direct antiviral activities against FluA including protein kinase R (PKR), RNaseL/2'5' oligoadenylate synthetase (OAS), and myxovirus resistance protein A (MxA) (17). Other antiviral proteins such as ISG15 and p56 are induced

by IFNαβ, but can also be independent of IFNαβ (18). Nitric oxide (NO) has also been shown to have antiviral effects (19). Previously, our lab showed that mast cells produce NO through the activity of endothelial NO synthase (eNOS/NOS3) (20) or other isoform of NOS. To our knowledge, the expression of these antiviral proteins has never been studied in human mast cells in response to FluA stimulation.

In addition to the possibility of direct infection of mast cells by FluA, the juxtaposition of mast cells to epithelial cells in the lungs suggests that communication likely exists between these two cell types during viral infections. Further, we have shown that mast cells produce cytokines in response to mediators generated by virus-infected epithelial cells, such as IFNαβ (Chapter 3). Supernatants from reovirus-infected mast cells induce NK cell chemotaxis, showing evidence that mast cells interact with other cell types during viral infections (21). Co-culture studies have shown that mast cells also influence the functions of epithelial cells, including effects on their proliferation and barrier function (22, 23). Thus, we postulated that during FluA infection, mast cells may exert effects on epithelial cells to limit the spread of the virus.

To study whether mast cells mount an antiviral response against FluA, we tested the effects of FluA infection on the expression of MxA, PKR, ISG15, p56, and eNOS in human mast cells at the gene and protein level. We also investigated the effect of placing mast cells in co-culture with epithelial cells on FluA titres released from epithelial cells. Our results show that antiviral proteins are expressed following FluA infection of human mast cells. Also, we show

preliminary evidence that mast cells interact with epithelial cells in co-culture to protect against FluA infection of epithelial cells. These results suggest that mast cells possess antiviral mechanisms to restrict the FluA replication cycle.

3. Materials and Methods

Reagents

TLR agonists used were LPS (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and polyI:C (InvivoGen, San Diego, CA, USA).

Cell Culture

Cell culture was performed as described previously in Chapter 2 and 3 for the human mast cell line Laboratory of Allergic Diseases 2 (LAD2) (a generous gift from Drs. A.S. Kirshenbaum and D.D. Metcalfe, National Institutes of Health, Bethesda, MD, USA), and primary human cultured mast cells (HCMC) developed from $CD34^+$ peripheral blood mononuclear cells (PBMC) (24).

Influenza Virus and Ultraviolet (UV)-Inactivation

As described in more detail in Chapter 2, influenza A virus (FluA) was of the A/PR/8/34 strain (H1N1) (kind contribution from Dr. K.P. Kane, University of Alberta, Edmonton, AB, Canada) and UV-inactivation was performed using a UV lamp (ENF-280C, Spectroline, Westbury, NY, USA) at 254 nm. Hemagglutination units (HAU) for the stock FluA virus was determined using the hemagglutination assay as described in Chapter 2 (25).

Virus Infection

FluA infection was performed as described in Chapter 2 for RT-PCR and Western blot experiments with the exception of experiment shown in Fig. 4.3B. Briefly, FluA or media only (sham) was added to LAD2 or HCMC cultures for 1 h to allow for virus adsorption. Cells were then washed 3 times with PBS and fresh media was added to the cells for specific times (0 to 6 d). For the experiment performed in Fig. 4.3B, LAD2 were treated for 48 h with a combination of LPS, PIC, FluA, and/or IFNαβ without washes.

Transcriptome Data from GEO Datasets

Transcriptome data was obtained from the Gene Expression Omnibus (GEO) Datasets (26), a public repository for genomic data that is provided by the National Center for Biotechnology Information (NCBI). We searched for experiments performed on the GPL96 platform. We identified mRNA expression of the antiviral proteins listed in Table 4.1 in three GEO series: GSE1982 (27), GSE1933 (28), and GSE1848 (29). Within these series, six sets of data were obtained from resting human cord blood derived mast cells (CBMC) (accession # GSM90662, GSM90842, GSM26391, GSM26395, GSM34668, GSM34669), one set of data from IgE-treated CBMC (accession # GSM90665), one set of data from human lung mast cells (accession # GSM29629), two sets of data from human tonsil mast cells (accession # GSM29630, GSM29631), and one set of data from the human mast cell line LAD3 treated with *Escherichia coli*.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA transcription and reverse transcription for mRNA was performed as described in Chapter 2. We performed PCR for the mRNA of the following antiviral proteins: PKR, forward primer: 5'-CAG CAC ACT CGC TTC TGA AT-3', reverse primer: 5'-GCT CCG CCT TCT CGT TAT TA-3' (335 bp product); MxA, forward primer: 5'-ATA AAG CCC AGA ATG CCA TC-3', reverse primer: 5'-CCC TGA AAT ATG GGT GGT TC-3' (501 bp product); eNOS, forward primer: 5'-ACC TGC AAA GCA GCA AGT CCA CG-3', reverse primer: 5'-CCG AAC ACC AAA GTC ATG GGA GT-3' (837 bp product); ISG15, forward primer: 5'-GAC CTG ACG GTG AAG ATG CT-3', reverse primer: 5'-CAC CAG GAT GCT CAG AGG TT-3' (249 bp product); p56, forward primer: 5'-ACA CCT GAA AGG CCA GAA TG-3', reverse primer: 5'-TCG GTA AAA CTT GGC TGC AT-3' (601 bp product). We also used β-actin forward primer: 5'-GGCATC CTC ACC CTG AAG TA-3' and reverse primer: 5'-AGG GCA TAC CCC TCG TAG AT-3' (326 bp product) and GAPDH forward primer: 5'-GAG TCA ACG GAT TTG GTC GT-3' and reverse primer: 5'-AAA TGA GCC CCA GCC TTC T-3' (316 bp product). PCR amplification of genes was performed as described in Chapter 2 with modifications. Cycle numbers for PKR, MxA, eNOS, ISG15, p56, β-actin, and GAPDH were optimized to be 33, 28, 30, 28, 32, 28, and 28 cycles, respectively, to be within the exponential phase of amplification. Annealing temperature of 58^{o}C was used for all products except 56^{o}C was used for eNOS and 55^{o}C was used for β-actin. cDNA products were confirmed by size based on migration on agarose gels.

Western Blot

Western blot was performed as described in Chapter 2 with the following modifications. MxA protein was detected using a mouse monoclonal anti-human MxA antibody (generous gift from Dr. George Kochs, University of Freiburg, Germany) (30). eNOS protein was detected using a mouse monoclonal anti-human eNOS antibody (610296, BD Transduction Laboratories, BD Biosciences, Mississauga, ON, Canada). A rabbit monoclonal antibody against β-actin (sc-1616R, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for Fig. 4.3. A mouse monoclonal antibody (AC-15) against β-actin (sc69879, Santa Cruz Biotechnology) was used for Fig. 4.4. IRDye®680-conjugated polyclonal goat anti-mouse IgG and IRDye®800CW- conjugated anti-rabbit IgG were used with the Odysey® Infrared Imaging System for immunodetection of proteins (Li-cor Biosciences, Lincoln, NE, USA).

Mast Cell-Epithelial Cell Co-Culture Model

Calu-3 (seeded at 2.5 x 10^5 cells/insert) were grown on 0.4 μm pore size Corning® Snapwell® inserts (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) with MEM media containing 20% fetal bovine serum (FBS) (see Chapter 2 for supplements added, Invitrogen Canada Inc., Burlington, ON, Canada). 500 μl of media was placed in the apical (AP) or upper chamber and 2.0 ml was placed in the basolateral (BL) or lower chamber. Fresh media was replaced in both chambers every 2 d. At 6 d, LAD2 in Stempro media (see Chapter 2 for supplements added, Invitrogen Canada Inc.) or Stempro media only was added to

the BL chamber. At 7 d, 100 µl of FluA at 20 HAU/ml was added to the AP side of Calu-3 for 1 hour (h) to allow for virus adsorption. Calu-3 were washed with 500 µl of PBS, 3 times and subsequently 500 µl of fresh media was added to the AP side. After 5 d post-FluA treatment, media in both the upper and lower chambers were assayed for presence of FluA.

Quantification of FluA Titres

Quantification of total FluA particles was determined by the hemagglutination assay in HAU/ml and infectious FluA particles was determined by calculation of the tissue culture infectious dose ($TCID_{50}$)/ml based on results of the hemadsorption assay (see Chaper 2 for details).

Live/Dead Cell Assay

Cell viability from co-culture experiments was determined by the "LIVE/DEAD® Viability/Cytotoxicity Kit" for mammalian cells (L-3224, Invitrogen Canada Inc.) that uses two different fluorescent probes to detect live or dead cells. % live and % dead cells were calculated based on manufacturer's protocol. In Table 4.3, "% live 1" indicates the % live cells calculated from the live cell signal of a particular sample, and "% live 2" indicates % live cells calculated from the dead cell signal subtracted from 100% of the same sample. % live Ave is the average number calculated from the "% live 1" and % "live 2 values".

4. Results

Expression of mRNA for antiviral proteins in human mast cells from transcriptome data

To investigate whether human mast cells express antiviral proteins, we first analyzed transcriptome data from the public database "GEO Datasets" (26). Specifically, we searched all available mast cell transcriptomes for expression of antiviral proteins previously shown to be important in FluA infection, as well as the NOS isoforms (Table 4.1). Human primary mast cells cultured from umbilical cord blood (cord blood-derived mast cells - CBMC) constitutively express mRNA for ISG15, four different isoforms of OAS, PKR, MxA, and p56 in at least one of six datasets. Primary mast cells isolated from human lungs and tonsils showed similar constitutive expression of mRNA for ISG15, OASL, PKR, and MxA. The mast cell line LAD3 stimulated with *Escherichia coli* (E. coli) expressed ISG15 and OAS3 transcripts. mRNA for three isoforms of NOS, namely neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), were not identified in any of the human mast cell datasets under the conditions listed. This database search reveals that human mast cells constitutively express mRNA for several antiviral proteins that are important against FluA infection.

Transcription of antiviral proteins induced by FluA in human mast cells

We investigated the transcriptional expression of various antiviral proteins in human mast cells in response to FluA treatment. The human mast cell line LAD2 was treated with sham or 20 HAU/ml of FluA on 0 to 5 d and samples were analyzed by RT-PCR. Representative gels for expression of PKR, MxA,

eNOS, ISG15, and p56 are shown in Fig. 4.1A. While mRNA levels appeared unchanged by sham treatments, FluA induced all tested antiviral proteins in LAD2 (Fig. 4.1A). Densitometric analyses are shown in Fig. 4.1B-F. PKR and MxA, two IFNαβ-dependent antiviral proteins, were induced from 0.25 to 1 d of FluA treatment (Fig. 4.1B-C). The IFNαβ-independent ISG15 and p56 were induced over a longer time period, from 0.25 to 4 d of FluA treatment for ISG15, and from 0.25 to 5 d of FluA treatment for p56 (Fig. 4.1E-F). eNOS was induced at a later time point by FluA treatment, from 4 to 5 d (Fig. 4.1D). Expression of nOS and iNOS mRNA were also tested, but not detectable in sham or with FluA treatment (data not shown).

We also investigated the mRNA expression of MxA, PKR, and ISG15 in primary human cultured mast cells (HCMC) (Fig. 4.2A). Similar to LAD2, PKR was upregulated at 0.25 to 1 d of FluA treatment, while MxA was upregulated at 0.25 to 2 d of FluA treatment in HCMC (Fig. 4.2B-C). Again, ISG15 was induced by FluA treatment over a longer time period, from 0.25 to 5 d (Fig. 4.2D). eNOS expression was not detectable in HCMC treated with FluA (data not shown). These results show that transcripts of antiviral proteins are upregulated by FluA in a human mast cell line and primary cultured human mast cells.

Protein expression of MxA and eNOS induced by FluA in human mast cells

Next, Western blot analysis was used to determine protein expression of MxA (Fig. 4.3A) and eNOS (Fig. 4.3B) in human mast cells treated with FluA. UV-inactivated FluA is a replication-incompetent virus used to assess whether induction of antiviral proteins in mast cells is dependent on FluA replication. The

epithelial cell line Calu-3 shows upregulation of MxA at 4 and 6 d of FluA treatment compared with sham, which showed no detectable MxA. Expression of MxA was also observed at 6 d of UV-FluA treatment in Calu-3, although less expression than for FluA treatment (Fig. 4.3A). Both LAD2 and HCMC showed induction of MxA expression with FluA, but not with UV-FluA or sham. FluA treatment induced MxA expression at 4 d in LAD2 and at 2 d in HCMC (Fig. 4.3A). MxA mRNA expression was detectable at 0.25 d post-FluA infection, with protein expression occurring at 2 to 4 d. These results suggest that upregulation of MxA in human mast cells is dependent on exposure to replication-competent FluA.

We also tested eNOS expression in LAD2 in response to 2 d of treatment with FluA as well as other pathogen-associated stimuli, including lipopolysaccharide (LPS), polyinosinic:polycytidilic acid (polyI:C), and IFNαβ (Fig. 4.3B). IFNαβ pretreatment was used since IFNαβ is produced early during FluA infections prior to the first cycle of FluA production by epithelial cells. FluA with 1 d IFNαβ pretreatment was the only tested condition that induced eNOS expression in LAD2. FluA, UV-FluA, polyI:C alone, polyI:C in combination with LPS, and polyI:C pretreated with IFNαβ did not induce eNOS expression. Human umbilical vein endothelial cells (HUVEC) were used as a positive control for eNOS expression. These results show that eNOS is expressed upon specific stimuli in mast cells, in this case by FluA treatment with prior IFNαβ exposure. While eNOS mRNA expression was detectable at 4 d post-FluA infection, eNOS protein expression was undetectable up to 6 d post-infection. MxA protein was

detectable approximately 2 to 4 d after the expression of MxA mRNA. If the timing of eNOS protein expression in relation to mRNA expression followed similar trends as that for MxA, later time points up to 8 d may need to be evaluated for eNOS protein expression. In our experiments, we only investigated antiviral protein expression up to 6 d post-FluA infection.

Human mast cells inhibit FluA protein expression in epithelial cells in a co-culture model

To investigate the influence of mast cells on FluA infection of epithelial cells, we used a transwell system where Calu-3 were exposed to FluA with or without mast cells in co-culture. Calu-3 were grown on inserts of the upper chamber of the transwell apparatus while LAD2 were placed in the lower chamber. FluA was added to the upper chamber on Calu-3 monolayers. Western blot analysis was performed to assess the effect of mast cells on FluA protein synthesis in infected epithelial cells. Expression of FluA proteins in LAD2 and Calu-3 from the co-culture is shown on the Western blot (Fig. 4.4A). Hemagglutination and hemadsorption assays for the presence of virus showed that FluA did not cross into the lower chamber (data not shown); thus, LAD2 in co-culture with FluA-infected Calu-3 did not express FluA proteins. The expression of FluA polymerase proteins (Ps), hemagglutinin (HA), nucleoprotein (NP), and matrix protein 1/non-structural protein 1 (M1/NS1) were measured in FluA-infected Calu-3 co-cultured with LAD2 compared to FluA-infected Calu-3 alone (Fig. 4.4B). M1 and NS1 cannot be distinguished because they have similar

molecular weights. Expression of FluA HA was significantly decreased when FluA-infected Calu-3 were co-cultured with LAD2 compared to FluA-infected Calu-3 alone ($p < 0.01$). Expression of the other FluA proteins in Calu-3 was not significantly different between treatments (Fig. 4.4B). This is evidence that mast cells in co-culture inhibit FluA protein expression in infected Calu-3.

Human mast cells inhibit FluA release from epithelial cells in a co-culture model

With evidence that FluA protein expression was downregulated, we also investigated whether release of FluA particles from Calu-3 was affected by mast cells in co-culture. LAD2 and another human mast cell line HMC-1 were used in co-culture with Calu-3. Hemagglutination assay was used to measure the amount of total new FluA particles produced. An end-point dilution assay using hemadsorption was used to determine the amount of infectious FluA particles calculated as 50% tissue culture infectious dose ($TCID_{50}$). For the infection protocol, Calu-3 monolayers were incubated with FluA for 1 h to allow virus adsorption, washed three times with three volumes of PBS, then incubated with fresh media for 5 d. The third PBS wash for all FluA-treated Calu-3 tested negative for FluA (data not shown). Both total FluA and infectious FluA release from Calu-3 at 5 d post-infection are shown in Table 4.2. Calu-3 produced 2560 HAU/ml of total FluA and 5.68 $logTCID_{50}$/ml (n=2) of infectious FluA without mast cells in co-culture. When LAD2 were added to the co-culture, the amount of total FluA decreased to 640 HAU/ml and the amount of infectious FluA decreased

to 5.49 log$TCID_{50}$/ml (n=2). With HMC-1 in co-culture, the amount of infectious FluA decreased to 4.30 log$TCID_{50}$/ml (n=1). These preliminary results show that mast cells in co-culture inhibit viral production in FluA-treated epithelial cells.

We also measured the viability of FluA-treated Calu-3 in co-culture with HMC-1 using a fluorescent live/dead cell assay (Table 4.3). There was little difference in the viability of Calu-3 alone (83.6%) compared to Calu-3 co-cultured with HMC-1 but without exposure to FluA (86.1%). FluA treatment decreased Calu-3 viability to 74.9% and this recovered to 95% when FluA-infected Calu-3 were co-cultured with HMC-1. This provides preliminary evidence that mast cells may protect epithelial cells against cell death from FluA exposure.

5. Discussion

We found that human mast cells express antiviral proteins at the mRNA and protein levels in response to FluA treatment. Specifically, human mast cells upregulate the transcriptional expression of MxA, PKR, ISG15, p56, and eNOS when exposed to FluA (Fig. 4.1-2). Induction of mRNA expression of all the antiviral proteins was seen by 0.25 d, except for eNOS mRNA expression, which was seen at 4 d. The duration of mRNA expression also varied, from approximately 1 d for PKR, MxA and eNOS to 4 to 5 d with ISG15 and p56. This suggests different regulatory mechanisms for induction of antiviral transcripts by FluA in mast cells. Also, the protein level of MxA was induced by FluA in both LAD2 and HCMC (Fig. 4.3A). In contrast, eNOS was not induced by FluA

treatment alone, but was detected in LAD2 pretreated with IFNαβ and subsequently treated with FluA (Fig. 4.3B). Thus, it appears that particular cytokine environments allow mast cells to respond in a specific manner to FluA treatment.

The ability of mast cells to express antiviral proteins led to the idea that mast cells may play a protective role against FluA infection of epithelial cells, which are the primary target of FluA. To test this hypothesis, we set up a co-culture model using human epithelial cells and human mast cells in a transwell system. Our co-culture experiments showed preliminary evidence that addition of mast cells to the lower chamber inhibited FluA replication of epithelial cells in the upper chamber (Table 4.2). We also found that mast cells inhibited FluA HA expression in infected epithelial cells and reduced the percentage of cell death in epithelial cells caused by FluA (Fig. 4.4 and Table 4.3).

Although preliminary, these are promising results suggesting that mast cells protect the host from FluA infection by inhibiting the development of FluA infection in epithelial cells. Our laboratory as well as others have shown that mast cells are susceptible to infections by viruses such as FluA, HIV, and dengue virus (Chapter 2, (3, 31)). Mast cells also respond by secreting inflammatory factors such as the cytokines IL-1β and IL-6 as well as the chemokines CCL5, CCL3α and CCL3β when exposed to dengue virus. Another study reported that human mast cells respond to UV-FluA and respiratory syncytial virus (RSV) by secreting IFNα (7). This is the first study to show that expression of MxA, PKR, ISG15, p56, and eNOS is upregulated in human mast cells in response to FluA

stimulation. In Chapter 2, we showed that human mast cells undergo transcription and translation of FluA proteins, but produce little infectious virus. Mast cell induction of antiviral proteins may provide a possible explanation for the restriction of the FluA replication cycle. Interestingly, evidence suggests that mast cells have chemotactic activity on CD8+ T cells and NK cells, immune cells that are important in immunity against viral infections (9, 21). In the first study, mast cells were shown to be important for the recruitment of CD8+ T cells to the peritoneum of mice injected with polyI:C (9). In the latter study, release of CXCL8 from mast cells in response to reovirus infection was shown to mediate the chemotaxis of NK cells (21). To our knowledge, no study thus far has investigated the effect of mast cells on epithelial cells during viral infection. Although a few studies of mast cell-epithelial cell interactions exist, none address this interaction in the context of innate immunity or viral infections. Our data supports current literature demonstrating that mast cells are critical effector cells in innate immunity and provides additional evidence that mast cells are active against viral infections.

To follow up on this study, we need to identify which of the antiviral proteins may be involved in restricting FluA infection in mast cells. It would be interesting to look at the signaling pathways involved in the inductions of these antiviral proteins in mast cells. There may be mast cell pathways that differ from other cell types, demonstrating unique abilities of mast cells to respond to FluA. Furthermore, we plan to investigate the mechanisms by which mast cells exert their inhibitory effects in co-culture with epithelial cells.

In summary, this study shows potential human mast cell antiviral mechanisms against FluA infection, including upregulation of antiviral proteins in response to direct FluA infection as well as inhibiting FluA production in epithelial cells in co-culture. In addition to our previous study showing that FluA-infected mast cells produce little infectious virus, this research suggest that mast cells may limit the host cell range of FluA in the infected host. In determining the mechanism of FluA restriction in human mast cells, novel antiviral processes that apply to other viral infections may be revealed. These unique processes could provide important targets for future development of antiviral prophylaxis and therapy for FluA infections. Our research substantiates a significant role for mast cells in innate immunity and strengthens the growing evidence for mast cells in defense against viruses.

6. Figures

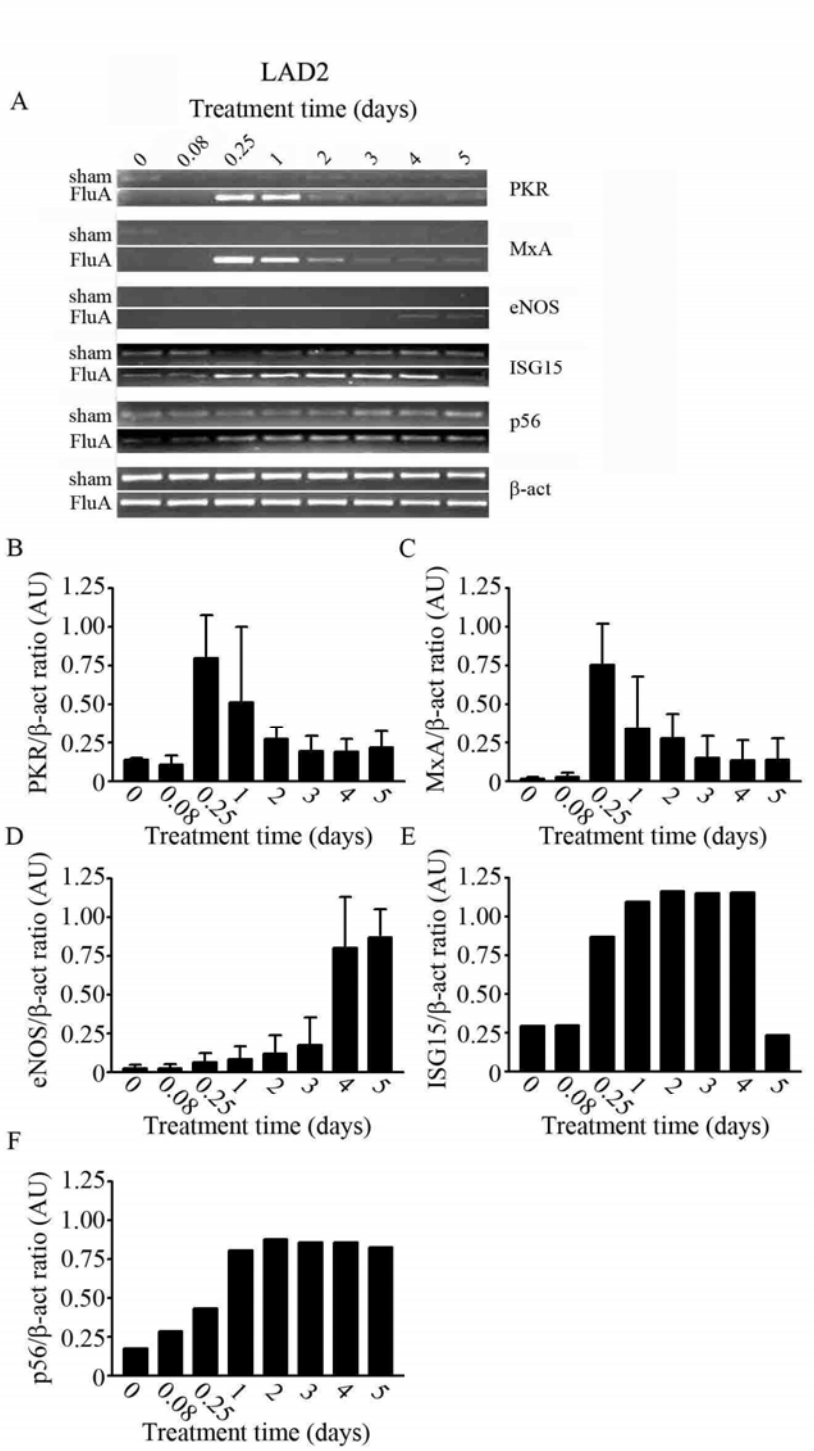

Figure 4.1: Gene expression of antiviral proteins in the human mast cell line LAD2 in response to FluA from 0 to 5 d. PCR analysis of mRNA expression of

the antiviral proteins PKR, MxA, eNOS, ISG15, and p56. Expression of β-actin (β-act) mRNA was used as a loading control. Densitometry data is expressed in arbitrary units (AU) based on antiviral mRNA to β-actin mRNA ratios. LAD2 were treated with sham or 20 HAU/ml of FluA and samples were collected after 0 to 5 d post-treatment. A) Time course of mRNA expression of antiviral proteins in LAD2. Densitometric analysis of mRNA expression of B) PKR, $n = 2$; C) MxA, $n = 2$; D) eNOS, $n = 2$; E) ISG15, $n = 1$; and F) p56, $n = 1$.

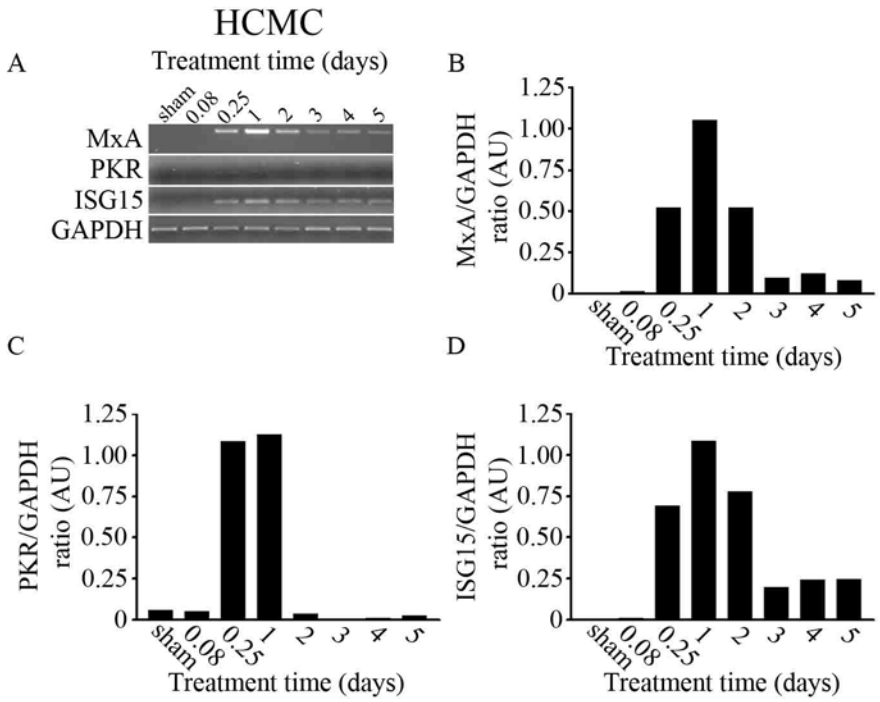

Figure 4.2: Gene expression of antiviral proteins in HCMC in response to FluA from 0 to 5 d. PCR analysis of mRNA expression of the antiviral proteins MxA, PKR, and ISG15. Expression of GAPDH mRNA was used as a loading control. Densitometry data is expressed in arbitrary units (AU) based on antiviral mRNA

to GAPDH mRNA ratios with *n*=1. HCMC were treated with sham or 20 HAU/ml of FluA and samples were collected after 0 to 5 d post-treatment. A) Time course of mRNA expression of antiviral proteins in HCMC. Densitometric analysis of mRNA expression of B) MxA, C) PKR, and D) ISG15.

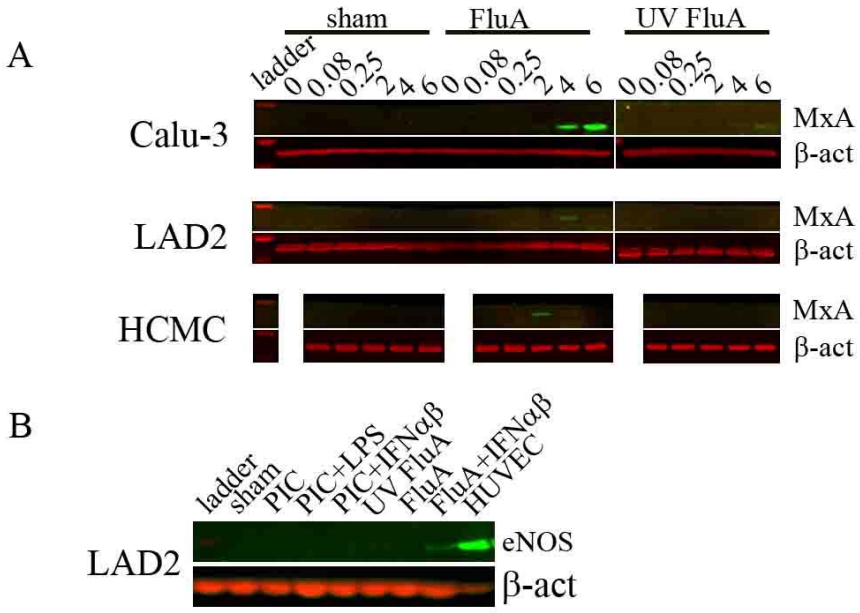

Figure 4.3: Protein expression of MxA and eNOS in human mast cells in response to FluA. Western blot analysis was used to detect proteins. Expression of β-actin (β-act) was used as a loading control. MxA and eNOS appear in the green channel and β-actin appears in the red channel. A) Time course expression of MxA post-FluA treatment in Calu-3, LAD2, and HCMC (*n* = 1). Calu-3 has been shown to express MxA in response to FluA (32). Cells were treated with sham, 20 HAU/ml of FluA, or 20 HAU/ml of UV-inactivated (UV) FluA. Samples were collected at 0 to 6 d post-treatment. B) eNOS expression in response to viral-associated stimuli in LAD2 (*n* = 1). LAD2 were treated with sham, or a combination of 10

μg/ml of polyI:C (PIC), 10 ng/ml of lipopolysaccharide (LPS), 80 ng/ml of IFNα and IFNβ (IFNαβ), and/or 20 HAU/ml of FluA. Primary human umbilical vein endothelial cells (HUVEC) were used as a positive control for eNOS expression. Samples were collected at 2 d post-treatment.

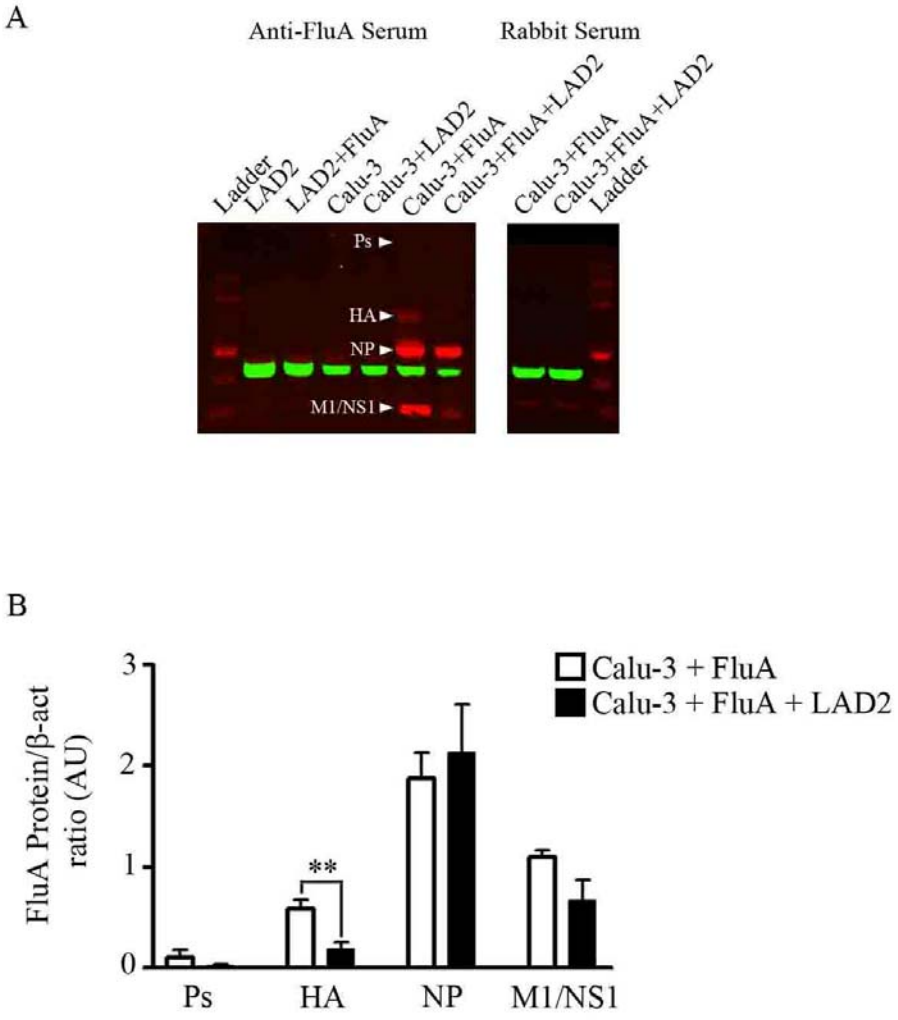

Figure 4.4: FluA protein expression in Calu-3 co-cultured with LAD2. Western blot analysis was used to detect FluA proteins. FluA proteins appear in the red channel and β-actin appears in the green channel. FluA proteins detected were Ps

(polymerase proteins), HA (hemagglutinin), NP (nucleoprotein), M1 (matrix protein 1), and NS1 (non-structural protein 1). Calu-3 were grown on inserts in the co-culture chamber where FluA was added to the apical (AP) side and LAD2 was added to the basolateral (BL) side. A) Representative western blot showing expression of FluA proteins in infected Calu-3 5 d post-FluA treatment with or without co-culture with LAD2. FluA proteins were detected using an anti-FluA rabbit serum. Control blot is shown using normal rabbit serum. B) Densitometric analysis of A) as measured by FluA protein to β-actin ratio in arbitrary units (AU) ($n = 3$), *$p < 0.05$.

7. Tables

Proteins	CBMC	CBMC+ IgE	Lung MC	Tonsil MC	LAD3+ Ecoli
ISG15	Y6	Y	Y	Y2	Y
OAS1	N2 Y4	N	nd	nd2	nd
OAS2	N2 Y4	N	nd	nd2	nd
OAS3	N2 Y4	N	M	N2	Y
OASL	Y6	Y	N	Y M	nd
PKR	N Y5	Y	Y	Y2	nd
MxA	N Y5	Y	Y	N Y	nd
p56	N5 Y	N	nd	nd2	nd
nNOS	N5 M	N	nd	nd2	nd
iNOS	N6	N	nd	nd2	nd
eNOS	N6	N	nd	nd2	nd

Table 4.1: Transcriptome data of antiviral proteins in human mast cells from "GEO datasets".Five different human mast cell subsets were found with this

database: cord blood derived mast cells (CBMC), CBMC + IgE (primed with α-IgE for 18 h, then stimulated with IgE for 2 h), lung mast cells (lung MC), tonsil mast cells (tonsil MC), and LAD3 + E.coli. We reviewed the mRNA expression of antiviral proteins previously shown to be important in FluA infection, which include ISG15, four isoforms of OAS (OAS1, 2, 3, and L), PKR, MxA, and p56. We also reviewed the mRNA expression of the three isoforms of NOS (nNOS, iNOS and eNOS). For expression of transcripts, "Y" indicates yes, "N" indicates no, "M" indicates moderate, and "nd" indicates not done. The *n*-value for each particular experimental setup is indicated by the numbers next to the letter.

	HAU/ml	**log $TCID_{50}$/ml**
+Flu	2560	5.68
+Flu +LAD2	640	5.49
+Flu +HMC-1	n/d	4.30

Table 4.2: Production of total and infectious FluA particles from infected Calu-3 co-cultured with the human mast cell lines, LAD2 and HMC1. Calu-3 were grown on inserts in the co-culture chamber where FluA was added to the apical side. Media, LAD2, or HMC-1 were added to the basolateral (BL) side. Supernatants from the apical side were collected at 5 d post-FluA treatment and assayed. Hemagglutination assay was used to determine total FluA particles in hemagglutination units (HAU)/mL and hemadsorption assay was used to determine infectious FluA particles in log$TCID_{50}$/ml. The logTCID50/ml for +FluA and +FluA+LAD2 samples are the average of $n = 2$ experiments. All other

values are from n = 1 experiments. “n/d” indicates experiments that were not done.

	%live 1	%live 2	%live Ave
	84.2	83.1	83.6
+HMC-1	89.8	82.4	86.1
+Flu	64.7	85.2	74.9
+Flu+HMC-1	100.0	90.1	95.0

Table 4.3: Viability of FluA-infected Calu-3 in co-culture with HMC-1. A live/dead cell assay with fluorescent dyes was used to determine the viability of intact Calu-3 monolayers on inserts (n = 1). Calu-3 were grown on inserts in a co-culture chamber where media or FluA was added to the apical (AP) side. Media or HMC-1 were added to the basolateral (BL) side. The table shows the percentage of live Calu-3 determined by two different approaches as described in materials and methods (%live 1 and %live 2) and the average live Calu-3 (%live Ave).

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Chapter V: General Discussion and Conclusions

1. Summary of Major Findings

We established that influenza A virus (FluA) infects human mast cells as evidenced by transcription of viral mRNA and translation of viral proteins, with relatively little detectable release of new FluA particles compared with infected human epithelial cells. Treatment of human mast cells with FluA resulted in detection of FluA mRNA in mast cells, showing that FluA is capable of using mast cell transcriptional machinery. Expression of FluA mRNA followed similar time courses in both primary human mast cells and human mast cell lines when compared with human epithelial cells. We also observed that FluA protein expression is sustained in mast cells, while production of FluA virus is decreased in mast cells compared with epithelial cells. This led us to investigate whether localization of FluA proteins differed between mast cells and epithelial cells. We showed that FluA hemagglutinin protein is present in the nucleus and cytoplasm of mast cells, but did not localize to the plasma membrane where new FluA particles are assembled.

With the knowledge that FluA infects mast cells, we studied the secretory response of human mast cells to viral-associated stimuli i.e. factors expected to be present in the surrounding environment during a viral infection. Mast cells were treated with the TLR3/MDA5 agonist PIC, IFNα and β, and FluA to investigate the release of various cytokines from mast cells, including proinflammatory and immunomodulatory cytokines as well as chemokines. Mediators released from

human mast cells in response to the different viral-associated stimuli include TGF-β, IL-6, CXCL8, and CCL5. The profile of mediators released from mast cells differed between PIC, IFN, and FluA treatment, with each stimulus generating a specific secretory response.

In addition to their role as secretory cells, we investigated whether mast cells had antiviral mechanisms to limit FluA infection. This would help explain our observation that mast cells produced comparable amounts of FluA protein but showed decreased release of FluA particles in comparison with epithelial cells. This blockage of the FluA life cycle in mast cells occurs at a post-translational step, and could potentially involve inhibition of FluA protein transport, assembly of viral particles, or lack of cleavage of HA by proteases to allow release of progeny viruses. Our results showed that at the mRNA and/or protein level, mast cells express MxA, PKR, ISG15, and p56, proteins which have been shown to be important in suppression of FluA infectivity (1-5). FluA treatment also induces mast cells to express eNOS, which has been shown by previous researchers in our lab to produce biologically active NO in human mast cells (6). Since mast cells express antiviral proteins, we used a co-culture model to test whether mast cells protect epithelial cells from infection by FluA. Our preliminary results showed that addition of human mast cells to the co-culture system decreased the infectious titre released from FluA-infected human epithelial cells.

2. Conceptual Model

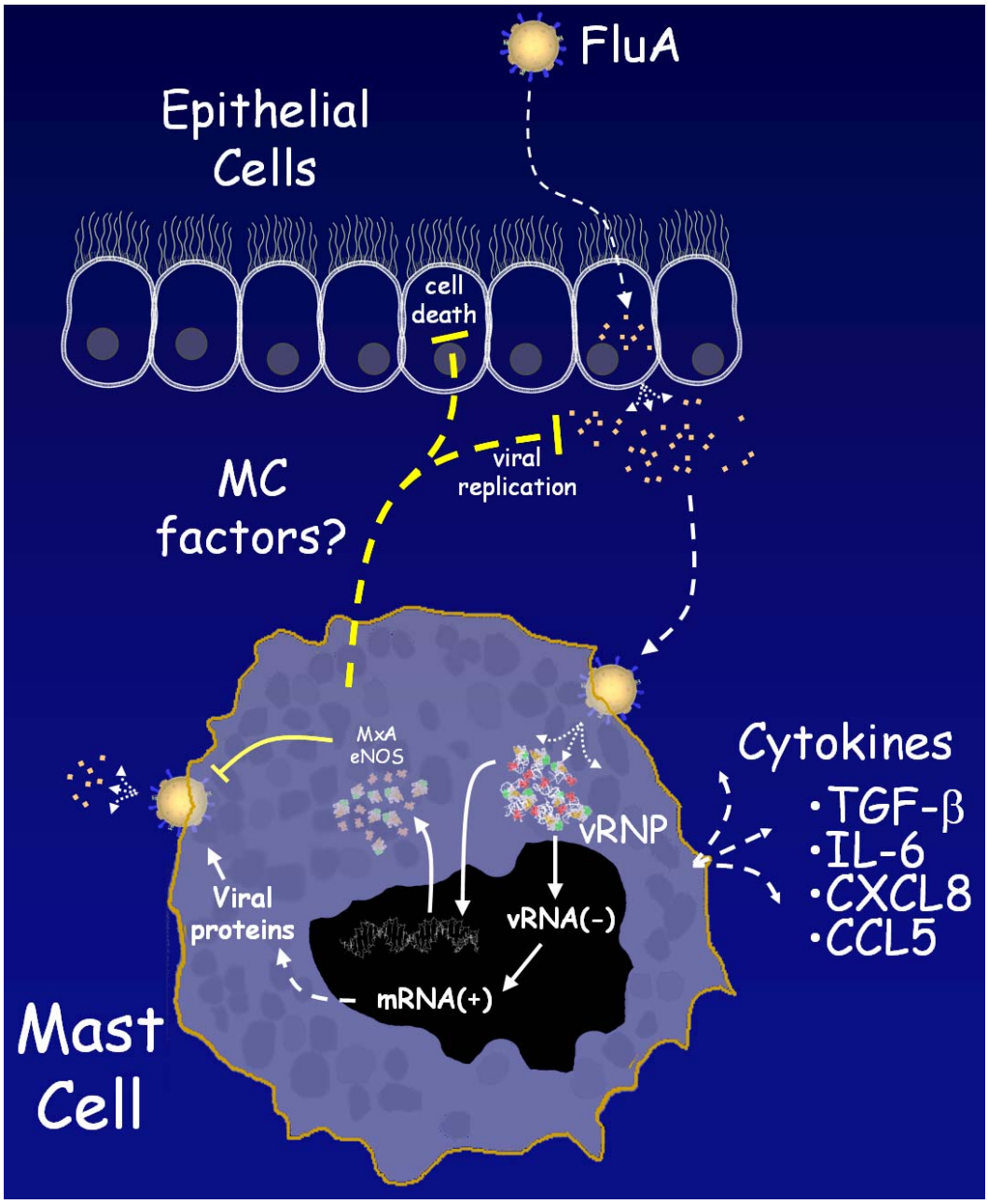

Figure 5.1: Conceptual Model (original figure).

Mast cells are located in close proximity with epithelial cells, mostly beneath the epithelial lining, in the respiratory tract. When FluA infects epithelial cells, the virus undergoes cycles of replication with multiple copies of progeny

virions released into the cellular environment. Epithelial cells respond to FluA infection by releasing antiviral factors such as IFNα and β, which act on surrounding mast cells. FluA also causes damage of the epithelial lining, leading to exposure of mast cells to FluA and dsRNA (TLR ligand) in the lungs. FluA RNA replication and protein synthesis occurs in mast cells. However, in contrast to productive FluA infection of epithelial cells, a near absence of progeny FluA is released from infected mast cells. Infection of mast cells by FluA leads to mast cell secretion of cytokines and chemokines, which contribute to the host innate immune response. FluA infection induces mast cell expression of antiviral proteins, such as MxA, PKR, ISG15 and eNOS, which potentially contribute to limiting infection of the host at the epithelial border. Signals from mast cells also protect epithelial cells from FluA infection by decreasing FluA infectivity by an unknown mechanism (blue arrow).

3. Results and Their Relation to Original Objectives

1. To characterize the FluA replication cycle in human mast cells.

Our primary aim for this objective was to determine whether FluA infects human mast cells and subsequently to characterize the life cycle of FluA infection in mast cells. We tested the infectivity of FluA in two human mast cell lines HMC-1 and LAD2 in addition to primary mast cells cultured from human peripheral blood (HCMC). We found that human mast cells produced FluA mRNA (Fig. 2.2), FluA

proteins (Fig. 2.3), and occasionally released new infectious FluA particles (Table 2.3) when infected with FluA.

2. To compare FluA replication in human mast cells versus human epithelial cells. As epithelial cells are the primary target of FluA infection in the human host and have been extensively studied in various experimental models, we compared the replication of FluA in human mast cells and epithelial cells. We found that transcription of FluA mRNA occurred in a similar time course in mast cells and epithelial cells (Fig. 2.3), whereas protein was detectable at later time points in mast cells compared to epithelial cells (Fig. 2.4). We were unable to detect significant release of total FluA particles as determined by hemagglutination assay from mast cells (Fig. 2.7) Release of infectious FluA particles as determined by hemadsorption assay from mast cells was only detectable at > 4 log higher initial FluA dose than that used for epithelial cells (Table 2.2). We observed with confocal microscopy that FluA hemagglutinin protein was expressed in the cytoplasm and nuclei of mast cells, but did not localize to the plasma membrane as in epithelial cells. These results together suggested to us that FluA mRNA and proteins were produced in mast cells, but viral release was inhibited compared with epithelial cells due to a lack of protein transport to the plasma membrane for viral assembly.

3. To measure cytokine release from human mast cells treated with viral-associated stimuli: intact and UV-inactivated FluA, IFNα and β, and TLR ligands.

In chapter 3, we wanted to compare the secretory response of human mast cells, using both cell lines and primary cultured cells, to different viral-associated stimuli. We measured a variety of factors released from mast cells including growth factors, immunomodulatory cytokines, pro-inflammatory cytokines, and chemokines. Activation by the TLR3 agonist PIC, and both IFNα and β induced HMC-1 release of IL-6 and TGF-β, with polyI:C also inducing CXCL8 release (Fig. 3.1 and 3.3). In a pilot study using a multikine array for 36 cytokines, we also showed preliminary evidence that LAD2 secretion of IFNγ, CD40 ligand, CCL5, and PAI-1 were increased with either UV-FluA or FluA treatment, but not with polyI:C (Fig. 3.5). Further ELISA experiments confirmed that FluA, but not UV-FluA, induced LAD2 secretion of CCL5, but not IFNγ or CXCL8, at 2 d and 4 d post-treatment (Fig. 3.7 and 3.8). These experiments showed that different viral-associated stimuli elicit specific responses from human mast cells, demonstrating that intact FluA virus activate mast cells in a unique manner compared with factors that are generated during the host immune response against FluA.

4. To study the expression of antiviral proteins in FluA-infected human mast cells. Since FluA-infected human mast cells produced few progeny FluA particles, we investigated whether mast cells expressed antiviral proteins, which could help explain the limited infectivity of FluA in mast cells. The transcriptional expression of MxA, PKR, ISG15, p56, and eNOS are upregulated in human mast cells exposed to FluA (Fig. 4.1 and 4.2). At the protein level, mast cell expression

of MxA was induced by FluA alone, while eNOS was induced by FluA with IFNαβ pretreatment (Fig. 4.3).

5. To determine the effect of mast cells on FluA infection of epithelial cells in a human mast cell-epithelial cell co-culture system.

We investigated whether human mast cells had protective effects on human epithelial cells infected with FluA. Our preliminary evidence showed that when either HMC-1 or LAD2 were placed in co-culture with epithelial cells infected with FluA, the amount of infectious FluA virus released from epithelial cells was inhibited (Table 4.2). Mast cells added in co-culture also decreased FluA protein expression and percentage of cell death in infected epithelial cells (Fig. 4.4 and Table 4.3). These results suggest that mast cells may protect the host from FluA infection by inhibiting the development of FluA infection in epithelial cells.

4. Results and Their Relation to the Literature

Our report showing that mast cells respond to FluA infection supports data in the literature that mast cells are important in the innate immune response against various pathogens. The majority of the published studies on mast cell responses to viral stimuli have focused on the ability of mast cells to secrete mediators as opposed to investigating potential antiviral responses of mast cells. One study showed that infection by reovirus or activation by polyI:C induces mast cell secretion of CXCL8, which mediates the chemotaxis of NK cells, which

suggests a potential involvement of mast cells in recruiting NK cells during a viral infection (7). In terms of mediator secretion, our results that mast cells release CCL5 in response to FluA support previous studies showing that mast cells release histamine, cytokines, and chemokines in response to viruses such as HIV and dengue virus (8-10). Prior studies have used viral models to confirm the effects of TLR3 activation in mast cells, but did not compare differences between TLR3 activation and viral activation (11, 12). From our studies, we have preliminary evidence that the cytokines released from mast cells differ depending on whether FluA is replication-competent (FluA vs. UV-FluA), or whether mast cells were pretreated with IFNαβ. These findings are important because it illustrates that mast cells do not respond in a general manner to similar triggers once a threshold is met; rather, mast cell responses appear to be specific for each viral-associated stimuli. This is in agreement with studies on IgE activation of mast cells that show modulation of responses by different factors in the mast cell environment. Previously in our lab, we have shown under video microscopy that individual mast cells under the same IgE treatment condition have dichotomous responses, with mast cells degranulating in the absence of NO production and NO-producing mast cells refractory to degranulation (6).

Mast cells are productively infected by both HIV and dengue virus, while RSV is unable to infect mast cells (8, 9, 13). Our results show that mast cells produce newly formed FluA particles, but only at 4 log higher doses of initial infection than for epithelial cells. Since the authors of the other studies did not directly compare the viral titres from virus-infected mast cells versus the primary

target cell type, it is not possible to determine whether production of virus from mast cells was also diminished in these viral models. Together, the established literature and our findings suggest that mast cells are permissive to infection by select viruses, which may reflect interplay between counteracting viral and host responses.

We found that mast cells express antiviral proteins in response to FluA infection, an observation that has not been published to our knowledge. Our findings are in agreement with a recent publication (May 2010) showing that hantaviruses induced transcriptional expression of MxA and ISG15 in human skin mast cells (14). So far, there is little evidence that mast cells respond directly to FluA, other than one study mentioning that UV-inactivated FluA induce IFNα release from human mast cells (11). IFNα has inhibitory effects on mast cells, by decreasing IgE-mediated TNF secretion and decreasing the adhesion of mast cells to extracellular matrix proteins (15, 16). IFNα has been shown to upregulate the mRNA expression of the IFN-inducible gene OAS (2'5'-oligoadenylate synthetase), which has been shown to inhibit FluA infectivity (17, 18). However, the effect of IFNα on the development of viral infection in mast cells has not been studied. The inhibitory effect of mast cells on FluA-infection of epithelial cells in our co-culture experiments suggests reciprocal signals between mast cells and epithelial cells. As FluA was not detectable in the mast cell-containing chamber of the co-culture system, it is likely that FluA-induced epithelial-derived factors, such as IFNα, mediate the generation of the suppressive effect of mast cells. Secondly, mast cells primed by epithelial signals release factors that in turn affect

the progression of FluA infection in epithelial cells. Thus, our results support a role for mast cells in innate immunity against viruses.

5. Relevance/Significance of Findings

To our knowledge, our findings are the first to demonstrate that mast cells are infected by FluA. It is interesting that mast cells do not undergo a productive FluA infectious cycle at doses that result in 2 log multiplication of viral titres in epithelial cells. To further support the data that mast cells are indeed infected by FluA, we could perform experiments such as flow cytometry or immunostaining to co-localize mast cell proteins and FluA proteins inside mast cells. Immunolabelled electron microscopy of FluA-infected mast cells would provide more information as to where the FluA proteins are being transported and whether budding of virus occurs at the plasma membrane.

As part of the initial investigation into whether mast cells had mechanisms to inhibit FluA infection, we discovered that mast cells expressed several antiviral proteins, at the mRNA and/or protein level, that have been shown to have direct antiviral effects against FluA. Expression of antiviral proteins in response to respiratory viruses has mostly been studied in epithelial cells. To our knowledge, the expression of these antiviral proteins – MxA, PKR, ISG15, p56, and eNOS – had not been demonstrated in mast cells in response to FluA exposure. Our phenomenon may not be limited to FluA infection, but instead, be applicable to mast cell responses against a number of different viruses. However, it is important

to perform further studies, for example using siRNA knockdown strategies, to identify important roles for the expression of these antiviral proteins in mast cells. In addition, we did not study whether FluA induces mast cell expression of various receptors such as TLR3, RIG-I, and MDA-5 that detect viral RNA.

We showed preliminary data suggesting that mast cells are protective against FluA infection of epithelial cells, which may or may not be related to the mast cell expression of antiviral proteins. This is an exciting finding that demonstrates communication between mast cells and epithelial cells in the context of FluA infection. Follow-up studies need to be conducted to identify which soluble factors released from mast cells are responsible for the protective effects on FluA-infected epithelial cells. Whether the expression of antiviral proteins induced in mast cells contribute to their effects on epithelial cells also need to be determined. Of the various published studies of mast cell-epithelial cell interactions, there is little evidence of interactions between these two cell types that are relevant to viral infections. Thus, our studies may open a new area of research into mast cell signals that modulate viral infections of other cell types. These signals may not limited to mast cells, but instead be produced by other immune cells to limit viral infections. This could lead to new targets that could be developed as antiviral therapeutics against FluA and other viruses.

We also performed experiments that demonstrated the classic response of mast cells, as secretory cells, to FluA treatment that differs compared to UV-inactivated FluA. Our preliminary multiplex cytokine experiment brings forth insight into certain cytokines that are released by mast cells only in response to

intact FluA or only in response to UV-FluA. As only one multiplex cytokine experiment was performed as a pilot study, repeated experiments need to be conducted to make any conclusions. However, the possibility exists that intact FluA have mechanisms to counteract mast cell responses that are only evident when using UV-FluA, or it could be that mast cells detect specific FluA signals during viral replication that are not present with UV-FluA. As inactivated FluA is used for vaccine preparations, specific mast cell responses to inactivated FluA may be contributing to the development of FluA immunity without causing extensive inflammation in vaccinated patients. Also, our results that mast cells produce TGF-β in response to TLR3 agonists and IFNα demonstrate potential involvement of mast cells in the resolution of immune responses against viruses, as TGF-β is known for its role as an immunomodulatory cytokine involved in the termination of inflammatory responses (19).

6. Future Directions

We have shown that FluA-infected mast cells have diminished production of virus in comparison to epithelial cells, which may be related to our results that mast cells express antiviral proteins in response to FluA exposure. It is important to follow up on these observations by investigating the mechanisms of antiviral proteins in mast cells. Although transfection of human mast cells is challenging, it would be worthwhile to attempt siRNA knockdown experiments of antiviral proteins, such as MxA, and measure the effects of siRNA treatment on FluA infection in mast cells. If MxA is important for limiting FluA infection, we predict

that decreased expression of MxA would result in increased viral titres from infected mast cells. Murine mast cells obtained from MxA-knockout mice could also be used in *ex vivo* experiments to investigate the requirement of MxA in FluA-induced mast cell responses.

MxA knockdown of mast cells may also affect the suppressive effects of mast cells on FluA infection of epithelial cells in the co-culture experiments. Alternatively, the inhibitory effects of mast cells in co-culture could be attributed to soluble factors released from mast cells, such as CCL5 and TGF-β, which may or may not be dependent on MxA expression. Also, mast cells have been shown to affect neurons by N-cadherin interaction; thus, mast cells may exert effects on epithelial cells through physical interactions in addition to soluble factors. We could investigate whether soluble mediators contribute to mast cell effects by treating epithelial cells with media from FluA-treated mast cells. In addition, we could determine the importance of mast cell adherence to epithelial cells in FluA infection by placing mast cells in direct contact with epithelial cells and measure the release of FluA from epithelial cells after the systematic blockade of different adhesion molecules

It would also be valuable to test the importance of mast cells in FluA infection *in vivo* using a mast cell-deficient mouse model. We would expect FluA infection of mice lacking mast cells to result in higher viral titres, more epithelial damage, increased inflammatory infiltrates into the lungs, and increased mortality compared to wildtype mice. W/W^{v} and W^{sh}/W^{sh} are mast cell-deficient mice with mutations in the mast cell c-kit receptor. Both these mice have limitations as they

have abnormalities, other than decreased mast cell numbers, that are attributed to the genetic mutations (20, 21). Thus, use of "knock-in" models to demonstrate that reconstitution of mast cells can recover the effects of the genetic mutation would be essential in studying the role of mast cells in FluA infection.

7. References

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