Mast Cell Response to Influenza A Infection and Their Antiviral Activity in Co-culture with Epithelial Cells

by

Kurtis Wing Chun Ng

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Medicine

University of Alberta

©Kurtis Wing Chun Ng, 2018

Abstract

Well known their role in allergic inflammation, mast cells (MC) are also important in innate immunity against pathogens. MC are abundant in the respiratory tract and in close proximity to epithelial cells (EC) where they could be important in host defenses or the pathogenesis of viral infections such as with influenza A (FluA). However, little is known about the roles of MC in viral infections of the airways.

To investigate, the potential role of MC in FluA infections, we measured short-term mediator release of MC and found that they release histamine, β-hexosaminidase and prostaglandin D_2 following exposure to selected strains of FluA. Of several cytokines and chemokines studied, MC only released CCL-4 after FluA exposure, whereas EC released CCL-5, CXCL-10 and type III interferon. Since it was previously shown that following FluA infection, MC produce few new FluA progeny, we investigated expression of selected antiviral genes in MC and EC at several different time points. FluA exposure induced the expression of RIG-I and MDA5 mRNA in MC, but only Viperin in EC. Neither EC nor MC had increased expression of MAVS mRNA.

We co-cultured FluA-exposed EC with or without MC. When MC were present, FluA release from EC was decreased. In co-culture, this was associated with increased release of Flt-3L and CCL-4. We also determined that bottom chamber supernatants from co-cultures with FluA-exposed EC inhibited FluA release from EC. Heat treatment (100°C) and protease digestion eliminated the antiviral activity in supernatants derived from MC. We determined the molecular size of the antiviral activity to be greater than 10 kDa and to have a relatively strong positive charge with a weak negative charge. Preliminary results from mass spectrometric studies of MC-derived antiviral supernatants showed strong signals of keratin. Despite not precisely identifying the nature

of the antiviral activity, we gained a better understanding of MC in antiviral immunity and their collaboration with EC.

Preface

Parts of the research conducted for the thesis involve research collaborations led by Dr. A. Dean Befus at the University of Alberta. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, entitled: Mast Cell Populations in Human (Pro00000859).

Chapter III of this thesis is a manuscript draft of "Mast Cells' and Epithelial Cells' Response to Influenza A Virus Exposure," that will be a multi-authored document. I was responsible for data collection and analysis as well as writing the manuscript. I performed the experiments for measuring the release of Flt-3L, GM-CSF, CCL-3, CCL-4, CCL-5, CXCL-10, IFN-λ1, IFN-λ2 and IFN-λ3. Chris St. Laurent and Tae Chul Moon prepared the samples for the 41 cytokine/chemokine multiplex assay screen by Eve Technologies. Chris St. Laurent measured IFN-α and IFN-β. Javeria Raheem performed the experiments for short-term mediator release and antiviral gene upregulation. Tae Chul Moon, Harissios Vliagoftis and A. Dean Befus were involved with concept formation and editing the manuscript.

Chapter IV of this thesis is a manuscript draft of "Human Mast Cell Mediators Have an Antiviral Effect on Influenza A Virus in Airway Epithelial Cells," will be multi-authored as well. This manuscript is part of a collaboration with Dr. Richard Fahlman and his trainee, Ramanaguru Piragasam, at the University of Alberta. I was responsible for data collection and analysis of co-culture experiments, cytokine and chemokine measurements, and FluA release assays along with writing the manuscript. Ramanaguru Piragasam conducted the anionic and cationic exchange studies along with protein mass spectrometry. Tae Chul Moon, Harissios Vliagoftis and A. Dean Befus were involved with concept formation and manuscript composition.

Acknowledgements

I would like to thank all those who were instrumental in helping me during my MSc and allowed this thesis, along with its associated projects, to be possible.

It was an honour to be able to work with my supervisors, Dr. A. Dean Befus and Dr. Harissios Vliagoftis for the duration of my MSc. They provided me with much needed guidance in my academic work and support throughout my research. I am forever thankful for their mentorship and life lessons that I learned from them. They were incredibly generous with their time, energy and patience to guide and teach me. They are truly wonderful mentors and friends that I will remain forever grateful to.

Thank you to all the members of the Alberta Respiratory Centre for providing a supportive infrastructure for developing academic discussions as well as networking. I would like to give additional thanks to the members of the Airway Inflammation Team – Dr. Paige Lacy, Iris de Guzman, Vivek Gandhi, Eduardo Reyes-Serratos, Nami Shrestha, Sarah Almas, Drew Nahirney, Ramses Illarraza, Narcy Arizmendi Puga, Yahya Fiteih, Samineh Kamravaei, Lindsey Felix, with a special mention to Dr. Tae Chul Moon, who not only assisted me on numerous occasions, but also advised and guided me throughout my studies.

I wish to acknowledge the members of my supervisory committee – Dr. A. Dean Befus, Dr. Harissios Vliagoftis, Dr. David Marchant, and Dr. Tae Chul Moon for providing me invaluable advice throughout my MSc project.

I want to thank my family for their continuous support in my pursuit of this MSc degree. Thank you, Mom, for working hard so that I could have the opportunity to pursue my dreams. Thank you to my sisters; Fiona, for always keeping me motivated; Bianca, for sharing your laughter and joy; and Alvina, for always being there for me in Edmonton. I could never thank you all enough for all the things you have done for me.

Table of Contents

List of Figures

List of Tables

Abbreviations

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
B- hex	B-hexosaminidase
BSA	Bovine Serum Albumin
Calu-3	Cultured Human Airway Epithelial Cell Line 3
CCL	CC Chemokine Ligand
CCL-2 / MCP-1	Monocyte Chemotactic Protein 1
CCL-3 / MIP-1α	Macrophage Inflammatory Protein- 1 alpha
CCL-4 / MIP-1β	Macrophage Inflammatory Protein- 1 beta
CCL-5 / RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
CCR	CC Chemokine Receptor
CD	Cluster of differentiation
cRNA	Complementary RNA (+ssRNA Template for FluA Replication)
CXCL	CXC Motif Chemokine
CXCL-10 / IP-10	CXC Motif Chemokine 10; Interferon gamma-Induced Protein 10
CXCR	CXC Chemokine Receptor
DGV	Dextrose Gelatin Veronal
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EC	Epithelial Cells
EDTA	Ethylenediaminetetraacetic Acid

ELISA	Enzyme-linked Immunosorbent Assay
eNOS	Endothelial NOS; Nitric Oxide Synthase 3
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FcεRI	High-affinity IgE Receptor
Flt-3L	Fms-related Tyrosine – Kinase 3 Ligand
FluA	Influenza A Virus
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GM-CSF	Granulocyte-macrophage Colony-stimulating Factor
HA	Hemagglutinin
HAU	Hemagglutination Units
HBSS	Hanks' Balanced Salt Solution
HEPES	2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid
HIV	Human Immunodeficiency Virus
HMC-1	Human Mast Cell Line 1
HRP	Horseradish Peroxidase
IFN	Interferon
IFNAR	Interferon α/β Receptor
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IRF	Interferon Regulatory Factor

ISG15	Interferon-stimulated Gene 15
JAK-STAT	Janus Kinase and Signal Transducer and Activator of Transcription
LAD2	Laboratory of Allergic Diseases 2; Human Mast Cell Line
LAIV	Live Attenuated Influenza Vaccine
LPS	Lipopolysaccharide
M1	Matrix Protein 1 of Influenza Virus
M2	Matrix Protein 2 of Influenza Virus
MAVS	Mitochondrial Antiviral-signaling Protein; also known as Virus-induced Signaling Adapter (VISA)
MDA5	Melanoma Differentiation-Associated Protein 5
MEM	Minimum Essential Medium
MC	Mast Cells
MCC	Chymase-Positive Mast Cell
MCT	Tryptase-Positive Mast Cell
MCTC	Tryptase/Chymase-Positive Mast Cell
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
mRNA	Messenger RNA
MxA	Myxovirus Resistance Protein A
MyD88	Myeloid Differentiation Primary Response Gene 88
NA	Neuraminidase
NEP	Nuclear Export Protein of Influenza Virus; also known as NS1
NES	Nuclear Export Signal

NFκB	Nuclear Factor kappa-light-chain-enhancer of Activated B Cells
NLS	Nuclear Localization Signal
NP	Nucleoprotein of Influenza Virus
ns	No Significant Difference
NS1	Non-structural Protein 1 of Influenza Virus
NS2	Non-structural Protein 2 of Influenza Virus; also known as NEP
p56	Viral Stress-inducible Protein p56
PA (-X, -N155, -N182)	Polymerase Acidic Protein; -X, ribosomal frameshift product; -N155, N-terminal 155-amino-acid-truncated form of PA; -N182, N-terminal 181-amino-acid-truncated form of PA
PB1 (-F2, -N40)	Polymerase Basic Protein 1; -F2, alternative reading frame of PB1; -N40, N-terminal 39-amino-acid-truncated form of PB1
PB2	Polymerase Basic Protein 2
PBMC	Peripheral Blood-derived Human Mast Cells
PBS	Phosphate-Buffered Saline
PKR	Protein Kinase R
PGD2	Prostaglandin D2
Poly I:C	Polyinosinic:polycytidylic Acid
PRR	Pattern Recognition Receptor
QIV	Quadrivalent Influenza Vaccine
qPCR	Real-time Polymerase Chain Reaction
RBC	Red Blood Cells
rhSCF/SCF	Recombinant human Stem Cell Factor

RIG-I	Retinoic Acid-Inducible Gene I
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
SEM	Standard Error of Mean
SFM	Serum Free Media
ssRNA	Single-stranded RNA
$Supernatant^{viral}$	Post-FluA-exposed LAD2 Supernatant with Antiviral Activity
TAME	*p*-toluene-sulfonyl-L-arginine methyl ester (TAME); a unit of measurement to describe enzymatic activity
TGF	Transforming Growth Factor
TIR	Toll-Interleukin Receptor Homology Domain
TIV	Trivalent Influenza Vaccine
TLR	Toll-like Receptor
TRIF	TIR-domain-containing Adapter-inducing Interferon-β
TNF	Tumor Necrosis Factor
UV	Ultraviolet
UVI	Ultraviolet Inactivated (Virus)
VEGF	Vascular Endothelial Growth Factor
VISA	Virus-induced Signaling Adapter; also known as Mitochondrial antiviral-signaling Protein (MAVS)
vRNP	Viral Ribonucleic Protein Particle
vRNA	Viral RNA (-ssRNA; FluA genome)

Chapter 1: Introduction

1) Influenza Virus

Influenza virus can infect humans and cause an acute respiratory disease, influenza. Influenza virus can be categorized into A, B and C types with influenza A virus (FluA), the subject of this thesis, being more frequently associated with human illness and outbreaks. Influenza B virus is also responsible for annual influenza outbreaks but is not associated with pandemics. Influenza C virus can cause influenza, but is usually associated with less severe symptoms. Influenza is prevalent in many countries in which local outbreaks or seasonal epidemics occur annually. Larger outbreaks, termed pandemics can also occur in which significant illness, death and economic loss occurs on a global scale. A notable example is the 1918 "Spanish flu" pandemic which caused an estimated over 50 million deaths, about 3% of the global population at that time. Fortunately, seasonal epidemics are more common than pandemics.

A) Influenza A Virology

The interaction between pathogenicity and host defense determines the ability of FluA to cause seasonal epidemics or pandemics. The annual impact of FluA on society is influenced by many factors such as the antigenic variation of the strains, relative virulence of the strains, degree of vaccination efficiency, and vaccination level in a population.

i) Virus Classification

FluA virus belongs to the *Orthomyxoviridae* family. FluA can be further classified into different subtypes based on serological and genetic differences of proteins on the virus envelope.

The surface proteins, hemagglutinin (HA) and neuraminidase (NA), are used for classification due to their significance as the main targets for neutralizing antibodies. HA and NA have been extensively characterized with currently 18 known HA subtypes and 11 NA subtypes[1]. However, only 3 subtypes of HA (H1, H2 and H3) and 2 subtypes of NA (N1 and N2) are commonly associated with human influenza virus. Strains of influenza viruses can be further differentiated from each other by their virus type, place of origin, strain number, year of isolation and subtype (e.g. A/Puerto Rico/8/34 (H1N1) vs. A/Hong Kong/8/68 (H3N2)).

ii) Virus Structure

The structure of FluA is depicted in Figure 1.1. FluA are enveloped viruses containing eight negative-sensed RNA segments and can be found in two distinct forms: an 80-120 nm spherical form and a > 300nm filamentous form. Each of the RNA segments encode for at least 1 viral protein and currently 15 viral proteins have been discovered. The lipid envelope is derived in part from the host cell membrane and contains viral hemagglutinin, neuraminidase and matrix protein 2 (M2). Underlying the lipid envelope is a protein layer formed by matrix protein 1 (M1) which anchors the viral ribonucleoprotein (vRNP) to the lipid envelope. The vRNP consists of one RNA segment protected with multiple copies of nucleoprotein (NP) along with small amount of nuclear export protein (NEP). A polymerase complex is also attached to the vRNP, consisting of polymerase proteins (PB1, PB2 and PA).

Hemagglutinin (HA)

HA is a viral glycoprotein found on the lipid envelope of FluA virus. HA is synthesized as a single polypeptide chain before being cleaved into two separate subunits, HA1 and HA2. The two subunits are covalently linked by disulfide bonds forming the viral spike used for host cell entry. HA plays two important roles for the virus. It is responsible for binding the virus to its target host cells, using the sialic acid-containing receptors on target host cells. Different forms of HA target different sialic acid-containing molecules, with human HA (H1, H2 and H3) specifically targeting α2-6 sialic acid-containing receptors which are abundant in human upper respiratory tract[2]. Whereas, avian HA targets α2-3 sialic acid-containing receptors which are found in birds and in the lower respiratory tract of humans[2]. After HA binds to a target host cell using the HA1 subunit, receptor-mediated endocytosis occurs and an endosome forms with the engulfed virus. The second function of HA involves the HA2 subunit which facilitates the fusion of the viral membrane with the host membrane. This occurs when the endosome acidifies which results in unfolding of HA and exposes a hydrophobic region of HA2 subunit. The hydrophobic region acts as a "fusion peptide" and inserts into the membrane of the endosome. This brings the viral membrane close to the host membrane and allows membrane fusion to occur. The fused membranes enable the emptying of viral content, including the RNA genome along with associated polymerases and proteins.

Neuraminidase (NA)

Another viral glycoprotein on the surface of FluA is NA. NA has enzymatic activity that cleaves sialic acid residue-containing structures on the surface of host cells. This is important for the release of viral progeny. NA cleavage of such sialic acid residues facilitates release of progeny

by preventing them from adhering to the host cell[3,4]. Furthermore, removal of sialic acid residues on host membrane also prevents aggregation of viral progenies[3,4].

Matrix Protein 2 (M2)

M2 is a proton-selective ion channel protein on the FluA viral envelope. It plays an essential role in FluA pathogenesis since it is involved with acidification of the virion core when FluA virus is endocytosed[5,6]. The selective transfer of protons during endosomal acidification into the virion core causes dissociation of vRNP from the M1 allowing the entry of viral RNA into the host cell during viral uncoating[5,6].

Matrix Protein 1 (M1)

M1 is a matrix protein of FluA that links the viral envelope with the vRNP. M1 contains a binding site rich in basic amino acids allowing the non-specific binding of RNA. During the process of viral assembly and budding, M1 forms a layer underneath the host cell membrane that is rich with viral HA, NA and M2[5,7]. The layer of M1 provides a platform for viral assembly by adhering vRNP to host membranes enriched with viral proteins[7,8].

Nucleoprotein (NP)

NP is a protein that binds to viral RNA (vRNA) to form vRNP that is localized in the virion core. Upon release of the vRNP from M1 after viral entry and uncoating, NP facilitates the transport of vRNP into the nucleus of host cell[9,10]. NP contains a nuclear localization sequence

which allows NP to carry the entire vRNP, which contains both the vRNA along with the necessary viral polymerases, into the nucleus to undergo viral replication and transcription[9,10].

Non-structural Protein 1 (NS1)

NS1 is a non-structural protein of FluA that is not essential for viral replication or assembly but plays a role in determining the virulence of a strain. NS1 contains a RNA-binding site that allows it to regulate cellular and viral protein expression[11-14]. It has been shown to inhibit polyadenylation, splicing and transport of host cellular mRNA[15-20]. In addition, NS1 enhances viral mRNA translation in the cytoplasm[18,21,22]. NS1 also affects host antiviral responses by inhibiting dsRNA protein kinase and affecting the interferon response[23].

Nuclear Export Protein (NEP)

Previously known as non-structural protein 2 (NS2), NEP contains a nuclear export signal (NES) that is essential for viral assembly. NEP facilitates the nuclear export of vRNP using the NES domain and transports the vRNP towards the host membrane[24]. NEP also contains a M1 binding domain which facilitates binding of vRNP to M1 during viral assembly[24,25].

Polymerase Basic Protein 1 (PB1)

PB1 is part of the vRNA polymerase complex. It contains a nuclear localization signal (NLS) for nuclear transport, along with binding domains to PA and PB2 subunits to form a polymerase complex[26,27]. PB1 catalyzes RNA synthesis and is necessary for vRNA initiation and elongation[28-30]. PB1 also exists in two other isoforms, PB1-F2 and PB1-N40[31]. PB1-F2 is an

alternative frameshift transcript of PB1 and is associated with strain virulence and polymerase regulation[32]. PB1-N40 is a N-terminally truncated form of PB1 and is associated with regulating PB1 and PB1-F2 expression[33].

Polymerase Basic Protein 2 (PB2)

PB2 is part of the vRNA polymerase complex involved with recognition of 5'capped host mRNA. It is a cap-binding protein associated with recognition of host mRNAs that are used to generate viral cap primers[34-37]. PB2 contains a nuclear localization signal and binding sites for PB1 to form a polymerase complex that regulates viral transcription and replication, along with two binding sites for NP with regulatory interaction potential[38-41].

Polymerase Acidic Protein (PA)

PA is part of the vRNA polymerase complex, hence it also contains a NLS required for transport into the nucleus for viral transcription and replication[27,42]. PA contains a RNA endonuclease activity site for cleavage of host mRNA to create primers used for viral transcription[43,44]. PA exists in 4 isoforms: PA, PA-X, PA-N155 and PA-N182[31]. PA-X is associated with the regulation of host immune response against viral infection[45]. Specifically, PA-X was shown to mediate degradation of host mRNAs and shut down host cell gene expression[45]. PA-N155 and PA-N182 are N-terminally truncated forms of PA and their specific roles are unknown[46].

iii) Antigenicity

Antigenicity of influenza virus could change by two different processes, "antigenic drift" and "antigenic shift". "Antigenic drift" is the process in which accumulation of mutations due to faulty proofreading of RNA polymerase results in new variation of viral proteins and genes[47]. The new variations of viral proteins and genes will result in new variant viral progenies that could be capable of evading host defence and immunity[48]. "Antigenic shift" is the process in which two or more different strains of influenza infect one single host cell and exchange genetic materials to produce new viral progenies[48,49]. The new viral progenies will be a genetic re-assortment of the original strains of virus with a mixture of surface and internal proteins of different origins. This is possible because influenza A virus is composed of eight RNA segments allowing viral progenies to be assembled from RNA segments of mixed origin, creating a new viral strain in the process. The new viral strain will have characteristics from both parental strains, potentially allowing the virus to evade existing host defenses.

iv) Transmission

Transmission of FluA are predominantly facilitated by respiratory droplets from sneezing, coughing and respiration of infected individuals. These droplets infect other individuals by three different methods, direct transmission, airborne transmission and hand-to-self transmission. Direct transmission refers to infected individuals directly sneezing or coughing onto an individual's eyes, nose or mouth. Airborne transmission is the process of individuals inhaling the respiratory droplets of the infected person leading to infection. Hand-to-self transmission refers to an individual being in contact with contaminated surfaces and transferring the virus by hand to their own mouth, nose or eyes. The half-life of influenza virus in the respiratory droplets ranges from 1 – 16 hours, with

environmental factors such as humidity and UV exposure affecting viral particle stability[50]. Typical influenza infection results in the host being contagious from 1 day pre-symptomatic to 5 days post-symptomatic.

v) Virus Reservoirs

Influenza A virus does not establish a sustained infection in human hosts, hence infections are transient within local populations. Influenza A virus is a zoonotic virus, a virus that is normally found in animals but can be transmitted and infect humans. The natural reservoir of influenza A virus is aquatic birds (see review[51]). Viral strains that originate from aquatic birds are normally H5 and H7 influenza strains, which are not efficient in infecting humans. However, it is frequently transmitted to domestic poultry which increases the risk of exposure to humans[51,52]. Furthermore, there are several strains that are known to be highly pathogenic within humans despite being avian influenza. In addition to transmission from poultry to humans, influenza A virus could also infect other animals, such as pigs, dogs, horses and cows before transmission to humans. The most well-studied intermediate host for influenza A virus is domesticated pigs. The reason pigs are a focus for influenza virus transmission is that they are susceptible to both avian and human strains of influenza A virus[51]. Thus, pigs are an ideal host for multiple re-assortment events to occur where highly pathogenic avian strains and human influenza strains infect the same host[51]. This could result in new influenza strains that contain attributes from both avian and human influenza strains, leading to the establishment of novel viral strains with potentially high pathogenicity[51].

B) Prevention and Treatment

Currently there are multiple approaches in prevention and control of influenza A virus transmission. When new avian influenza strains are detected in domestic poultry or livestock, eradication of all affected animals could be used to reduce the risk of exposure and transmission to humans[53].

i) Vaccination

Prevention strategies in human populations typically involve the use of vaccines to prevent the spread and development of local outbreaks. Annual vaccinations are recommended for elderly, children, pregnant women, immunocompromised individuals and individuals who are in frequent contact with previously mentioned groups, such as healthcare workers and teachers (see immunization guideline[54]). There are three different types of vaccines used in Canada: trivalent inactivated influenza vaccine (TIV), quadrivalent inactivated influenza vaccine (QIV) and live attenuated influenza vaccine (LAIV). TIV consists of a mixture of three inactivated influenza virus particles, two influenza A strains and one influenza B strain. These strains are selected annually based on global monitoring and predictions of circulating strains. TIV can reach up to 90% efficacy in the population depending on the accuracy of the predictions[54]. TIV is approved for all individuals ≥ 6 months of age in Canada[54]. QIV protects against the same strains found in TIV but also against another strain of influenza B virus. Due to the similarity of TIV and QIV, both vaccines are equally recommended for individuals ≥ 18 years of age[54]. However, for individuals between 6 months of age and 17 years of age, QIV is recommended due to the improved efficacy against circulating strains of influenza B virus[54]. LAIV is cold-adapted and temperature sensitive live attenuated influenza virus re-assortment strains which do not produce classical influenza

infection and illness. LAIV is not injected intramuscularly like TIV and QIV, but given through intranasal sprays. LAIV has low virulence and replicates in the nasal mucosa rather than the lower respiratory tract creating a localized infection. LAIV provides better cross-protection against drifted strains compared to QIV and TIV but increases cost due to LAIV requiring refrigeration when in transport and storage. LAIV is approved for individuals between 2 years of age to 59 years of age who do not have immune compromised conditions[54].

ii) Antiviral Drugs

Oseltamivir and zanamivir are antiviral drugs that are approved for use in Canada to treat influenza infection. Both drugs are NA inhibitors and are recommended for prophylaxis in high-risk individuals during community outbreaks[55,56]. Oseltamivir and zanamivir work by inhibiting the activity of the NA protein. This blocks NA cleavage of host cell sialic acid residue which inhibits the release of new viral progenies to spread infection. Administration of oseltamivir and zanamivir are recommended within 2 days of the onset of illness[55]. However, individuals with severe (hospitalization), progressive, or complicated illness, and individuals with pre-existing immune compromise are recommended for antiviral treatment regardless of illness onset[55]. These treatments are administered irrespective of influenza vaccination status to prevent development of severe illness[55].

There is another class of antivirals that can be administered to FluA infected patients. In Canada, only amantadine, a derivative of adamantane compound, is approved in this class of antivirals[55]. Amantadine is a M2 inhibitor, that works by binding to the M2 ion channel of FluA virus, preventing proton transfer into the viral core. This prevents acidification of the viral core

and inhibits the release of vRNA into the cytoplasm. However, amantadine is no longer recommended by Health Canada due to its high incidence of resistance[55,56].

C) Viral Pathogenesis

i) Replication Cycle

FluA replication cycle is initiated by the binding of HA to cell surface sialic acid. After binding of HA to sialic acid residues, the virus enters the host cell through receptor-mediated endocytosis in an endosome. The endosome will undergo acidification resulting in lowering of pH which facilitates the fusion of the virus to the endosomal membrane. Low pH will induce a conformational change in HA which will expose a fusion peptide domain to insert into the endosomal membrane[57]. The binding of the fusion peptide to the endosomal membrane brings both viral and endosomal membrane into contact with each other. Furthermore, the low pH of the endosome will also activate the M2 ion channels on the virus surface. M2 ion channels are proton-selective ion channels which acidify the viral core allowing the vRNP to dissociate from the structural M1 and freely enter the cytoplasm of the host cell[57].

FluA vRNP contains nucleoproteins and viral polymerases. All of these proteins have NLS that can use host cell nuclear import machinery to enter the nucleus where FluA transcription and replication occurs. Because FluA is a negative stranded RNA virus, it must first be converted to positive stranded RNA for the genome to be replicated. Replication of the FluA genome is facilitated by viral RNA polymerases to transcribe the RNA genome into complimentary RNA (cRNA), vRNA and mRNA. The cRNA will be used as a template to produce vRNA that will be used for packaging into new virions. The FluA genome is unable to produce the 5' methylated cap that is necessary for mRNA transcripts for viral proteins. Instead, it was found that PB2 has endonuclease activity and will bind to 5' methylated caps of host cell mRNA and cleave the methylated cap structure off the mRNA[34,43]. The methylated cap fragment is then used as a primer to initiate transcription of viral mRNA. Despite FluA genome being composed of eight RNA

segments, FluA uses different mechanisms to increase viral protein function and diversity[57]. M2 and NEP are spliced products of M1 and NS1 respectively. FluA uses host cell machinery to splice M1 and NS1 mRNA to express M2 and NEP, albeit in much lower abundance. Furthermore, there exist many instance of frameshift transcripts of various viral proteins (PA-X, PB1-F2, etc.) with unconfirmed functionality. Newly translated viral nucleoprotein and polymerase proteins are imported into the nucleus and associate with replicated vRNA to assemble vRNP. The vRNP are exported out of the nucleus to be packaged into newly formed virions. Translated M2, NA and HA viral proteins are targeted to endoplasmic reticulum (ER) for post-translational modification before being directed to cell surface for virion formation[57]. The virion formation site is where budding of FluA particles occurs. Virion formation occurs with HA, NA and M2 accumulating on host cell membrane with M1 present underneath the lipid bilayer. vRNP will then bind to the M1 layer before the virion closes and buds off the host cell surface. Prior to budding, NA are responsible for cleaving sialic acid residues from glycoproteins and glycolipids on host cell surface to facilitate the release of virions from host cells.

ii) Innate Host Cell Response to Influenza A Infection

Despite current approaches to control influenza infection and spread, infections are common during seasonal outbreaks. The host immune response plays a critical role to limit and prevent the spread of the virus. Many cells are susceptible to FluA infection, such as epithelial cells, dendritic cells, neutrophils and macrophages and have various methods of detecting and responding to the virus. Cells respond to FluA infection by producing and releasing various cytokines and chemokines to initiate inflammation and facilitate recruitment of immune cells to

the infection sites. Upregulation of antiviral genes is a major component of the host response used to restrict viral replication.

Pattern recognition receptors (PRR) are used to detect FluA infection and activate various signalling pathways to initiate antiviral responses. Toll-like receptor 7 (TLR7) is a PRR that recognizes single-stranded viral RNA and initiates the nuclear translocation of interferon regulatory factor 7 (IRF7) to activate type I IFN production[58-60]. Toll-like receptor 3 (TLR3), which recognizes dsRNA, also recognizes FluA RNA despite it being ssRNA[61]. The FluA RNA segments contains complementary sequences at the 5' and 3' ends, forming panhandle structures similar to dsRNA allowing recognition by TLR3[61,62]. TLR3 activates IRF3 to activate IFN-α and β production. Retinoic acid inducible gene-I (RIG-I) also recognizes the panhandle structures of the FluA genome[63,64]. In epithelial cells, TLR3 and RIG-I recognition result in a pro-inflammatory response along with the activation of IRF3 dependent IFN production[65]. It was also shown that RIG-I dependent responses occur in macrophages and dendritic cells suggesting that similar FluA recognition systems exist in different cell types[64,66,67].

iii) Inflammatory Responses

During the development of FluA infection in humans, cytokine and chemokine productions are upregulated as part of the host cell antiviral response. The inflammatory response is often beneficial and helps promote the recruitment of various immune cells to the infection site and aids in viral clearance. However, severe complications of FluA infection often involve development of "cytokine storm", where the production of various inflammatory cytokines and chemokines are elevated and uncontrolled[68,69]. This dysregulation of cytokine and chemokine production disrupts the balance of pro- and anti- inflammatory responses in the surrounding areas of inflammation.

"Cytokine storms" can lead to permanent damage to tissues, systemic sepsis or even death[68,69]. Hence, it is important to understand the various cytokine and chemokine mediators that are involved with FluA infection.

As the major replication host for FluA virus, epithelial cells release cytokines and chemokines such as TNF, IL-1β, IL-6, CCL-2 and CXCL-10[69-71]. However, production of cytokines and chemokines are relatively low in epithelial cells and the escalation of cytokine storms are dependent on the induction of secondary inflammatory cascades in other cells[68,69]. Macrophages, dendritic cells and neutrophils are considered to be major contributors to the release of inflammatory cytokines and chemokines during FluA infection[60,72,73]. However, the variability of host inflammatory cytokine and chemokine responses suggests that many factors can regulate the development or clearance of inflammatory cascade that leads to "cytokine storms".

Beyond the initiation of the inflammatory cascade caused by FluA infection, various antiviral pathways are activated in an attempt to control and resist the spread of viral infection. Type I IFN, a family of antiviral proteins that interfere with viral replication and suppresses viral processes within host cells, are critical in restricting virus replication during FluA infection[69]. Type I IFN-α and-β bind to a common IFN-α/β receptor (IFNAR) which activates a JAK/STAT-dependent pathway to induce the transcription of several antiviral genes[74,75]. These IFN-stimulated genes have various direct and indirect antiviral activities which affects FluA replication. Protein Kinase R (PKR), one of the direct antivirals induced by type I IFN, is activated by binding dsRNA which is present during FluA infections. Activated PKR inhibits mRNA translation, thus preventing viral protein synthesis in host cells[76]. Furthermore, activated PKR facilitates the activation of transcription factor NFκB which upregulates IFN expression[77,78]. Lastly, activated

PKR is a pro-apoptotic signalling molecule which can induce cellular apoptosis to prevent FluA replication[77].

FluA can counteract and suppress antiviral mechanisms. For example, FluA protein, NS1, acts in several ways to block antiviral activities. NS1 has a domain which binds to and masks dsRNA regions of the FluA genome, which inhibits TLR3 recognition and PKR activation[79]. NS1 can also bind to RIG-I and inhibit RIG-I-induced IFN production[80]. NS1 also affects cellular mRNA transport from the nucleus to the cytoplasm, effectively suppressing production of IFN-stimulated genes[17-19].

iv) Adaptive Host Response to Influenza A Infection

The adaptive host response to FluA is initiated when dendritic cells present FluA antigens to naïve and memory T cells (see review[81]). Dendritic cells are professional antigen presenting cells, that initiate and activate the adaptive immune system. During FluA infection, dendritic cells can acquire viral antigens through direct infection or through phagocytosis of virus particles / apoptotic FluA-infected EC. The dendritic cells then present viral proteins on their major histocompatibility complex (MHC) class I and II molecules. Antigens presented on MHC class I are used to activate virus-specific CD8+ cytotoxic T cells. Cytotoxic T cells will target FluA-infected cells and induce apoptosis to inhibit viral progeny production. MHC class II/peptide complexes are used to activate CD4+ T helper cells. Activated CD4+ T helper cells will initiate B cell proliferation and maturation into antibody-producing plasma cells. Mature B cell activation will result in the production of antibodies against different FluA antigens. Neutralizing antibodies predominantly target either the HA or the NA viral proteins on the lipid envelope of FluA. HA-specific antibodies deter virus attachment to host cell receptors, effectively preventing the spread

of viral infection. NA-specific antibodies inhibit enzymatic activity of NA which limits virus spread. However, the effectiveness of these antibodies is affected by antigenic drift and shifts of FluA proteins. Hence, a combination of both innate and adaptive immunity is desirable to facilitate viral clearance.

2) Mast Cells

Mast cells (MC) are secretory cells that are critical sentinel and effector cells involved in immune and inflammatory diseases. MC respond to various pathogens and environmental stimuli through selective release of stored and newly synthesized mediators. Upon stimulation, the MC response can lead to changes in vascular permeability and effector and immune cell recruitment and mediate both innate and acquired immunity. Conventionally known for their role in allergic inflammation and helminth immunity, the roles of MC in innate and acquired immunity against microbial pathogens are emerging.

A) Biology

MC are granulocytes derived from pluripotent $CD34^+$ hematopoietic stem cells from bone marrow[82]. Mature MC do not circulate, instead $CD34^+$ hematopoietic MC progenitors migrate from bone marrow to peripheral tissues before differentiation and maturation into MC. Human MC depend on stem cell factor (SCF) as a determinant during the differentiation and maturation process[83]. MC are found in nearly all vascularized tissues but are prominent at barrier interfaces, such as skin and mucosal surfaces[84-87].

i) Morphology

MC are heterogeneous differing in morphology and function. MC morphology such as granularity differs based on factors such as species, age, anatomical location, and microenvironmental conditions[87,88]. The functional responses of MC to environmental stimuli are

varied and in part reflect stimulus-specific differences in mechanisms and nature of mediator secretion[87].

Human MC can be categorized into three major subsets based upon their serine protease content. Immunohistochemical staining of granule proteases characterizes MC into tryptase-positive MC (MCT), tryptase/chymase-positive MC (MCTC) and chymase-positive MC (MCC)[88-90]. MCT are found in abundance in lungs, whilst MCTC are concentrated in the skin and submucosa[91]. MCC are not found in great abundance throughout the body but the largest concentrations of MCC can be detected in intestinal submucosa[92].

Although MCT are abundant in lung tissue, lung MC also include MCTC and MCC phenotypes, albeit at lower frequencies. This classification does not adequately define the complexity of MC heterogeneity in the lung. In fact, the proportion and distribution of the three MC phenotypes are influenced dynamically by local microenvironments[93,94]. Furthermore, each MC phenotype can be further distinguished into different subtypes based on expression of various receptors and proteins, such as IgE receptors and leukotriene C_4 synthase[93]. When investigating MC responses in pulmonary diseases, it is important to understand the complexity of MC heterogeneity within the lungs and how local microenvironments may differ and affect MC populations.

ii) Function

Most well-known for its role in allergic inflammation, MC can be activated by the crosslinking of IgE antibodies bound on FcεRI receptors present on the cell surface[95]. However, other stimuli, such as complement components, microbes, neuropeptides, cytokines, hormones and

chemical reagents are capable of activating MC. MC activation is characterized by immediate degranulation, the process in which MC release mediators stored within the granules. The magnitude of MC degranulation is dependent on the signal intensity and stimuli received[96]. Extensive MC degranulation is often associated with IgE crosslinking of a high proportion of receptors, whereas weaker stimulation may result only in partial degranulation. Other stimuli, such as viral infections, may result in selective release of various stored mediators, hence MC activation and responses are highly variable.

MC are involved with many physiological functions, such as vasodilation, microbial elimination, angiogenesis and immune cell regulation. MC can produce many powerful mediators, such as histamine, proteases, arachidonic acid metabolites, heparin, growth factors along with a variety of cytokine and chemokines making them a significant player in the regulation of growth, repair and remodeling of tissues[82,84-87,96,97]. MC can enhance angiogenesis by releasing pro-angiogenic factors, such as VEGF, TNF, and IL-8[96,98-101]. MC play a significant role in homeostasis of commensal gut bacteria. MC situated beneath the epithelial layer, help maintain the equilibrium between gut bacteria and immune cells by regulating the permeability of epithelial cells and the maturation of IgA responses to the microflora[102-104].

In innate immunity, MC can recognize harmful antigens through various receptors such as TLRs. Once MC recognize the target, they release various inflammatory mediators to help suppress the pathogen that it detected. The nature of the specific response is dependent on the receptor that is activated. For example, TLR2 can be activated by Gram-positive bacteria and result in the MC release of cytokines such as IL-4 and degranulation of mediators such as histamine in humans[105-108]. However, TLR4 activation by LPS results in release of TNF and IL-6 but does not induce degranulation[109-111]. MC release inflammatory mediators that promote microbial clearance,

such as mediators leading to enhanced vascular permeability and chemokine production for immune cell recruitment. Additionally, MC can produce antibacterial products such as cathelicidins and defensins to suppress bacterial infections[112,113]. Furthermore, MC are important immune effector cells that can also act against viral infections, such as by recruiting $CD8^+$ T cells, which can produce type I IFN that can suppress viral replication[114].

iii) Mast Cells Used in Research

Rat and murine MC have been used extensively in functional studies because of their ease of acquisition and relatively low cost compared to larger animal alternatives, such as pigs. The numbers of primary MC that can be isolated and enriched to near purity from rodent tissues are limited, and instead, *in vitro* cultured IL-3 dependent MC derived from bone-marrow progenitors are often used[115]. Although the use of animal-derived MC has provided countless valuable studies on MC biology and function, there are important functional differences between human and animal MC. Furthermore, MC *in vivo* are a heterogeneous population that makes extrapolation and generalization of MC studies across instances of disease difficult. There are many established methods used to study roles of human MC in disease including: long-term MC lines, primary cultures from progenitors, and primary cells isolated and enriched to near purity from tissues. All these approaches have different strengths and limitations.

HMC-1

One of the earliest developed human MC lines, HMC-1, was derived from a leukemia patient[116]. The cell line possesses a c-kit mutation that allows it to be growth factor independent,

specifically SCF-independent[117]. This is an important advantage, because HMC-1 replicates rapidly and large numbers can be cultured relatively inexpensively. However, HMC-1 are immature transformed MC that lack FcεRI receptors and are poorly granulated[116]. This makes HMC-1 a model MC with significant limitations when studying allergic responses and degranulation. However, other MC secretory events such as cytokine production do not seem to be restricted[117].

LAD2

Laboratory of allergic diseases 2 (LAD2) MC line was derived from bone marrow aspirates of a MC sarcoma-leukemia patient. Unlike HMC-1, LAD2 does not have a c-kit mutation, thus similar to primary MC cultures from progenitors, requires the presence of SCF for differentiation and survival[117]. LAD2 are heavily granulated and express FcεRI receptors which makes them an ideal candidate for studies of MC allergic responses. However, LAD2 replicates much slower than the HMC-1 cell line, with doubling times of up to a week and has higher maintenance costs due to the need for SCF supplemented media. LAD2 are a heterogenous population of MC, with ~98% positive for tryptase and ~37% positive for chymase and tryptase[118].

Primary Mast Cell Cultures

$CD34^+$ progenitors from human peripheral blood or umbilical cord blood can be used to develop primary MC cultures in the presence of SCF and other cytokines such as IL-6 and IL-4[117]. Although primary cultured MC are preferred over cell lines in MC research, current culture protocols are limited in providing both quality and quantity of MC for functional studies. Culturing

of progenitor cells takes 6-12 weeks with the use of expensive cocktails of cytokines and growth factors and produces a limited number of cells for experimentation (e.g. 3-10 × 10^8 MC by 8 weeks cultured from 1 × 10^7 $CD34^+$ progenitors from 100 mL of peripheral blood)[117]. Furthermore, the MC do not fully mature *in vitro* as evidenced by functional studies and cell surface marker expression. Primary MC can also be isolated from tissues but require extensive isolation and purification procedures to produce limited quantity (~ 1-3 × 10^5 MC/g of tissue) of cells for research[119]. Both culture and isolation of primary MC require careful assessment of the quality and purity of MC isolated or derived in cultures.

B) Mast Cells and the Antiviral Response

As part of the innate immune system, tissue-resident MC in proximity to the EC layer are in a prime location to recognize and respond against invading pathogens. MC are equipped with a large inventory of pre-synthesized mediators in their granules, allowing for a rapid response and release of granule contents upon activation. Furthermore, MC can selectively release different mediators from their granules depending on the intensity and type of stimuli received. MC may also respond to external stimuli by *de novo* synthesis and release of various lipid mediators, cytokines and chemokines that have pro- and anti- inflammatory effects.

The innate immune system is largely dependent on pattern recognition receptors (PRR) that can recognize pathogen-associated molecular patterns (PAMP). TLRs are a subset of PRR found in MC and have a broad range of specificity and targets, allowing for detection and responses to various PAMPs such as from bacterial cell walls, fungal cell walls, and viral RNA. Specifically, TLR 3, 7, 8 and 9 are known to detection viral infections through the recognition of viral RNA and DNA. Human MC express TLR 1-9 and can respond accordingly to many PAMPs[120,121].

TLR activation can be segregated into two different signalling pathways: myeloid differentiation primary response 88 (MyD88) dependent pathway and TIR-domain-containing adapter-inducing IFN-β (TRIF) dependent pathway (Figure 1.2). TLR 3 can detect double-stranded RNA (dsRNA) such as the genome of dsRNA viruses and the replication intermediates of single-stranded RNA (ssRNA)[61,120]. TLR 3 can also recognize the synthetic dsRNA analogue, polyinosinic-polycytidylic acid (poly:IC), which is commonly used to study TLR 3 activation. Upon TLR3 activation, human MC will initiate type I IFN production *via* a TRIF-dependent pathway. By contrast, TLR 7, 8 and 9 use a MyD88-dependent pathway to induce the expression of inflammatory mediators and type I IFN[122,123]. Both TLR 7 and 8 recognize ssRNA within the

endosome. TLR 9 can recognize motifs commonly found in bacterial and viral DNA but not in vertebrate genome allowing it to selectively recognize foreign, microbial dsDNA[123]. TLR3, 7, 8 and 9 can induce the release of TNF, IL-6, CCL-3 and CCL-5 when activated by viral PAMPs resulting in the initiation of an inflammatory response[123,124].

The roles of MC in viral infections are diverse and may have beneficial or deleterious effects. In human immunodeficiency virus (HIV) infections, HIV can productively infect human MC. HIV enters MC through the binding of CD4 and CXCR4 receptors, which induces the release of histamine from MC[125,126]. In Dengue virus, MC can recognize infection and release IL-6, CCL-3, CCL-4 and CCL-5, which suggests that MC plays a role in the inflammatory response against Dengue infection[127,128]. However, in patients with severe Dengue infection (hemorrhagic fever and shock syndrome), VEGF, tryptase and chymase are significantly increased, suggesting a possible harmful role that MC play in the development of dengue pathogenesis[129]. In Sendai virus infections, MC are activated by Sendai virus and produce Type I and III IFN[130]. Despite multiple instances of MC activation and release of mediators, the exact role of MC in viral infections is poorly defined.

Recent studies have shown MC recognition and response to FluA infection. In murine MC, FluA induces release of histamine, TNF, CCL-2, CXCL-10, IL-1β, IL-6, IL-12 and IFN-γ[131-134]. Both TLR 3 and RIG-I were shown to play a role in FluA-induced MC mediator release (Figure 1.3) [131,132]. IL-6 release in MC varied when infected with different strains of FluA, which suggests MC responses are dependent on viral strain[131]. In porcine MC, it was shown that histamine, IL-1α, IL-6, CXCL-10 and CXCL-11 release were upregulated by FluA[135]. Furthermore, in MC-deficient mouse models, inflammatory cell (macrophages, neutrophils, and lymphocytes) recruitment was decreased in the bronchoalveolar lavage fluid following FluA infection when compared to wildtype mice[131]. Another study showed that when FluA-infected mice were treated with ketotifen,

a mast cell degranulation inhibitor, there were less inflammatory cell infiltration and only mild pathological bronchiolitis[133]. This suggests that MC may play an important role in the development of FluA-induced inflammation and disease pathogenesis. Human MC are susceptible to FluA infection, albeit with limited virus release[136]. Moreover, in preliminary studies, FluA induced IFN-γ and CCL-5 release, and upregulated PKR, MxA, eNOS, ISG15 and p56 mRNA expression in human MC[137]. Furthermore, in EC-MC co-cultures, MC restricted FluA replication and release in EC[137]. In summary, these studies showed that MC can detect and respond to FluA infection and play a role in viral inflammation in animal models. Furthermore, there is evidence that MC responses vary depending on the viral strain and species origin of MC, suggesting that further study in human MC are needed to characterize MC roles in FluA infection.

C) Interactions Between Mast Cells and Epithelial Cells

There are limited studies performed on interactions between MC and EC during viral infections, but there is evidence that MC functions can be influenced by EC. In co-culture experiments with both EC and MC, MC survival was enhanced compared to in single cultures[138]. Furthermore, primary MC populations undergo phenotypic changes from uniformly MCTC, to predominantly MCT when co-cultured with EC[138]. This suggests that EC modulate MC function and are involved in the development of mucosal-associated MCT in the human respiratory tract. Furthermore, MC degranulate in the presence of RSV-infected EC when in co-culture, but were not directly activated by RSV alone[139]. This suggests that there is active communication between EC and MC during viral infections of EC. Direct MC and EC adhesion in co-cultures show reciprocal communication between EC and MC. EC modulate MC degranulation by suppressing IgE-dependent activation, whereas MC inhibit EC release of secretory leukocyte protease inhibitor[140,141]. MC can be infected by FluA and can limit viral replication[136]. This suggests that MC respond to FluA infections and given the proximity of tissue resident MC to lung EC, MC may interact with EC during FluA infections *in vivo*. Interestingly, MC are reactive to several inflammatory mediators produced by EC during viral infections, further supporting my postulate that they are a prime candidate to respond to FluA infection of EC. However, evidence of MC and EC interactions are limited in scope and further elucidation is required to understand the role of MC in innate immunity against pathogens in EC.

i) Response of Mast Cells to Epithelial Cell Mediators

EC are often first cells to interact with foreign pathogens and an important target for viral replication. EC act as the barrier interface between internal and external environment, hence they

express various receptors that recognize invading pathogens. Human lung EC express mRNA for all 10 known human TLRs which can detect viruses, fungi and bacteria[142]. Of all the TLRs expressed by EC, only three are predominantly known to be associated with viral recognition, namely TLR3, 7 and 9. EC TLR3 can be activated by FluA virus, specifically by dsRNA moieties that exist in the RNA genome and induce the production of type I IFN[143]. TLR7 and 9 similarly induce IFN production upon activation by viral antigens[144,145]. In addition to IFN production, EC also produce cytokines and chemokines that will help recruit and activate immune cells, including MC[143-145].

Moreover, MC functions can be altered by soluble mediators released by EC following viral infections. Type I IFN produced and released by EC can inhibit the release of TNF and histamine from MC[146-148]. Furthermore, when infected with respiratory viruses, EC also produce nitric oxide (NO) which can inhibit release of MC mediators, such as histamine, arachidonic acid metabolites, cytokines and chemokines[149]. However, the effect of virus-induced EC mediators on the MC antiviral response requires further investigations.

ii) Changes in Epithelial Cells by Mast Cells

MC activation is an important component in the regulation of host defense against pathogens invading EC. MC tryptase is a serine protease that can induce EC to release cytokines and chemokines such as CCL-2 and CXCL-8[150-152]. Tryptase can also activate the protease-activated receptor family, resulting in the production of various inflammatory mediators such as GM-CSF, eotaxin, and MMP-9[153,154]. EC production of pro-inflammatory cytokines results in recruitment of macrophages and neutrophils that aid in the clearance of FluA. MC histamine upregulates TLR3 expression in EC, which suggests that MC may enhance viral recognition by

EC[155]. MC chymase induces TGF-β from EC, which may be involved with both pro- and anti-inflammatory responses[156]. All this evidence suggests that MC can influence EC function and play a role in host defense against viral infections. However, the exact roles that MC and their products may play is unknown.

3) Rationale

MC are significant producers of several mediators including arachidonic acid metabolites, cytokines and chemokines. Given their location close to the EC layer, MC can encounter and respond to foreign pathogens early after exposure. Moreover, MC can also be infected by FluA, but limit FluA replication and release of infectious progenies. Thus, it is possible that MC can also suppress FluA replication in other cells as well, such as EC. Indeed, using an *in vitro* co-culture system, it was demonstrated that when EC were exposed to FluA in the presence of MC, there was decreased virus release from EC[137]. This observation provided the basis for further investigation into the mechanisms that MC use to affect FluA replication in EC, a core component of this thesis.

To investigate the mechanisms employed by MC to protect EC against FluA infection, we used co-cultures with the human MC line, LAD2, the human lung adenocarcinoma EC line Calu-3, and the FluA strain Puerto Rico/8/1934 (H1N1)[137]. We studied the responses of the MC alone to FluA, measuring: cytokines, chemokines, arachidonic acid metabolites, degranulation and expression of antiviral genes. We also measured FluA release from EC using hemagglutination assays following EC-virus cultures alone, or in co-culture with MC. We investigated cytokine and chemokine release in co-culture to identify possible antiviral candidates. However, given that cytokine and chemokine studies exhibited considerable variability and we could not identify a known antiviral candidate, we focused on alternative methods, namely fractionation of the supernatants containing antiviral activity to identify the active factor(s). Finally, we attempted to isolate and identify possible antiviral candidate(s) using protein mass spectrometry.

4) Hypothesis

MC respond to FluA infection of EC by releasing various cytokines and chemokines, and in co-cultures, MC reduce the release of infectious FluA from EC. Because the co-culture system is a membrane separating two chambers, MC have limited ability to contact the EC. Thus, it is likely that the MC suppress FluA release in EC through a soluble factor(s) and that supernatants of co-cultures, antiviral supernatant (supernatant^{viral}), will possess antiviral activity after the MC have been removed. As proteins are one of the major products produced by MC, and many signalling pathways are dependent on various cytokines and chemokines, the antiviral activity is likely to involve proteins. Hence protease digestion should inhibit the MC-derived antiviral activity. In this thesis, soluble factors are defined as molecules or entities that are released and could include factors such as proteins, lipid products, glycoproteins, etc.

5) Objectives

1. To characterize FluA-induced mediator secretion and antiviral gene expression in MC and EC.

2. To characterize cytokine and chemokine release in EC-MC FluA co-culture systems.

3. To characterize the antiviral activity in EC-MC co-cultures.

6) Figures

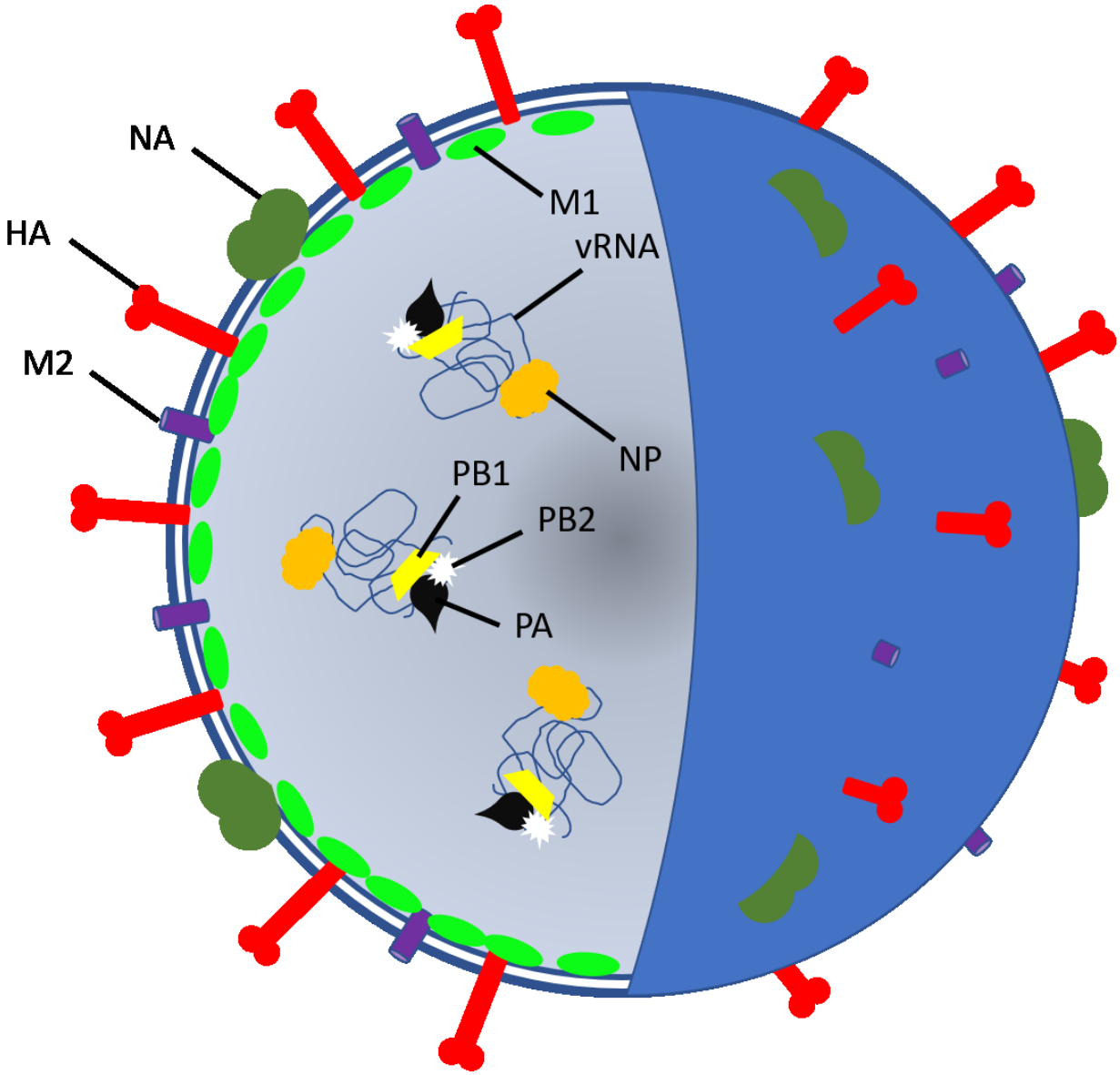

Figure 1.1 Depiction of Influenza A Virus. Viral envelope proteins, hemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2), are depicted on the surface of the virus particle. Matrix protein 1 (M1) lines the interior surface of the virus particle. Viral RNA (vRNA) segments are negative-sense single stranded RNA (ssRNA) stored inside the virus particle. The polymerase proteins, polymerase acidic protein (PA), polymerase basic protein 1 (PB1) and polymerase basic protein 2 (PB2) form a complex that binds to RNA segments. Viral ribonucleoprotein complexes (vRNP) consist of the polymerase complex binding to RNA segments with multiple copies of NP (only 1 copy shown) and small amounts of nuclear export protein (NEP; not shown).

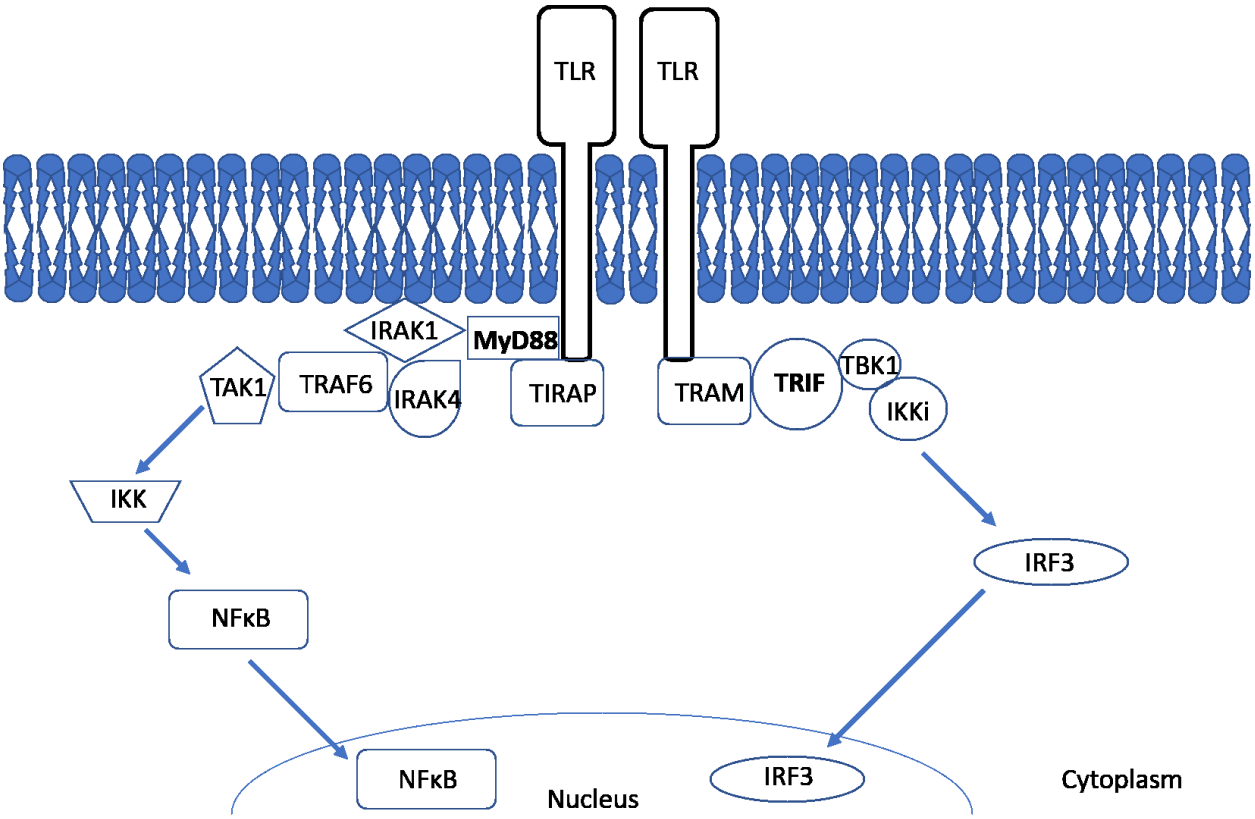

Figure 1.2 Simplified Toll-like Receptor Signalling Pathway. MyD88-dependent pathway results in the activation of NFκB. Activated NFκB will then translocate into the cell nucleus, activate transcription and induce inflammatory cytokine production. TRIF-dependent pathway results in the phosphorylation of IRF3, allowing it to translocate into the nucleus. Upon entry of the nucleus, IRF3 induces type I IFN transcription. In viral recognition, TLR 3 uses the TRIF-dependent signalling pathway; TLR7, 8 and 9 uses the MyD88-dependent signalling pathway.

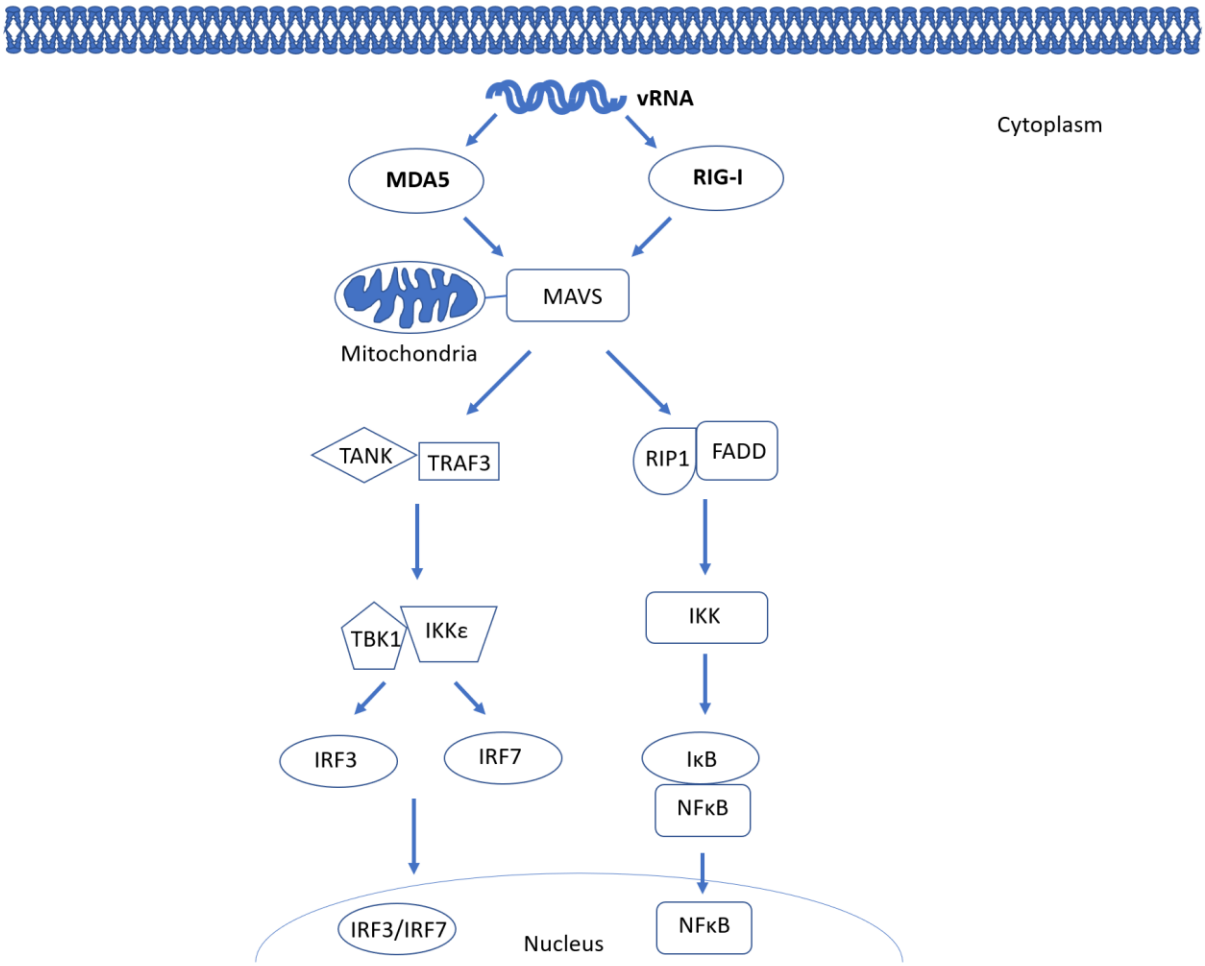

Figure 1.3 Simplified Retinoic Acid-inducible Gene-I-like Receptors' Signalling Pathway. Retinoic acid-inducible gene-I-like receptors (MDA5 and RIG-I) recognize viral dsRNA. After recognition of the viral ligand, RIG-I and MDA5, through MAVS, activate various transcription factors such as IRF3, IRF7 and NFκB. The activated transcription factors translocate into the nucleus. IRF3 and IRF7 activation induces type I IFN expression, while NFκB regulates production of inflammatory cytokines.

Chapter II: Materials and Methods

1) Materials

All materials/chemicals were used according to suppliers' guidelines and in compliance with the guidelines of Environmental Health and Safety, University of Alberta and Work Hazardous Materials Information System (WHMIS).

A) Cell Culture

- Human Mast Cell Line, Laboratory of Allergic Diseases 2 (LAD2), from Dr. D. D. Metcalfe and A. Kirshenbaum, National Institutes of Health, Bethesda, Maryland, United State of America (USA).
- Human Lung Adenocarcinoma Epithelial Cell Line (Calu-3), from American Type Culture Collection (ATCC® HTB-55), Manassas, Virginia, USA.
- Stempro®-34 SFM (Cat # 10640-019) supplemented with Stempro®-34 Nutrient Supplement (Cat # 10641-025), 2 mM L-glutamine and 100 U/mL Penicillin and 100 µg/mL Streptomycin (Cat # 15140-122).
- Recombinant Human Stem Cell Factor (rhSCF) (Cat # 300-07) from Peprotech Inc., Rocky Hill, New Jersey, USA.
- Minimum Essential Medium (MEM) and Earle's salts (Cat # 10370-021), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Cat # 15140-122), 2 mM L-glutamine (Cat # 25030-081) and 1mM sodium pyruvate (Cat # 11360-070) from Gibco® by Thermo Fisher Scientific, Waltham, Massachusetts, USA.

- Fetal Bovine Serum (FBS); Hyclone Defined (Cat # SH30070.03) and Gibco® (Cat # 12483-020).
- 0.25% Trypsin – 1mM EDTA Solution (Cat # 25200-072), from Gibco®.
- Tissue Culture Flask – 75 cm^2 (Cat # 353136), 25 cm^2 (Cat # 353108) and Tissue Culture Plate – 12 well (Cat # 353043) from Falcon® by Thermo Fisher Scientific.
- Snapwell® Culture Inserts for 6-well plates (Cat # C3407) from Corning®, Corning, New York, USA.
- Hanks' balanced salt solution (HBSS), (Cat # 14175-095) from Gibco®.
- Trypan Blue Stain 0.4% (Cat # 15250) from Gibco®.

B) Influenza Virus Treatment

- A/PR/8/34 (H1N1) propagated from duck allantois; courtesy of Dr. K. P. Kane from University of Alberta.
- A/WS/33 (H1N1) (ATCC® number: VR-1520) and A/HK/8/68 (H3N2) (ATCC® number: VR-1679) from American Type Culture Collection.
- UV lamp (ENF-280C) from Spectroline, Westbury, NY, USA
- Thermo™ Scientific 96-well round bottom plates (Cat # 12-565-65) from Thermo Fisher Scientific.
- Dextrose Gelatin Veronal (DGV) buffer (Cat # 10-539B) from Lonza, Basel, Switzerland.
- Phosphate Buffered Saline (PBS) pH 7.4 (Cat # P-5368), from Sigma-Aldrich, St. Louis, Missouri, USA.

- One-step Antibody Biotinylation Kit (Cat # 130-093-385), from Miltenyi Biotec, Auburn, California, USA.
- Human IgE (Cat # 401152), from EMD Millipore, Darmstadt, Germany.
- Streptavidin (Cat # S0677), from Sigma-Aldrich.
- A23187, Calcium ionophore, (Cat # C7522) from Sigma-Aldrich.
- Normal Goat IgG control, (Cat # AB-108-C) from R&D Systems, Inc.
- Human CCL-4/MIP-1β polyclonal antibody, (Cat # AB-271-NA) R&D Systems, Inc.
- Trypsin, Sterile, Irradiated (Cat # T8675) US Biologicals, Salem, MA, USA.
- Centricon Plus-70 Centrifugal Filter Units (10 kDa and 30 kDa; Cat # UFC701008 and Cat # UFC703008) from EMD Millipore.
- Amicon Ultra-15 Centrifugal Filter Unit (50 kDa; Cat # UFC905024) from EMD Millipore.
- Hi-Trap Q Sepharose XL anion exchange column and SP Sepharose XL cation exchange columns (Cat # 17515801 and Cat # 17516001) from GE Healthcare, Chicago, Illinois, USA.

C) Enzyme-linked Immunosorbent Assay

- ELISA kits containing: Capture Antibody, Detection Antibody, Standard, and Streptavidin-HRP; from R&D Systems, Inc.
    - Flt-3L Duoset™ (Cat # DY308)
    - GM-CSF Duoset™ (Cat # DY215)
    - CCL-2/MCP-1 Duoset™ (Cat # DY279)
    - CCL-3/MIP-1α Duoset™ (Cat # DY270)

- CCL-4/MIP-1β Duoset™ (Cat # DY271)
- CCL-5/RANTES Duoset™ (Cat # DY278)
- IFN-λ1 Duoset™ (Cat # DY7246)
- IFN-λ2 Duoset™ (Cat # DY1587)
- IFN-λ3 Duoset™ (Cat # DY5259)

- Verikine ELISA Kits from PBL Assay Science, Piscataway, New Jersey, USA.
  - IFN-α Multi-subtype ELISA Kit (TCM) (Cat # 41105)
  - IFN-β ELISA kit (Cat # 41410)
- Substrate Solution: Color Reagent A (Cat # 895000), and Color Reagent B (Cat # 895001), from R&D Systems, Inc**.**
- Stop Solution (Sulfuric Acid solution 1N, H_2SO_4).
- Phosphate Buffered Saline (PBS) pH 7.4 (Cat # P-5368), from Sigma-Aldrich.
- Tween® 20 (Cat # BP337-500) from F Fisher Scientific by Thermo Fisher Scientific.
- Thermo Scientific™ Clear Flat-Bottom Immuno Nonsterile 96-Well Plates (Cat # 12-565-135), from Thermo Scientific™ by Thermo Fisher Scientific.
- Autowash II Microplate Washer WellWash ™ from Labsystems by Thermo Fisher Scientific.
- PowerWave XS Microplate Spectrophotometer from BioTek, from Winooski, Vermont, USA.
- Bovine Serum Albumin (BSA) (cat # A3059-50G), from Sigma-Aldrich.
- Reagent Diluent: 1% BSA in PBS, pH 7.2-7.4, 0.2 μm filtered
- Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4.

2) Methods

A) Cell Culture

LAD2 cells were cultured in Stempro®-34 SFM with 2 mM L-glutamine, nutrient supplement, penicillin-streptomycin and 100 ng/mL rhSCF[117]. LAD2 were passaged on a weekly basis using hemi-depletion of media (only half of the volume of cell culture was replenished with fresh media). LAD2 concentrations are maintained between 1.0 – 6.0 x 10^5 cells/mL with a doubling time of approximately a week.

Calu-3 cell line (passage 11) were previously preserved and stored in liquid nitrogen. All experiments were conducted using Calu-3 passages 15-35. Calu-3 cells were cultured (5 x 10^5 cells / 75 cm^2) in MEM (20 mL) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL/100 µg/mL penicillin-streptomycin and 10% heat-inactivated FBS at 37°C, 5% CO_2, and 90% humidity. Calu-3 cells were passaged when flask reaches 80-95% confluency.

B) Single Cell Line Virus Exposure

LAD2 were plated at 5.0 x 10^5 cells/mL in 2 mL of media and Calu-3 were plated at 5.0 x 10^5 cells/cm^2 in 2 mL of media in 6-well tissue culture plates. Mast cells were rested for 1 hour prior to FluA exposure. Calu-3 were rested overnight prior to FluA exposure to allow monolayer formation. FluA (200 µL) was added to the cells at various concentrations for 1 hour to allow for virus adsorption before being removed by three successive washes with 2 mL of HBSS. Cell culture media were replenished. UV-inactivated virus and chicken allantoic fluid were used as negative controls for the effects of viral infection, whereas A23187 and biotinylated IgE were used as positive controls for MC activation. Biotinylated IgE were obtained using one-step biotinylation

kit (Miltenyi Biotic) on human IgE (EMD Millipore). LAD2 were sensitized with biotinylated human IgE (100 ng/mL) overnight, and streptavidin (100 ng/mL; Sigma-Aldrich) was used to activate cells. Supernatants and/or cells were collected at specified time points.

C) Epithelial Cell-Mast Cell Co-culture System

Experimental set-up is depicted in Figure 2.1. This method was adapted from a previous study in MC response to FluA infection[137]. Briefly, Calu-3 (seeded at 5.0 x 10^5 cells/insert) were grown on the 0.4 µm pore size membrane of the Snapwell® inserts with MEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL/100 µg/mL penicillin-streptomycin. 0.5 mL of media was used to fill the cavity of the Snapwell® insert (the top side of the membrane) and 2.6 mL of media was used to fill the lower chamber (the bottom side of the membrane). The Calu-3 were incubated for 4 days to allow for monolayer formation on the membrane. At day 3, all media were removed from both the top and bottom chambers. Fresh media was used to replenish the top chamber. LAD2 in complete Stempro® media (hemi-depleted or fresh media with all supplements added) or Stempro® media (hemi-depleted or fresh) alone was added to the bottom chamber. The co-culture system was exposed to FluA only in the top chamber for 1 hour to allow for viral adsorption. Afterwards, the chamber was washed three times with HBSS before media was replenished. Supernatant and cell samples were collected 1-5 days post-infection.

D) Assessment of Antiviral Activity of LAD2 Supernatants

As shown in Figure 2.2, LAD2 supernatants were isolated from the co-culture system 3 days after FluA exposure and re-introduced into the bottom chamber of a new co-culture plate. The isolated FluA-exposed LAD2 supernatant will be denoted as supernatant^{viral}. The top chamber of the co-culture plate was set-up as previously described with Calu-3. The top chamber was exposed to FluA for 1 hour before three successive washes with HBSS. Media was replenished and supernatant and cells were collected 3 days post-infection.

E) Investigations of LAD2 Supernatants to Characterize the Antiviral Activity

Supernatant^{viral} was isolated from the co-culture system and processed to characterize the antiviral effect of the supernatant.

- LAD2 supernatant^{viral} were heat treated either for 30 minutes at 65°C or 100°C.
- LAD2 supernatant^{viral} were treated with trypsin for 1 hour. FBS was used to neutralize trypsin activity.
- LAD2 supernatant^{viral} were fractionated using 10 kDa, 30 kDa and 50 kDa cut-off centricon/amicon filter units from EMD Millipore according to the manufacturer's protocols. Retentate and filtrate from each filter were adjusted to original volumes using fresh Stempro® media.
- LAD2 supernatant^{viral} were fractionated using QXL and SPXL ion exchange columns. Samples were concentrated 5-fold using a 10 kDa filter prior to fractionation using ion exchange columns. Elution was performed using a NaCl salt buffer at increasing

concentrations ranging from 0.2 M – 1.0 M. Flow through and elution were all collected and stored separately to assess potential antiviral activity.

After subjecting supernatant^{viral} to the above treatment conditions, the potential antiviral activity of the modified supernatant^{viral} was measured and compared to unmodified supernatant^{viral} in the system as depicted in Figure 2.2. A co-culture plate is set-up with epithelial cells grown in a monolayer on top chamber. Stempro® media in the bottom chamber was used as negative control in antiviral activity. MC and unmodified supernatant^{viral} was used as positive control in antiviral activity. The modified supernatant^{viral} was tested in the co-culture system, and supernatant were harvested 3 days-post-infection. FluA release in all wells was measured.

F) Quantification of Influenza A Virus Release

Quantification of total FluA release was determined by measuring the amount of hemagglutination activity in supernatants[157]. Briefly, a human red blood cell (RBC) sample (courtesy of Airway Inflammation Team, University of Alberta) was washed with PBS three times before stored for 24 hours in DGV buffer. Using a 96-well round bottom plate, 50 µL PBS with 0.1% of BSA was added to each well. Samples (50 µL) were added on the short-axis of the plate (8 wells). Two-fold serial dilution was performed by diluting samples with adjacent wells by moving samples along the long-axis of the plate. 50 µL of 0.05% RBC in PBS is added to each well. The plate was incubated for 2 hours at room temperature. Positive hemagglutination was determined by the presence of lattice formation on the bottom of the well. Negative hemagglutination was verified by pellet formation of RBC at the bottom of the well. The lowest dilution at which hemagglutination was present was used to define the hemagglutination units for

the sample. Each sample was done in triplicate and each plate contained FluA stock sample and PBS for both positive and negative controls respectively. FluA release was expressed in HAU/mL.

G) Cytokine and Chemokine Release

To investigate cytokine and chemokine release in single culture and co-culture experiments, supernatants were sent to Eve Technologies (Calgary, AB) to select possible candidates for further analysis. The human cytokines / chemokines 41-plex assay was used for this screening process. The cytokine/chemokine screened were:

EGF, FGF2, Eotaxin, TGF-α, G-CSF, Flt-3L, GM-CSF, Fractalkine, IFNα2, IFNγ, GRO pan, IL-10, MCP-3, IL-12, MDC, IL-12 P70, PDGF-AA, IL-13, PDGF-BB, IL-15, sCD40L, IL-17A, IL-1RA, IL-1a, IL-9, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, CXCL-10, CCL-2, CCL-3, CCL-4, CCL-5, TNF, TNFβ, VEGF-A

Afterwards, The R&D Systems, Inc. DuoSet™ ELISA kits were used to measure Flt-3L, GM-CSF, CCL-2, CCL-3, CCL-4, CCL-5, IFN-λ1, IFN- λ2, and IFN- λ3. All measurements were conducted based on supplier's protocols. Briefly, 96-well immunosorbent plates were coated with capture antibody overnight at room temperature. Plates were washed three times and then treated for one hour with reagent diluent to block non-specific binding sites in wells. Plates were washed three times again and samples were loaded along with specified standards for 2 hours. After another three washes, detection antibodies were added for 2 hours. The plates were washed again, and streptavidin-HRP was added to each plate for 20 minutes in the dark. Afterwards, three final washes were performed, and substrate solutions were added to develop the plates in the dark. After 20 minutes, stop solution was added to terminate reactions and the plates were read using a microplate reader. Optical density readings were measured at 450 nm and 540 nm. A seven-point

standard curve was generated for each plate and used to interpolate the amount of respective protein in the sample.

For blocking CCL-4 experiments, anti-CCL-4 IgG or goat IgG (20 μg/mL) control were added to FluA exposed co-cultures every 24 hours starting on day 0. Concentration of antibodies used were in excess of manufacturer's recommended dosage (1.5 - 9.0 μg/mL antibody per 5 ng/mL protein). Our co-cultures produced less than 1ng/mL CCL-4 when exposed to FluA. On day 5 post-FluA infection, supernatants were harvested.

H) Statistical Analysis

Using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA), statistical analysis was performed using paired *t*-test, one-way analysis of variance (ANOVA) and two-way ANOVA where appropriate. Post-tests were used and reported when necessary. Data shown are mean ± SEM. A p value <0.05 was considered significant.

3) Figures

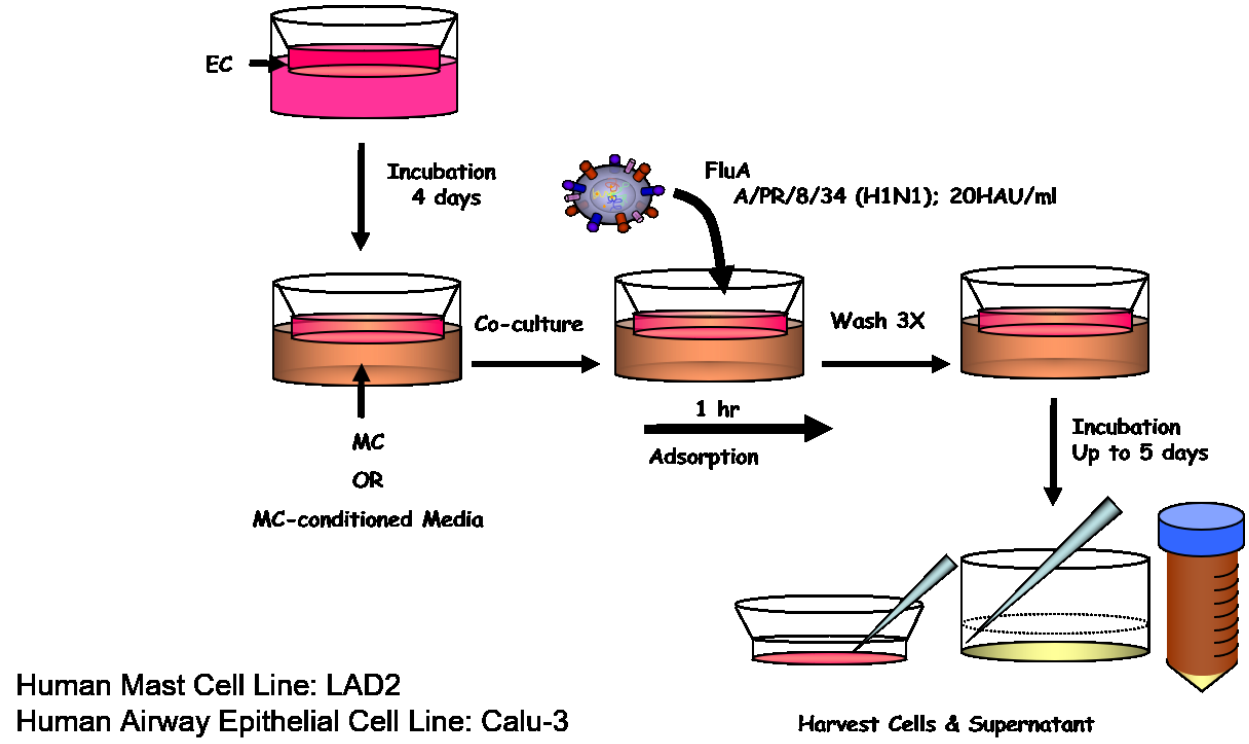

Figure 2.1 Experimental Design of Influenza A Infection of Epithelial Cell-Mast Cell Co-culture. Briefly, EC were grown on membrane inserts for 4 days. MC were added to bottom chambers (or MC-conditioned media as control). FluA (0.04 MOI) was added to top chamber for 1 hour. Excess FluA was removed from co-culture. FluA-exposed co-cultures were incubated for up to 5 days before supernatants were harvested.

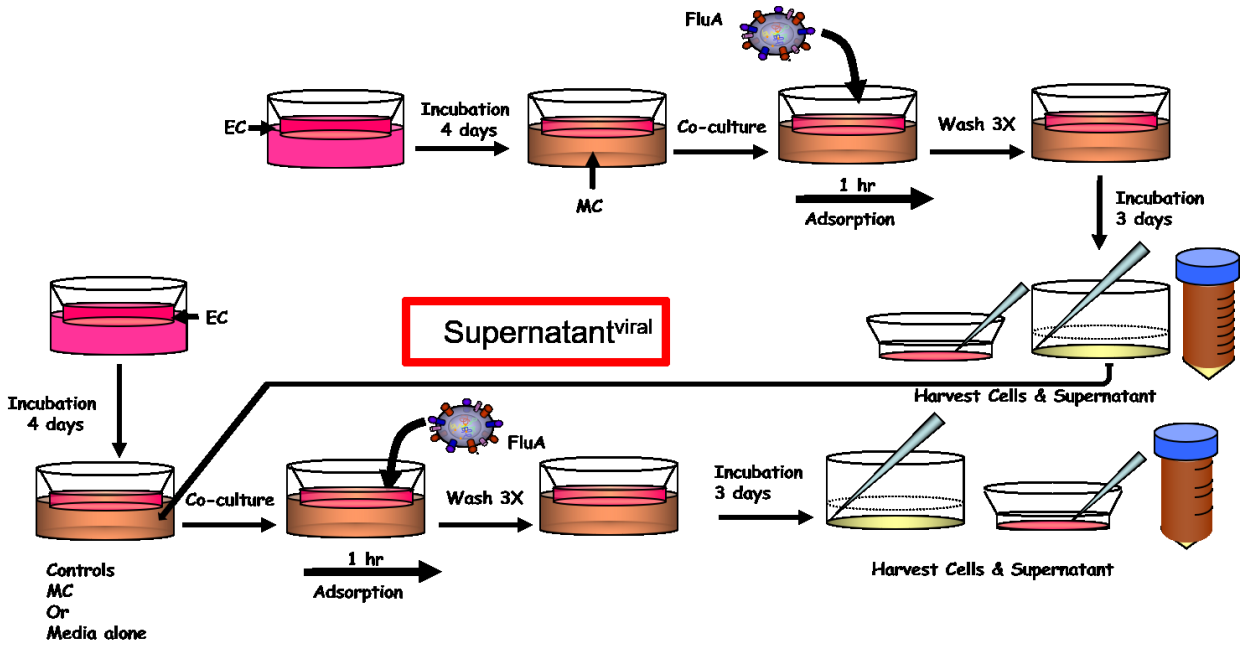

Figure 2.2 Experimental Design of Investigating Antiviral Activity of Supernatant^{viral}. Briefly, after harvesting MC supernatant from a 3-days-post-FluA-EC-MC-co-culture, MC were removed from suspension to create supernatant^{viral}. Supernatant^{viral} was then added to the bottom chamber of a new co-culture well with EC grown in top chamber. FluA exposure was performed as previously described. Supernatants were collected 3 days post-infection.

Chapter III: Responses of Mast Cells and Epithelial Cells Following Exposure to Influenza A Virus

1) Authors

Kurtis Ng, Javeria Raheem, Chris St. Laurent, Tae Chul Moon, Harissios Vliagoftis, A. Dean Befus

2) Introduction

Influenza A virus (FluA) causes one of the most common respiratory diseases. Despite advances in vaccine development and health care systems, influenza affects millions of individuals annually and pandemics, such as that of H1N1 in 2009 remain a major concern. FluA primarily infects and replicates within airway epithelial cells (EC), but host defences against influenza involves many cell types, including monocytes, dendritic cells and neutrophils[60,72,73]. By investigating the response of different cell types to FluA infection, we can gain a better understanding of the mechanisms of host resistance and viral clearance against FluA.

The role of mast cells (MC) in anti-viral immunity is emerging but still incompletely understood. They are enriched near mucosal surfaces, and thus in a prime location to respond to infections and help initiate and boost host defences[96,158]. Best known for their classical role in allergic inflammation, evidence has emerged over the last two decades that MC play critical roles in bacterial, fungal and viral infections, as well as protection against helminths[85,86,96,98,121,158]. MC can be activated through IgE-dependent or IgE-independent mechanisms, resulting in the release of mediators stored in granules as well as newly synthesized cytokines, chemokines and

arachidonic acid metabolites that appear to be important in viral pathogenesis and host defences. For example, respiratory syncytial virus, vesicular stomatitis virus and dengue virus induce various cytokine, chemokine and anti-viral gene responses in MC [121,127-129,139].

Porcine and murine MC release histamine upon FluA infection and may affect FluA pathogenesis, but it is unknown whether human MC respond similarly[131,135]. Graham *et al.* showed that infecting murine MC with FluA can induce release of IL-6 that varies depending on the strain of virus used[131]. Thus, despite commonalities in genetic sequence and viral structure, host responses can differ significantly depending on the strain of FluA present. Interestingly, mice with MC deficiency exhibit reduced respiratory pathology in FluA infection and lower levels of inflammatory cytokines and chemokines in their bronchoalveolar lavage fluid compared to normal mice[131]. This suggests that MC play a significant role in innate immune responses to FluA and help direct the inflammatory cascade during infection.

It has been previously established that the MC inflammatory response to FluA was mediated by RIG-I signalling, but it is unknown whether MDA5 may also play a role in FluA recognition in MC as there are evidence of MDA5 signalling in other cell types[65,66,131,159]. Furthermore, it had been shown that MC undergoes non-productive FluA infection, despite producing viral proteins which suggests MC may have antiviral mechanism(s) that suppress FluA assembly or budding[136]. Viperin was found to interfere with FluA release in other cell types, which could also be involved in the control of FluA release in MC[160,161]. Investigating antiviral gene expression in MC during FluA infection may enhance understanding of innate immune responses of MC during infections.

To further characterize the response of MC to FluA, we investigated the release of mediators including histamine, β-hexosaminidase (β-hex), cytokines, chemokines, and

arachidonic acid metabolites, as well as the induction of selected anti-viral genes following exposure to three strains of FluA.

3) Materials and Methods

A) Cell Culture

The human mast cell line, LAD2 (Laboratory of Allergic Diseases 2, generously provided by Dr. D.D. Metcalfe and A Kirshenbaum, National Institutes of Health, Bethesda, MD) was cultured as previously described[117]. The human lung adenocarcinoma epithelial cell line, Calu-3 (ATCC® number: HTB-55), was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured with minimum essential medium and Earle's salts supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies, Burlington, ON). Calu-3 cells were harvested at ~80% confluency using 0.05% Trypsin-EDTA (Life Technologies) and subcultured at a seeding concentration of 7.0×10^3 cells/cm^2.

B) Influenza Virus Strains and Ultraviolet Inactivation

Influenza A/PR/8/34 virus strain (H1N1), propagated in duck eggs, was obtained from Dr. K. P. Kane from the University of Alberta. FluA/WS/33 (H1N1) (ATCC® number: VR-1520) and FluA/Hong Kong/8/68 (H3N2) (ATCC® number: VR-1679) were obtained from ATCC and propagated with the help of Dr. Katharine Magor (University of Alberta) in chicken eggs. UV inactivation of all virus strains was performed using a UV lamp (ENF-280C, Spectroline, Westbury, NY) as previously described[136].

C) Influenza A Virus Infection

LAD2 were seeded at 5×10^5 cells/mL in 2 mL of media in 6-well tissue culture plates and rested for 1 hr prior to FluA exposure. Calu-3 were incubated 24 hr prior to FluA exposure to allow formation of monolayers at 9.0×10^4 cells/cm^2 based on our growth curves in 6-well plates. Two hundred µL of FluA virus were added to the cells at 0.1 and 1.0 multiplicity of infection [MOI]

for 1 hr to allow virus adsorption. UV-inactivated virus for each strain were also used at 1.0 MOI. Cells were then washed three times with HBSS before cultured in fresh media. No FluA was detected in third wash supernatant by hemagglutination assay. Hemagglutination assay was used to quantify viral particles in samples as previously described[136].

D) β-hexosaminidase and Histamine Measurements

β-hex assay was performed in HEPES-buffered Tyrode's solution as previously described with modifications[162]. Briefly, LAD2 cells (1×10^5) were incubated with different doses of FluA virus (A/PR/8/34, A/WS/33, A/HK/8/68; 1.0 MOI, 5.0 MOI) for 30 min at 37°C. Cells were centrifuged (5 min, 300 *g*) after incubation and supernatants were collected and stored at 4°C. Cell pellets were resuspended in HEPES buffer and lysed by three freeze-thaw cycles using sonication and liquid nitrogen. Results are expressed as % secretion using formula [(β-hex in supernatant) / (β-hex in supernatant + β-hex in cell pellet)] × 100. Histamine was measured by automated fluorometric histamine assay as previously described[162]. Chicken allantoic fluid (virus propagation medium) was used as negative control and A23187 (Sigma Aldrich, St. Louis, MO) was used as positive control for MC activation and these controls produced results as expected.

E) Arachidonic Acid Metabolite, Cytokine and Chemokine Measurements

PGD_2 and leukotriene C_4 release was measured using a commercially available enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI) using the infection protocol as decribed in previous section. However, no significant release was observed using leukotriene C_4 assay. Human cytokine array/ chemokine array 41-plex performed by Eve Technologies (Calgary, AB) was used for screening differential release of cytokines and chemokines between LAD2 and Calu-3 (refer to Appendix A). Following this screen, GM-CSF, Flt-3L, CCL-3, CCL-4, CCL-5, CXCL-10, IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ1/3 release was measured in LAD2 and Calu-3 culture

supernatants at 1, 2 and 4 days post virus exposure using commercially available enzyme-linked immunosorbent assays (ELISAs) according to the supplier's protocols (R&D Systems, Minneapolis, MN).

F) RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

Total RNA from LAD2 and Calu-3 cells were prepared using RNeasy Mini Kit (Qiagen, Germantown, MD) according to supplier's protocols. Reverse transcription was performed using SuperScript III First-Strand Synthesis SuperMix (Life Technologies). SYBR green (Life Technologies) was used for qPCR with the following primers: Viperin, forward 5'- TGG TGA GGT TCT GCA AAG TAG-3', reverse 5'- GTC ACA GGA GAT AGC GAG AAT G-3'; MAVS, forward 5'- TGC CGT TTG CTG GAG ACA A-3', reverse 5'- TTC GTC CGC GAG ATC AAC T-3'; GAPDH, forward 5'- CTG AGA ACG GGA AGC TTG TCA-3', reverse 5'- GCA AAT GAG CCC CAG CCT T-3'; MDA-5, forward 5'- TGG TCT CAC CAA TGA AA- 3', reverse 5'- CTC CTG AAC CAC TGT GAG CA-3'; and RIG-I, forward 5'- TGG CAT ATT GAC TGG ACG TG-3', reverse 5'- CAC TGG CTT TGA ATG CAT CC-3'. Forty-three cycles of qPCR were performed using 95°C for denaturation (15 seconds) and 60°C for annealing/extension (1 minute). qPCR results were analyzed using ΔΔCT method and relative quantification using human GAPDH as a housekeeping gene[163,164].

G) Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). One-way ANOVA with Dunn's Multiple Comparison and two-way ANOVA with Bonferroni post-tests were used for statistical analysis where appropriate and described in figure legends. Data shown are mean ± SEM. A p value <0.05 was considered significant.

4) Results

A) Release of Short-term Mediators from Mast Cells After Exposure to Different Strains of Influenza A Virus

We exposed LAD2 to three strains of Flu (A/PR/8/34, A/WS/33 and A/HK/8/68) and collected supernatants at 30 minutes post-infection to investigate short-term mediator release. Neither A/PR/8/34 nor A/WS/33 induced β-hex release compared to mock treatment (Figure 3.1a). However, A/HK/8/68 induced 8.5 ± 1.1% (p <0.05) β-hex release at 5 MOI compared to mock at 4.1 ± 0.6% (Figure 3.1a). This pattern was also seen in histamine release (Figure 3.1b). Only A/HK/8/68 induced statistically significantly histamine (175.5 ± 23.5 pg/mL) release at 5 MOI as compared to mock treatment (36.7 ± 13.8 pg/mL) (Figure 3.1b). When we measured the release of the arachidonic acid metabolite, PGD_2, by LAD2 after FluA exposure, we found that A/PR/8/34 did not significantly affect PGD_2 release from LAD2 (Figure 3.1c). However, both A/WS/33 and A/HK/8/68 induced statistically significant PGD_2 (1173.9 ± 194.7 pg/mL and 683.2 ± 43.4 pg/mL respectively) release at 5 MOI as compared to mock treatment (65.1 ± 10.3 pg/mL) (Figure 3.1c). These results suggest that release of MC mediators induced by FluA exposure varies among strains of FluA.

B) Influenza A Induced Cytokine and Chemokine Release in Epithelial Cells and Mast Cells

We next compared cytokine and chemokine release from Calu-3 and LAD2 after FluA infection, initially using the multiplex assay system performed by Eve Technologies (refer to Appendix A). To confirm those screening results that appeared to be positive, ELISAs were used to measure cytokine and chemokine release from LAD2 and Calu-3. In contrast to the screening

assays, using ELISA assays we could not confirm that the three FluA strains induced Flt-3L or CCL-3 release in Calu-3 or LAD2 (refer to Appendix B).

However, A/PR/8/34 induced statistically significant CCL-5 and CXCL-10 release at all time points in Calu-3, with peak levels detected on day 2 at 1077.6 ± 143.3 pg/mL and 2481.2 ± 95.8 pg/mL respectively compared to respective mock treatments (0 pg/mL, 14.4 ± 5.1 pg/mL) (Figure 3.2a, d, e). However, A/PR/8/34 did not induce CCL-4 release in Calu-3 (Figure 3.2a). In contrast to Calu-3, LAD2 released statistically significant levels of CCL-4 (1010.2 ± 306.5 pg/mL on day 2) compared to mock treatment of 15.2 ± 3.7 pg/mL but did not release statistically significant levels of CCL-5 or CXCL-10 (Figure 3.2a, d, e). A/WS/33 did not induce significant release of CCL-4, CCL-5, or CXCL-10 in LAD2 compared to its respective mock or UVI treatments (Figure 3.2b, e, h). Moreover, Calu-3 did not release CCL-4 when exposed to A/WS/33 but did release statistically significant levels of CCL-5 (812.4 ± 55.2 pg/mL; day 1 [peak]) and CXCL-10 (1943.5 ± 482.2 pg/mL; day 1 [peak]) beyond mock and UVI treatments (Figure 3.2b, e, h). A/HK/8/68 did not induce statistically significant release of CCL-4, CCL-5 or CXCL-10 from LAD2 during 4 days of infection (Figure 3.2c, f, i), but induced Calu-3 to release CCL-5 and CXCL-10, but not CCL-4. CCL-5 release peaked on day 4 (713 ± 104 pg/mL), whereas CXCL-10 peaked on day 2 (1668 ±129.3 pg/mL) in Calu-3 (Figure 3.2c, f, i). These results show significant differences in cytokine/chemokine release by MC when compared to EC, suggesting different viral response pathways in the two cell types and FluA strain dependency (refer to Table 3.1 for simplified peak releases of cytokines/chemokines).

We also examined whether there were differences in interferon release between FluA treated LAD2 and Calu-3. FluA exposure induced low but not statistically significant levels of IFN-α release by LAD2, whereas IFN-β and IFN-γ were not detected in LAD2 or Calu-3 (refer to

Appendix A and B). Similarly, LAD2 also did not release statistically significant levels of type III interferons after FluA exposure compared to mock and UVI treatments (Figure 3.3a-i). However, we found that Calu-3 released significantly higher levels of IFN-λ1, -λ2, and -λ3 in the presence of the three strains of FluA (1.0 MOI) compared to its respective mock and UVI controls (Figure 3.3a-i). In Calu-3, A/PR/8/34 induced release of IFN-λ1 peaked on day 1 (2302.9 ± 117.2 pg/mL), IFN-λ2 peaked on day 2 (6593 ± 582.7 pg/mL) and IFN-λ3 peaked on day 2 (2877.2 ± 415.4 pg/mL). A/WS/33 induced peak releases of IFN-λ1 (2446.15 ± 246.9 pg/mL) and IFN-λ2 (7274.7 ± 286.2 pg/mL) on day 1, and IFN-λ3 (2567.2 ± 738.4 pg/mL) on day 2. Peak releases of IFN-λ1 (712.2 ± 81.8 pg/mL), IFN-λ2 (4961.4 ± 297.7 pg/mL), and IFN-λ3 (1647.5 ± 517.3 pg/mL) were detected on day 2 for Calu-3 when infected with A/HK/8/68. These results suggest that LAD2 do not produce much IFN during FluA infections (refer to Table 3.2 for simplified peak releases of IFN-λs).

C) Upregulation of Antiviral Genes in Mast Cells and Epithelial Cells After Influenza A Exposure

We assessed the effect of the three strains of FluA on the expression of selected anti-viral genes in Calu-3 and LAD2. FluA infection did not affect MAVS mRNA expression in either Calu-3 or LAD2 compared to mock and UVI treatments for all three strains tested (refer to Appendix B).

However, RIG-I mRNA was significantly upregulated relative to GAPDH on day 1 and 2 by A/PR/8/34 infection compared to UVI treatment in LAD2 (Figure 3.4a). By contrast, A/PR/8/34 induction of RIG-I mRNA expression was not statistically significant in Calu-3 when compared to UVI treatment. RIG-I expression in LAD2 was statistically greater than in Calu-3 (day 1 & 2, *p*

<0.001) (Figure 3.4a). RIG-I mRNA expression in LAD2 was upregulated by A/WS/33 on day 4 compared to its UVI treatment (Figure 3.4b). As with A/PR/8/34, A/WS/33 regulation of RIG-I mRNA expression in Calu-3 was not statistically significant compared to UVI treatment and RIG-I mRNA expression levels in LAD2 were statistically greater than in Calu-3 (p <0.01) (Figure 3.4b). A/HK/8/68 upregulated RIG-I mRNA expression in LAD2 and Calu-3 compared to its respective UVI treatments and as with the other strains, upregulation of RIG-I mRNA was also higher in LAD2 than in Calu-3 (p <0.001) (Figure 3.4c).

A/PR/8/34 and A/HK/8/68 significantly upregulated MDA5 mRNA expression in LAD2 and Calu-3 compared to their respective UVI treatments. Moreover, upregulation of MDA5 mRNA was statistically higher in LAD2 compared to Calu-3 for day 1 (p <0.001), day 2 (p <0.01), and day 4 (p <0.001 for A/HK/8/68 only) (Figure 3.4d, f). A/WS/33 did not significantly affect MDA5 mRNA expression in LAD2 or Calu-3 (Figure 3.4e). Interestingly, FluA virus did not upregulate Viperin mRNA expression in LAD2, whereas A/PR/8/34 and A/HK/8/68 upregulated Viperin mRNA expression on day 1, 2 and 4 in Calu-3 compared to UVI treatment (p <0.001) (Figure 3.4g, i). A/WS/33 upregulated Viperin mRNA expression in Calu-3 on day 1 (p <0.001) and day 4 (p <0.01) compared to UVI treatment (Figure 3.4h). Refer to Table 3.3 for a simplified peak expression of selected antiviral gene expression.

5) Discussion

FluA primarily infects airway EC in human, but studies have also shown that FluA can infect and activate cytokine and chemokine release in monocytes, neutrophils and dendritic cells. However, the specific roles these cells may play in viral pathogenesis and innate immune responses remains unclear.

Resident pulmonary MC are located near EC in the lungs, allowing them to contact and respond to respiratory viral infections[86,87,98,121,158]. Porcine MC infected with A/California/07/2009 (H1N1) and mouse bone marrow-cultured MC release histamine upon infection with A/WS/33 (H1N1) [131,135]. In this study, we used LAD2, a human MC line, to examine the response of human MC to different strains of FluA (refer to Table 3.4). We show that A/HK/8/68 (H3N2) infection of LAD2 induces 2-fold and 3-fold higher β-hex and histamine release respectively, compared to mock infection, whereas A/PR/8/68 and A/WS/33 (H1N1) did not affect β-hex and histamine release (Figure 3.1a). We also demonstrated that PGD_2 secretion in LAD2 was increased when exposed to either A/WS/33 (H1N1) or A/HK/8/68 (H3N2) (Figure 3.1c). Although A/WS/33 did not induce degranulation of LAD2 (histamine and β-hex release), it induced the release of newly synthesized PGD_2. Our results differ from those with murine and porcine MC, perhaps because of differences in viral strain or dose, and species origin of the MC. Thus, differences in viral strain, host species and mediator response uncover diverse immunological responses to FluA.

After establishing that MC are activated by different strains of FluA, we investigated production of selected cytokines and chemokines that may play a role in the responses of MC and EC to FluA (refer to Table 3.1). We found that only A/PR/8/34 induced CCL-4 release in LAD2. There is also a time dependent trend (p <0.05) in which CCL-4 release increases until day 2 post infection before decreasing afterwards (Figure 3.2). This contrasts with no significant CCL-4

release in LAD2 after A/WS/33 and A/HK/8/68 infection. Furthermore, CCL-4 release was not detected when Calu-3 was infected by the three FluA strains. CCL-5, which attracts T cells, eosinophils and basophils; and CXCL-10, which attracts T cells, NK cells, dendritic cells and monocytes, were released by Calu-3 after FluA infection, but not by LAD2 (Figure 3.2). Lee *et al*. has demonstrated that CXCL-10 mRNA is upregulated at 12 h post infection in porcine MC, but our results do not show increased release of CXCL-10 protein in LAD2 post-FluA exposure[135]. In contrast to LAD2, Calu-3 released CXCL-10 after FluA exposure, further demonstrating host cell type variation in responses to FluA. Thus, there are clear differences in cytokine and chemokine release between MC and EC after FluA exposure demonstrating that FluA elicits a diverse response in different cells, and that recognition and response to infection may involve distinct signalling pathways in different cell types.

Despite type I interferons being an indicator of FluA-infection in EC and monocytes, we did not detect significant IFN-α or IFN-β release in LAD2 (refer to Appendix A and B)[165,166]. Low amount of Type I IFN release in LAD2 are consistent with a recent report that the murine MC line, P815, do not produce much IFN-α or IFN-β upon FluA infection[132]. We also investigated type II IFN release in MC and EC, but no IFN-γ was detected in our study. This was inconsistent with IFN-γ release seen in murine MC, but supports our evidence that MC response varied dependent on host species[132]. Finally, we investigated the release of type III IFN, IFN-λ1, -λ2 and -λ3. Our results show that all three FluA strains induced the release of IFN-λ1, -λ2 and -λ3 in Calu-3 but not in LAD2 (Figure 3.3, Table 3.2). This leads us to speculate that LAD2, human MC, is not a major source of IFN during FluA infection. However, it is unknown whether LAD2 will respond to exogenous IFNs during FluA infections and activate IFN-dependent pathways, hence it may warrant further studies in IFN-stimulated genes.

Our study also showed that antiviral gene regulation differs significantly between Calu-3 and LAD2 (refer to Table 3.3). RIG-I is a cytosolic receptor that recognizes FluA and docks on its adaptor MAVS to initiate IRF3- and NFκB-dependent signalling[64,65,120,131]. It is interesting to note that despite upregulation of RIG-I mRNA in LAD2 after FluA infection, there was no upregulation of MAVS mRNA expression, an associated adaptor to RIG-I. It was shown previously with FluA that RIG-I/MAVS interaction is involved in MC cytokine, chemokine and leukotriene production[131]. Thus, why RIG-I is upregulated but not MAVS mRNA is intriguing and requires further investigations. In contrast to LAD2, A/HK/8/68 was the only FluA strain that upregulated RIG-I expression in Calu-3. This emphasizes the diversity in antiviral responses that different viral strains may elicit in different host cells at different time points.

MDA5 is also another antiviral gene that is often associated with viral recognition and host defense. Our results showed that both Calu-3 and LAD2 respond to FluA infection by upregulating MDA5 mRNA expression. Similar to RIG-I, MDA5 uses MAVS as a downstream adaptor molecule in its signalling pathway[66]. Why would MDA5 and RIG-I be upregulated after FluA infection considering that MAVS, an associated adaptor molecule, is not similarly upregulated? Additional investigations are warranted about the role of MDA5 and RIG-I in antiviral defenses and whether a MAVS-independent pathway is involved with host antiviral signalling in MC.

Viperin is a cellular protein that disrupts modifications of plasma membrane fluidity which affect FluA viral budding and release. We found that Calu-3, but not LAD2 increased Viperin mRNA expression following FluA infection (Figure 3.4). Our previous study showed FluA transcription, replication and protein synthesis occur in LAD2, but viral release was limited[136]. Our results suggest that Viperin was not involved with limited FluA progeny release in MC,

instead a different antiviral mechanism is involved. Further studies are needed concerning the mechanisms MC use to interfere with FluA replication.

In conclusion, our results demonstrate that human MC responses differ depending on the strain of FluA used, and also differ from the responses of animal MC[131,135]. Also, MC respond differently than EC to FluA infection, suggesting that MC could potentially supplement the responses of EC, such as CCL-4 release to facilitate recruitment of various immune cells and the developing inflammatory responses (refer to Table 3.4). FluA infection of MC results in partial degranulation and release of newly synthesized PGD_2 along with CCL-4. Based on our preliminary results on MC response to FluA infection, we have a better understanding of MC and its role in response to FluA infections. However, there is much to learn about MC in viral immunity and pathogenesis, and about whether MC might provide a novel therapeutic target to help reduce the impact of seasonal and pandemic strains of FluA.

6) Figures

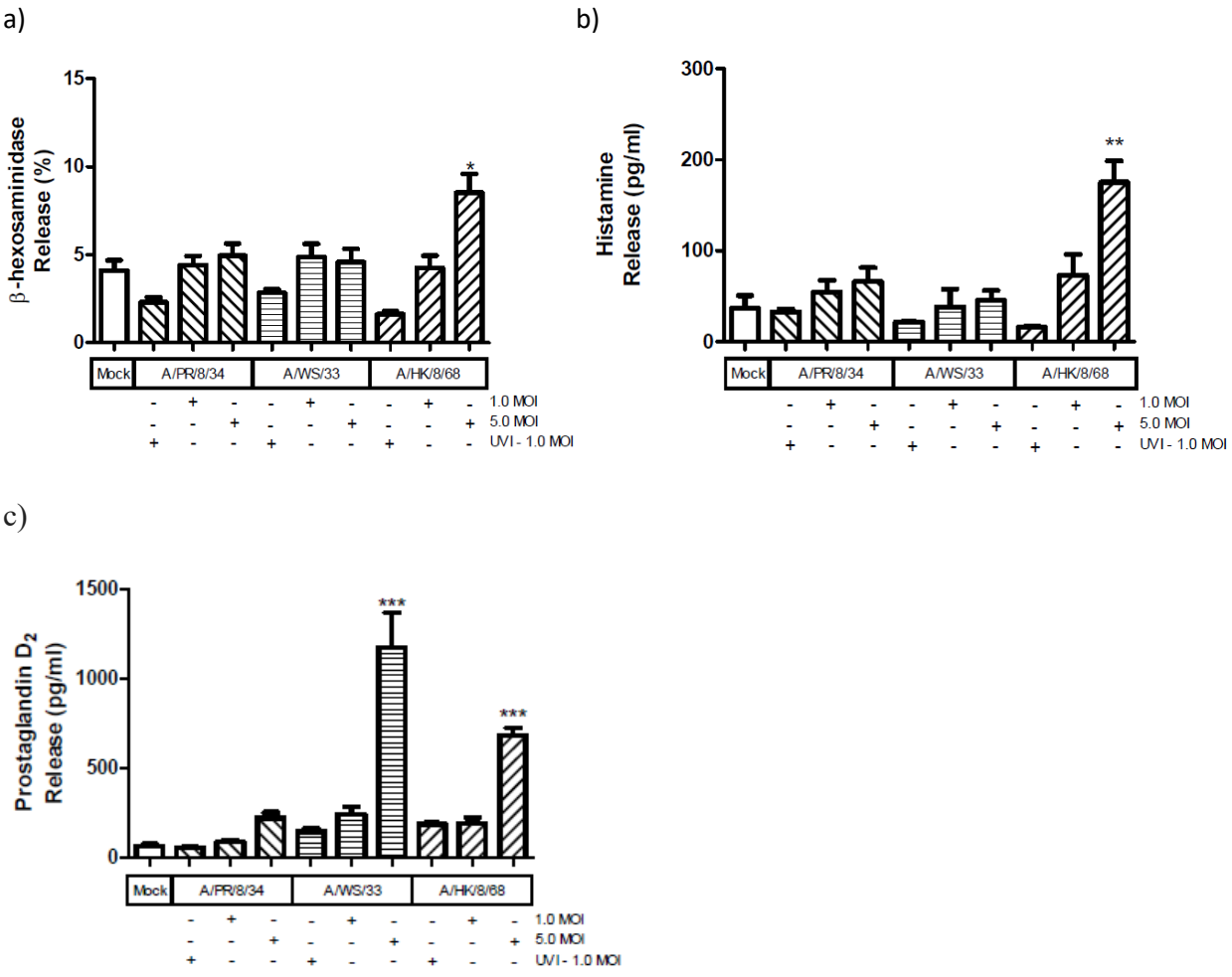

Figure 3.1 Selective Mediator Release in LAD2 After Exposure to Three Strains of Influenza A Virus; A/PR/8/34, A/WS/33 and A/HK/8/68. UV-inactivated virus was used as negative control and A21387 was used as positive control (not shown). Thirty minutes after inoculation, β-hex (A, n=5), histamine (B, n=3) and PGD_2 (C, n=3) were measured as described in methods. A/PR/8/34 did not affect β-hex, histamine or PGD_2 release. A/WS/33 did not affect β-hex or histamine release but did induce statistically significant levels of prostaglandin D_2 release. A/HK/8/68 infection resulted in statistically significant β-hex, histamine and prostaglandin D_2 release in LAD2. One-way ANOVA with Dunn's multiple comparison post-test was used for statistical analysis and represented as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ when compared to mock treatments.

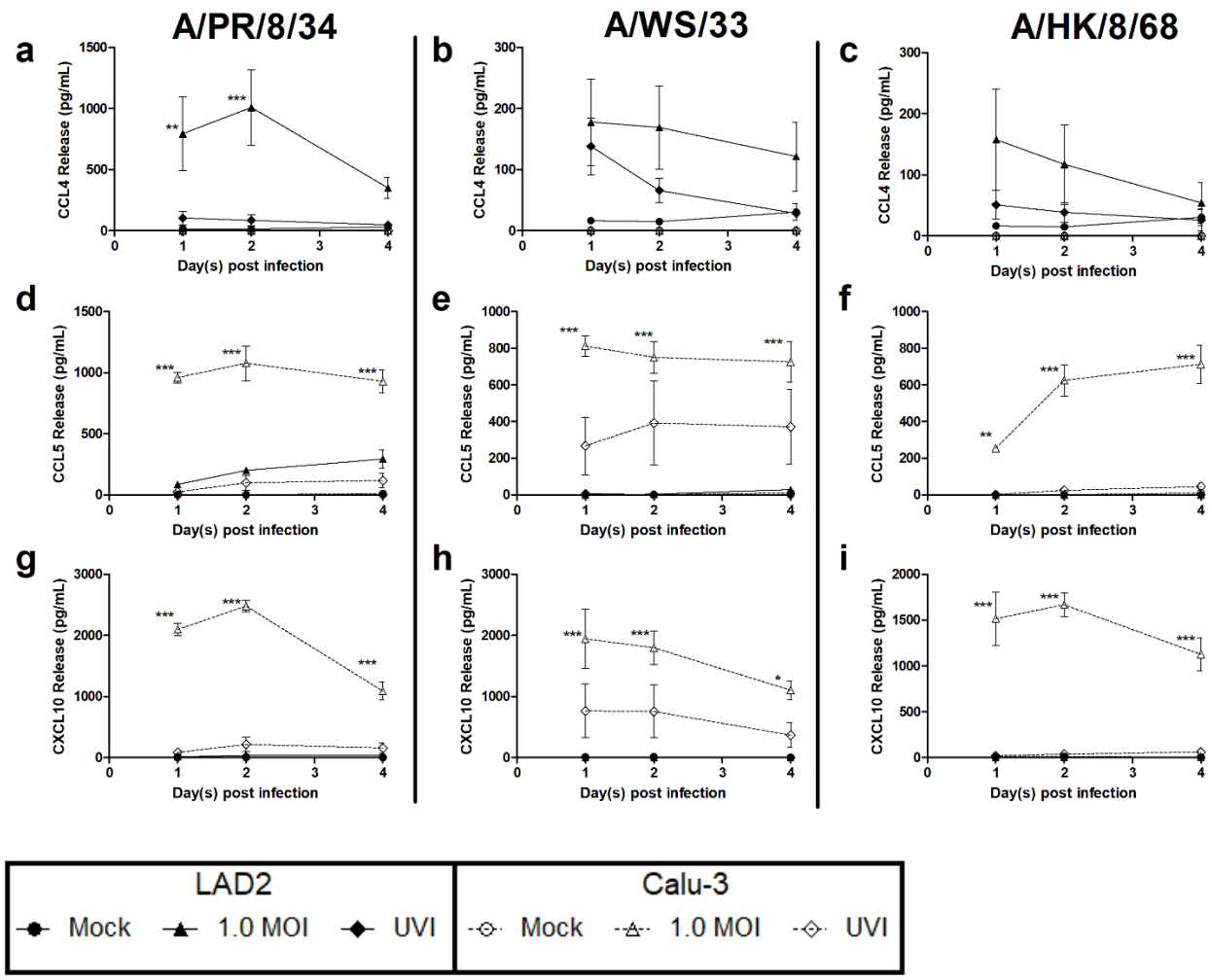

Figure 3.2 Cytokine and Chemokine Release in LAD2 and Calu-3 After Exposure to Three Strains of Influenza A Virus. Virus was used at 1.0 or 0.1 MOI. UV-inactivated virus was used as control. Cytokine/chemokine release was measured 1, 2 and 4 days post-FluA infection by ELISAs. CCL-3 and Ftl-3L release were not affected by FluA exposure (refer to Appendix B). Significant CCL-4 release was detected in LAD2 only and not Calu-3 when exposed to A/PR/8/34 (a); A/WS/33 (b) and A/HK/8/68 (c) did not induce significant release of CCL-4 in LAD2 or Calu-3. CCL-5 (d-f) and CXCL-10 (g-i) release were detected in Calu-3 for all three viral strains of Flu but not in LAD2. Two-way ANOVA with Bonferroni post-test was used for statistical analysis and represented as *p <0.05, **p <0.01 and ***p <0.001 when compared to respective mock treatments (n=4).

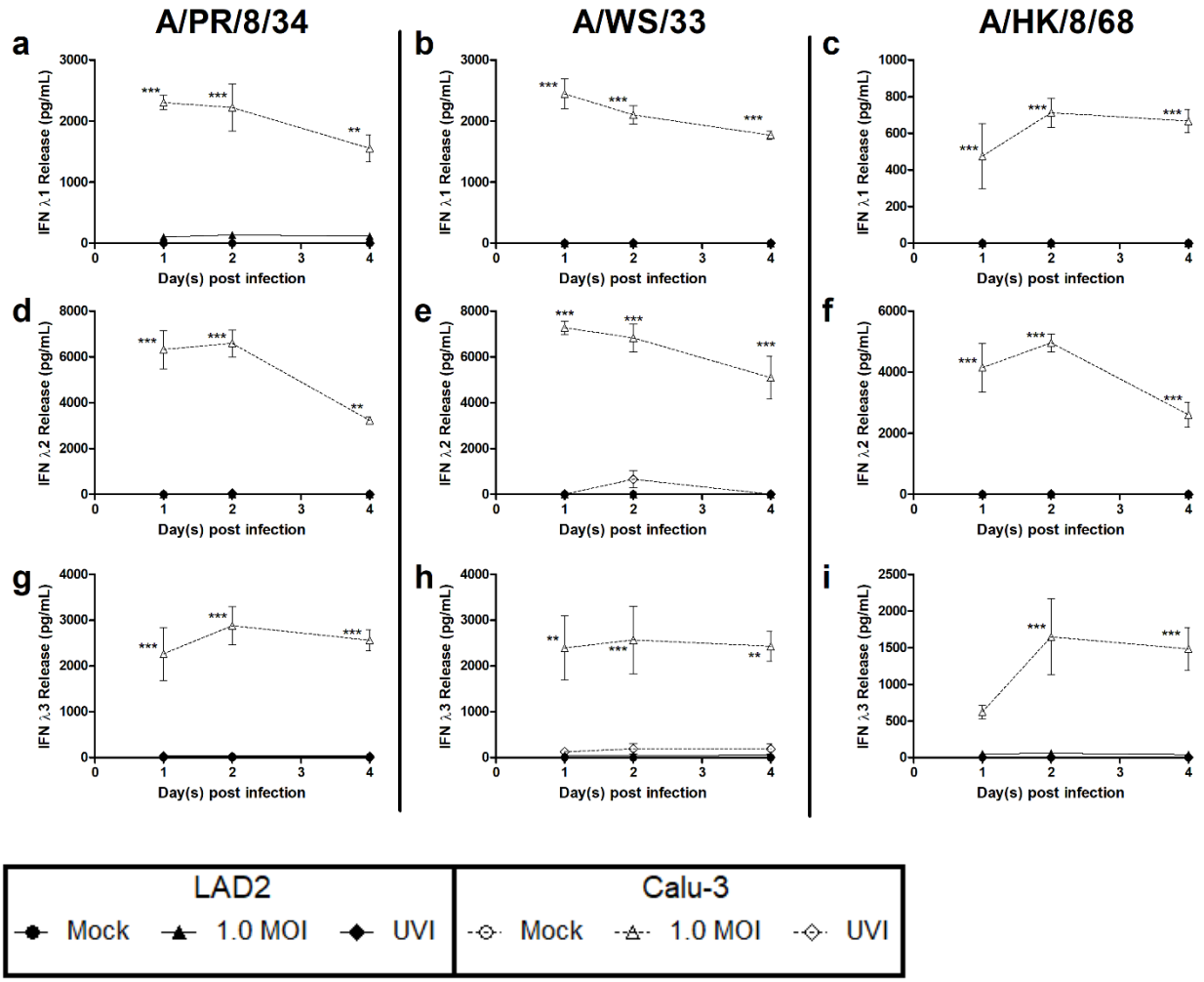

Figure 3.3 Type III Interferon Release in LAD2 and Calu-3 After Exposure to Three Strains of Influenza A Virus. Virus was used at 1.0 or 0.1 MOI. UV-inactivated virus was used as control. Interferon release was measured 1, 2 and 4 days post FluA infection by ELISAs. IFN-λ1 (a-c), IFN-λ2 (d-f), and IFN-λ3 (g-i) were measured for three FluA strains, A/PR/8/34, A/WS/33 and A/HK/8/68. Low IFN-α release was detected in LAD2; IFN-β and IFN-γ release were not detected (refer to Appendix A and B). IFN-λ1, IFN-λ2 and IFN-λ3 release were not detected in LAD2 when exposed to the three FluA viral strains. Calu-3 was found to release higher levels of IFN-λ1, IFN-λ2 and IFN-λ3 when compared to its respective mock and UVI treatments for all three FluA strains. Two-way ANOVA with Bonferroni post-test was used for statistical analysis and represented as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ when compared to respective mock treatments (n=4).

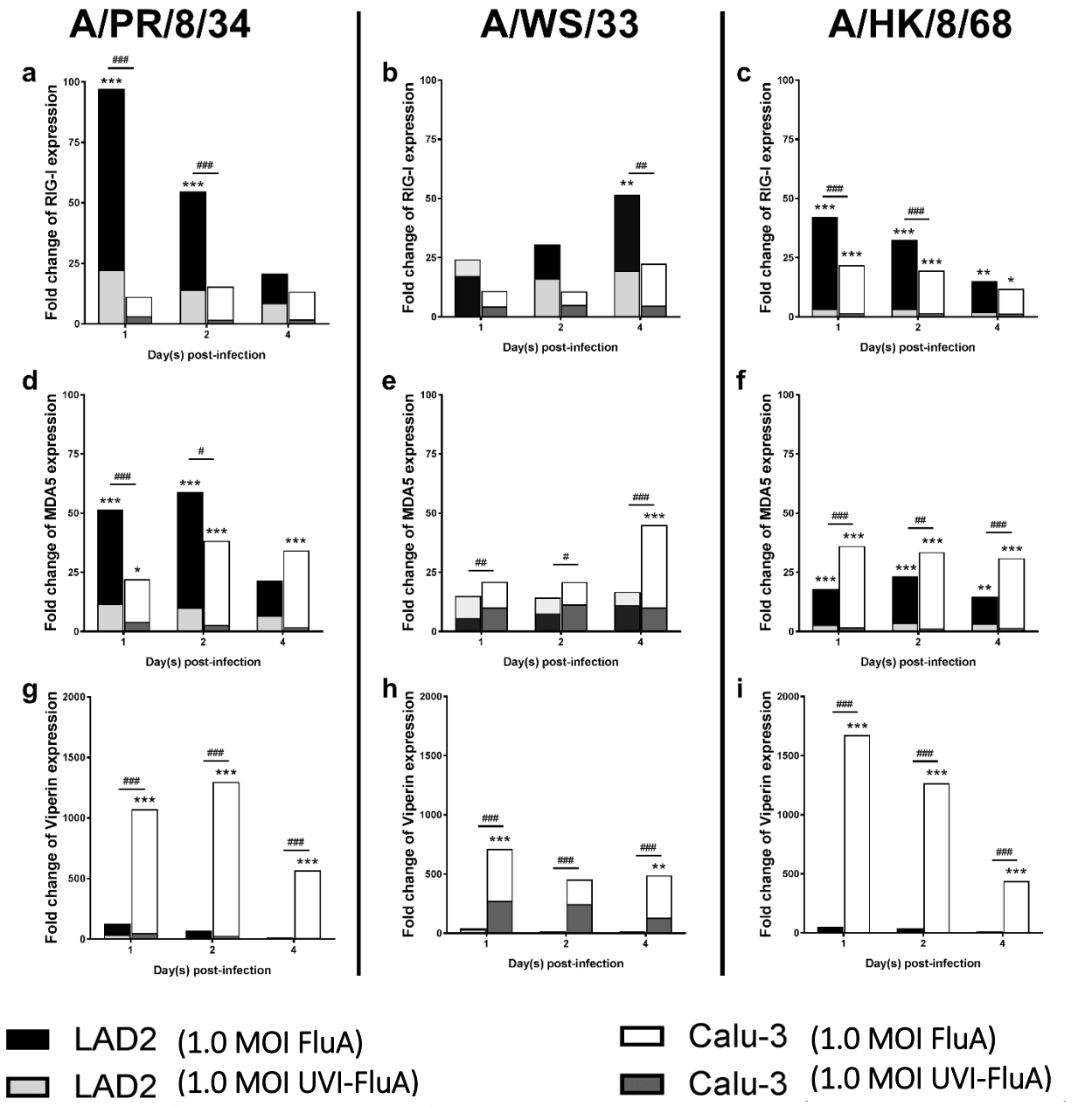

Figure 3.4 Antiviral Gene Expression in LAD2 and Calu-3 After Exposure to Three Strains of Influenza A Virus. Virus was used at 1.0 MOI. UV-inactivated virus was used as control. qPCR was conducted to determine mRNA expression levels in LAD2 and Calu-3. GAPDH was used as house-keeping gene to normalize data. All data are shown as relative fold change to naïve treatment (no viral exposure). RIG-I (a-c), MDA5 (d-f) and Viperin (g-i) were measured at 1, 2 and 4 days post infection. RIG-I mRNA was upregulated in LAD2 when exposed to the three FluA viral strains. MDA5 mRNA was upregulated only in LAD2 when exposed to either A/PR/8/34 or A/HK/8/68. Viperin mRNA was upregulated in Calu3 but not LAD2 for all three viral strains.

Two-way ANOVA with Bonferroni post-test was used for statistical analysis and represented as *p <0.05, **p <0.01 and ***p <0.001 denotes FluA vs. UVI-FluA; $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 and $^{\#\#\#}p$ <0.001 denotes MC vs. EC (n=4).

7. Tables

		Calu-3 only	LAD2 only
CCL-4	A/PR/8/34	*0 pg/mL	1010.2 ± 306.5 pg/mL (Day 2)
	A/WS/33	*0 pg/mL	178.0 ± 70.1 pg/mL (Day 1)
	A/HK/8/68	*0 pg/mL	157.8 ± 82.9 pg/mL (Day 1)
CCL-5	A/PR/8/34	1077.6 ± 143.3 pg/mL (Day 2)	298.9 ± 74.2 pg/mL (Day 4)
	A/WS/33	812.4 ± 55.2 pg/mL (Day 1)	29.1 ± 3.7 pg/mL (Day 4)
	A/HK/8/68	713.0 ± 104.2 pg/mL (Day 4)	*0 pg/mL
CXCL-10	A/PR/8/34	2481.2 ± 95.8 pg/mL (Day 2)	42.0 ± 25.5 pg/mL (Day 4)
	A/WS/33	1943.5 ± 482.2 pg/mL (Day 1)	*0 pg/mL
	A/HK/8/68	1668.0 ± 129.3 pg/mL (Day 2)	*0 pg/mL

Table 3.1 Peak Cytokine/Chemokine Release in Calu-3 and LAD2 After Influenza A Infection. Selected cytokine/chemokine release in EC and MC after exposure to three FluA strains. All values are reported as Mean ± SEM with day of peak release denoted within parentheses. Greyed boxes denote peak releases were not statistically different from respective mock controls. * Below detection limit of assay (CCL-4 and CCL-5 [15.6 pg/mL]; CXCL-10 [31.2 pg/mL]).

		Calu-3 only	LAD2 only
IFN-λ1	A/PR/8/34	2302.9 ± 117.2 pg/mL (Day 1)	135.5 ± 43.3 pg/mL (Day 2)
	A/WS/33	2446.2 ± 246.9 pg/mL (Day 1)	*0 pg/mL
	A/HK/8/68	712.2 ± 81.7 pg/mL (Day 2)	*0 pg/mL
IFN-λ2	A/PR/8/34	6593.0 ± 582.7 pg/mL (Day 2)	*0 pg/mL
	A/WS/33	7274.7 ±286.2 pg/mL (Day 1)	*0 pg/mL
	A/HK/8/68	4961.4 ± 297.7 pg/mL (Day 2)	*0 pg/mL
IFN-λ3	A/PR/8/34	2481.2 ± 95.8 pg/mL (Day 2)	40.5 ± 20.4 pg/mL (Day 2)
	A/WS/33	2567.2 ± 738.4 pg/mL (Day 2)	55.6 ± 5.3 pg/mL (Day 4)
	A/HK/8/68	1647.5 ± 517.3 pg/mL (Day 2)	58.7 ± 4.0 pg/mL (Day 2)

Table 3.2 Peak Interferon-λ Release in Calu-3 and LAD2 After Influenza A Infection. IFN-λ release in EC and MC after exposure to three FluA strains. All values are reported as Mean ± SEM with day of peak release denoted within parentheses. Greyed boxes denote peak releases were not statistically different from respective mock controls. * Below detection limit of assay (IFN-λ1 [62.5 pg/mL]; IFN-λ2 [125 pg/mL]).

		Calu-3 only	LAD2 only
RIG-I	A/PR/8/34	15.4 ± 2.1 (Day 2)	97.2 ± 5.1 (Day 1)
	A/WS/33	22.6 ± 0.8 (Day 4)	51.7 ± 18.4 (Day 4)
	A/HK/8/68	21.8 ± 3.0 (Day 1)	42.3 ± 3.6 (Day 1)
MDA5	A/PR/8/34	38.3 ± 0.9 (Day 2)	58.9 ± 11.0 (Day 2)
	A/WS/33	45.0 ± 1.4 (Day 4)	11.2 ± 2.5 (Day 4)
	A/HK/8/68	36.1 ± 2.7 (Day 1)	23.3 ± 4.0 (Day 2)
Viperin	A/PR/8/34	1299.1 ± 120.5 (Day 2)	128.2 ± 11.8 (Day 1)
	A/WS/33	711.8 ± 141.4 (Day 1)	38.5 ± 3.1 (Day 1)
	A/HK/8/68	1675.0 ± 126.5 (Day 1)	51.6 ± 9.2 (Day 1)

Table 3.3 Peak mRNA Fold Change of Selected Antiviral Gene Expression of Calu-3 and LAD2 After Influenza A Infection. Selected antiviral gene expression in EC and MC after exposure to three FluA strains. All values are reported as Mean ± SEM (Fold change relative to naïve after normalization with GAPDH expression) with day of peak expression denoted within parentheses. Greyed boxes denote peak releases were not statistically different from respective UVI controls.

	Calu-3 only	LAD2 only
Degranulation (Histamine/β-hex)	Not Applicable	Partial degranulation (strain-dependent)
Arachidonic Acid Metabolite (PGD_2)		Strain-dependent release
Cytokine/Chemokine Release	CCL-5 and CXCL-10 No CCL-3, CCL-4 or Flt-3L detected	CCL-4 (strain-dependent) No CCL-3, CCL-5, CXCL-10 or Flt-3L detected
Interferon Release	IFN-λ1, -λ2 and -λ3 No IFN-α or IFN-β detected	IFN-α (low; A/PR/8/34) No IFN-β or IFN-λ detected
Antiviral Gene Expression	Upregulation of Viperin (all strains), RIG-I and MDA-5 (strain-dependent) No MAVS upregulation detected	Upregulation of RIG-I and MDA5 (strain-dependent) No Viperin or MAVS upregulation detected

Table 3.4 Comparison Between Calu-3 and LAD2 Response to Influenza A Exposure. Summary of EC and MC response to the three FluA strains.

Chapter IV: Characterization of a Human Mast Cell-Derived Antiviral Effect on Influenza A Virus in Airway Epithelial Cells

1) Authors

Kurtis Ng, Ramanaguru Piragasam, Tae Chul Moon, Richard Fahlman, Harissios Vliagoftis, A. Dean Befus.

2) Introduction

Influenza A virus (FluA) is one of the main causes of seasonal influenza outbreaks. Despite advances in vaccination and health care, seasonal influenza causes significant morbidity and mortality. An improved understanding of host defence against FluA may aid further development of preventative and therapeutic approaches. The primary target of FluA replication is epithelial cells (EC). However, understanding the interactions of EC with immune cells may provide insights on host defenses and pathogenesis in FluA infections in humans.

Due to their close proximity to mucosal surfaces in lungs, pulmonary mast cells (MC) are in a prime location to respond to respiratory viral infections. MC respond to respiratory syncytial virus (RSV) infection and release CCL-4, CCL-5, CXCL-10 and type I interferons (IFN)[167]. MC are also permissive to rhinovirus (RV) infections and produce IFN-β, IFN-λ and interferon-stimulated genes[168,169]. Such responses to viral infections may promote immune effector cell recruitment and permit faster viral clearance. Collectively, these studies support that MC play a role in viral pathogenesis and host defenses.

MC are susceptible to FluA infection but release few infectious FluA progenies compared to EC, suggesting that there are antiviral mechanisms in MC[136]. However, mechanisms that MC use to restrict FluA replication are unknown, and whether these MC-derived antiviral mechanisms can affect surrounding cells has not been studied. There is a precedence for MC-EC interactions during viral infections as Shirato *et al*. showed that MC degranulate when exposed to RSV-infected EC, but not RSV alone[139]. This suggests that MC respond differently to viral infection in the presence of EC, and that EC-MC interactions could lead to the production and secretion of different MC mediators than MC-FluA interactions alone. By studying the interactions between MC and EC during FluA infections, we could investigate the potential actions of MC in FluA infections. Furthermore, we may discover the extent to which MC-derived antiviral mechanisms affect the primary replication host, EC. Thus, we investigated FluA production and release from EC in EC-MC co-cultures and the antiviral activity in co-culture supernatant.

3) Materials and Methods

A) Cell Culture

The human MC line, LAD2 (Laboratory of Allergic Diseases 2, generously provided by Dr. D.D. Metcalfe and A Kirshenbaum, National Institutes of Health, Bethesda, MD) was cultured as previously described[117]. The human lung adenocarcinoma EC line, Calu-3 (ATCC® number: HTB-55), was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured with minimum essential medium and Earle's salts supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies, Burlington, ON). Calu-3 cells were harvested at ~80% confluency using 0.05% Trypsin-EDTA (Life Technologies) and subcultured at a seeding concentration of 7.0 x 10^3 cells/cm^2.

B) Influenza Virus Strains and Ultraviolet Inactivation

Influenza A/PR/8/34 virus strain (H1N1) propagated from duck eggs was obtained from Dr. K. P. Kane from the University of Alberta. UV inactivation of A/PR/8/34 was performed using a ultraviolet (UV) lamp (ENF-280C, Spectroline, Westbury, NY) as previously described[136].

C) Epithelial Cell-Mast Cell Co-Culture Infection Model

Calu-3 (seeded at 5.0 × 10^5 cells/insert) were grown on Corning® Snapwell® inserts (0.4 µm pore size, 1.12 cm^2; Sigma-Aldrich Canada LTD., Oakville, ON, Canada) with culturing media (as previously described). At 4 days, LAD2 (1.0 × 10^5 cells/mL) in 2.6 mL Stempro media (as previously described) or Stempro media alone were added to the bottom chamber. Fresh media was replaced in top chamber with the EC. FluA (0.04 MOI) was added to the top chamber for 1 hour to allow for virus adsorption. Calu-3 were washed with 0.5 mL of HBSS, three times and

subsequently 0.5 mL of fresh media was placed in top chamber. After 1-5 days post-FluA exposure, media in both top and bottom chambers were harvested for further investigations.

D) Cytokine/Chemokine Measurements

Human cytokine/chemokine array 41-plex performed by Eve Technologies (Calgary, AB) was used for screening differential release of cytokines and chemokines in EC-MC co-culture versus EC only cultures (refer to Appendix C). Following pilot results, GM-CSF, Flt-3L, CCL-2, CCL-3 and CCL-4 release was measured in EC-MC co-culture supernatants 5 days post virus exposure using commercially available enzyme-linked immunosorbent assays (ELISAs) according to the supplier's protocols (R&D Systems, Minneapolis, MN). For blocking CCL-4 experiments, goat anti-CCL-4 IgG or goat IgG (20 µg/mL) control were added to FluA exposed co-cultures every 24 hours starting on day 0. Concentration of antibodies used were in excess of manufacturer's recommended dosage (1.5 - 9.0 µg/mL antibody per 5 ng/mL CCL-4). Without these antibodies, our co-cultures produced less than 1 ng/mL CCL-4 when exposed to FluA. On day 5 post-FluA infection, supernatants were harvested.

E) Assessment of Antiviral Activity of LAD2 Supernatant

LAD2 supernatant was isolated from multiple co-culture system (3-days post-FluA exposure) in bulk (up to 90 mL at one time). The LAD2 supernatant underwent centrifugation (300 g for 10 min) to remove LAD2 from suspension. LAD2-free supernatants (henceforth known as supernatant^{viral}) were routinely aliquoted (6.0 mL) and kept frozen in -80°C. Supernatant^{viral} was reintroduced into the bottom chamber of a prepared co-culture plate with Calu-3 in top chamber (grown as described in previous section). The top chamber was exposed to FluA in the same manner as described above. Bottom supernatants from an EC-MC co-culture (no FluA exposure)

and from an EC-MC co-culture exposed to UVI-FluA (0.04 MOI) were also tested for antiviral activity and were negative.

F) Quantification of Influenza A Virus Release

Quantification of total FluA particles released into the top (EC) chamber supernatant was determined by hemagglutination assay in HAU/mL. Briefly, human red blood cells (RBC) (courtesy of Airway Inflammation Team, University of Alberta) were washed with PBS three times before being stored in DGV (Lonza, Basel, Switzerland) buffer for 24 hours. Using 96-well round bottom plates, 50 µL of PBS with 0.1% of BSA was added to each well. Supernatant samples (50 µL) were added on the short-axis of the plate (8 wells). Two-fold serial dilution was performed by diluting samples with adjacent wells along the long-axis of the plate. RBC (0.05%) in PBS (50 µL) were added to each well and the plate was incubated for 2 hours at room temperature. Positive hemagglutination was determined by presence of lattice formation in a well, negative hemagglutination was identified by RBC pellet formation. The lowest dilution in which hemagglutination occurred was used to define hemagglutination units (HAU/mL) for each sample. No hemagglutination activity was detected in bottom (MC) chamber supernatant. Supernatant^{viral} did not interfere with hemagglutination assay when using known FluA concentrations.

G) Investigations of LAD2 Supernatant^{viral}

Supernatant^{viral} was subjected to various treatments to characterize the antiviral activity. Supernatant^{viral} was subjected to 65°C or 100°C for 30 minutes to test heat liability of antiviral effect. The newly heated supernatant^{viral} was used in the bottom chamber of the co-culture plate to test for antiviral activity. To test if the antiviral activity might be susceptible to a serine proteinase, supernatant^{viral} (2 mL) was treated with 100 µL of 1.0 µg/µL of trypsin (~180 TAME units/mg trypsin; US Biological, Salem, MA) for 2 hours. FBS (0.5 mL) was added to neutralize trypsin

activity for 30 minutes. We also attempted to estimate the molecular size of the antiviral activity by filtering supernatant^{viral} through 10 kDa Centricon, 30 kDa Centricon and 50 kDa Amicon centrifugal filter apparatus (EMD Millipore, Etobicoke, ON, supplier's protocol). All retentates and filtrates were collected and reconstituted to original volumes. To further characterize the antiviral activity, supernatant^{viral} was first concentrated using 10 kDa filters before fractionation using a Hi-trap SP XL cation exchange column (GE Healthcare, Chicago, Illinois) and a Hi-trap Q XL anionic exchange column. NaCl buffers (0.2 M - 1.0 M) were used at for elution purposes. All eluate and flow-through fractions were collected.

After subjecting supernatant^{viral} to the above procedures, the samples were tested in our co-culture system. The modified supernatant^{viral} was added to the bottom chamber of a pre-prepared co-culture plate with Calu-3 grown in top chamber. Stempro® media was used as negative control for antiviral activity. MC and supernatant^{viral} were used as positive controls for antiviral activity. FluA exposure was performed as described in previous section and supernatants in the top (EC) chamber were harvested 3 days post FluA exposure for determination of hemagglutination activity (viral release).

H) Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Paired *t*-test, one-way ANOVA with Dunn's Multiple Comparison post-test and two-way ANOVA with Bonferroni post-test were used for statistical analysis where appropriate and described in figure legends. Data shown are mean ± standard error of mean (SEM). A p value <0.05 was considered significant.

4) Results

A) Limited Influenza A Release in Epithelial Cells Due to Mast Cell Antiviral Activity

A co-culture system was used to investigate the possible role of MC in FluA infections of EC. EC were placed in the top chamber together with FluA, and MC in the bottom chamber. As one control, no MC were used in the bottom chamber, only EC and FluA were present in the co-culture system. Control treatment released 10069 ± 170.7 HAU/mL (n=5) of FluA in the top chamber 5 days post-FluA exposure. In the presence of MC, EC released significantly less FluA (2688 ± 298.7 HAU/mL, p <0.001) (Figure 4.1).

Time course of FluA release was investigated using the co-culture system with or without MC (Figure 4.2). There was no significant difference in FluA release between control (no MC) and MC treatment groups for day 1 and day 2 post FluA exposure. The presence of MC in the co-culture system began to significantly limit FluA release (1178 ± 203.6 HAU/mL) compared to control 4978 ± 142.2 HAU/mL, 3 days post-FluA exposure (Figure 4.2). By 5 days post-FluA exposure, 9956 ± 284.4 HAU/mL of FluA was detected in the positive control treatment (EC and FluA) and 2987 ± 426.7 HAU/mL of FluA in the MC treatment group (p <0.01).

To determine whether MC antiviral activity is soluble and stable, MC were removed from 3 days post-FluA-exposed co-culture supernatant (henceforth denoted as supernatant^{viral}) and this supernatant was re-introduced into the bottom well of a new co-culture system (no MC added) to test for antiviral activity. FluA release in the MC treatment group (1821 ± 156 HAU/mL) and in supernatant^{viral} (1707 ± 141 HAU/mL) group were comparable to each other and significantly different than compared to the positive control (6105 ± 453 HAU/mL; p <0.001) (Figure 4.3). Neither co-culture control (no FluA) nor UVI-FluA control (9813 ± 279 HAU/mL and 10027 ± 213 HAU/mL, respectively) limited FluA release when compared to the positive control (EC +

FluA; 10240 HAU/mL) (Figure 4.4). These results suggested that the antiviral activity is a soluble molecule(s) that requires a productive FluA infection of the co-culture for it to be produced.

B) Release of Selected Cytokines and Chemokines in Mast Cells and Epithelial Cells Co-culture

Cytokine and chemokine release in top and bottom chambers were measured for both treatment groups. Although GM-CSF, CCL-2 and CCL-3 release were detected, there were no significant differences in the levels of release between control and MC treatment groups (Figure 4.5, n=6). However, significantly increased Flt-3L (42.0 ± 4.1 pg/mL) release was detected in the bottom chamber of the MC treatment group compared to control group (13.4 ± 1.9 pg/mL; n=6, p <0.001). FluA also induced significantly increased CCL-4 release (493.2 ± 118.3 pg/mL) in co-culture when MC were present, whereas control only induced 170.5 ± 56.3 pg/mL.

C) CCL-4 as a Candidate for Mast Cell-derived Antiviral Activity

Given the significant increase in CCL-4 release in co-culture and evidence that CCL-4 has antiviral activities in other experimental systems[170,171], we tested if CCL-4 might be a candidate for the antiviral activity that we observed with FluA. We used anti-CCL-4 IgG antibody (20 µg/mL) in an effort to neutralize the MC-derived antiviral activity. Neither IgG control (3200 ± 334.2 HAU/mL) nor anti-CCL-4 IgG treatment (3627 ± 665.4 HAU/mL) inhibited the antiviral effect of MC (3200 ± 334.2 HAU/mL) when compared to control (10098 ± 142.2 HAU/mL) (Figure 4.6).

D) Strategies to Define the Molecular Characteristics of Mast Cell-derived Antiviral Activity

i) Reduction of Protein Content in Culturing Media

To facilitate the identification of possible protein candidates for the MC antiviral effect using mass spectrometry, we tested ways to reduce the protein complexity in the culture media used and yet still retain the ability of the MC to produce the antiviral activity. Thus, we tested if FBS in the EC culturing media, a source of increased protein complexity, was essential for MC to produce the antiviral activity. The MC-derived antiviral activity was comparable between FBS (1440 ± 78.1 HAU/mL) and no FBS (1333 ± 53.3 HAU/mL) treatment which was significantly less (p <0.001) when compared to control (no MC, 5120 HAU/mL) (Figure 4.7). This suggested that FBS can be removed from the upper chamber in the co-culture without affecting the production of MC-derived antiviral activity.

Another possible source of potentially unnecessary protein complexity in our system was in hemi-depletion of MC culturing media. MC were cultured in media composing of 1:1 ratio (hemi-depletion) of fresh Stempro-34 media and MC-experienced media. By removing MC-experienced media, we could potentially remove another source of proteins from our system without affecting MC-derived antiviral activity. Removal of FBS and using only fresh MC media (no hemi-depletion) in our co-cultures still allowed for the production of antiviral activity (limited FluA release, 1653 ± 204.6 HAU/mL of FluA, which was significantly [p <0.01] lower than positive control treatment [5120 HAU/mL]) (Figure 4.7). This allowed us to remove FBS and hemi-depleted MC media (using only fresh media) without affecting MC-derived antiviral activity, thereby reducing protein complexity in our supernatant^{viral}.

ii) Stability of Antiviral Activity in Supernatant^{viral}

In attempts to standardize multiple experiments over long time periods, storage stability of antiviral effects of MC supernatant was investigated. Both short-term 4°C and long-term -80°C storage did not affect antiviral activity (3072 ± 341.3 HAU/mL and 3584 ± 418 HAU/mL respectively) when compared with FluA release in the fresh EC-MC co-culture treatment group (2560 HAU/mL; $p < 0.001$ and $p < 0.01$ respectively; Figure 4.8).

iii) Effects of Heat Treatment and Exposure to Serine Proteinase on Supernatant^{viral} Antiviral Activity

After determining that the antiviral activity was soluble, various treatments were used to characterize the antiviral activity. Heat treated (65°C) supernatant^{viral} had no effect on the antiviral activity (FluA release, 1707 ± 196 HAU/mL) compared to positive control (6400 ± 838 HAU/mL) (Figure 4.9), whereas 100°C heat treated supernatant^{viral} lost the antiviral activity (5440 ± 702 HAU/mL). Thus, the antiviral activity was sensitive to heat treatment of 100°C, but not 65°C for 30 minutes. MC supernatant^{viral} treated with trypsin lost the antiviral activity (FluA release, 4693 ± 427 HAU/mL) compared to control treatment (5120 HAU/mL) (Figure 4.10). The release seen in the trypsin treatment was statistically ($p < 0.05$) greater than the supernatant^{viral} + FBS control group (1493 ± 213 HAU/mL). Collectively, these results suggest that the antiviral activity is propagated through a protein component, sensitive to both heat treatment and serine protease digestion.

iv) Size Fractionation of Supernatant^{viral} Antiviral Activity

Supernatant^{viral} was fractionated using centrifugal filters to estimate the range of size of the antiviral activity. Antiviral activity was not retained in the 10 kDa filtrate (FluA release, 4800 ± 224 HAU/mL, comparable to positive control, 5120 HAU/mL) (Figure 4.11). By contrast, 10 kDa retentate had antiviral activity, limiting FluA release to 1333 ± 53 HAU/mL, suggesting that the antiviral activity had a molecular size greater than or equal to 10 kDa. The 30 kDa retentate contained significant antiviral activity with 1600 ± 1256.1 HAU/mL of FluA release, compared to 5120 HAU/mL in the positive control treatment (Figure 4.12). Similarly, the 30 kDa filtrate also significantly limited FluA release to 1547 ± 78.1 HAU/mL. When using 50 kDa filters, only the 50 kDa filtrate contained significant antiviral activity, where only 1351 ± 71 HAU/mL of FluA was detected when compared to positive control treatment of 5120 HAU/mL (Figure 4.13). When using successive size fractionation, antiviral activity was detected within the 10 kDa – 50 kDa range (Figure 4.11 and 4.13).

v) Ion Exchange Chromatography Fractionation of the Antiviral Activity

After size filtration of supernatant^{viral}, the active fraction was passed through a cationic exchange column. The flow-through lacked antiviral activity as did the 0.4 M salt eluate (Figure 4.14). However, using 1.0 M salt buffer, the eluate contained antiviral activity (FluA release, 2480 ± 622 HAU/mL compared to control, 7893 ± 850 HAU/mL; p <0.05). Further fractionation was performed using anionic exchange columns, although this study was only done twice (no statistical analysis done). FluA release was found to be limited to 4096 ± 418 HAU/mL in the flow through and 0.2 M salt eluate (Figure 4.15); 0.4 M and 0.6 M salt eluate had an intermediate FluA release (7424 ± 969.2 HAU/mL and 8192 ± 836.1 HAU/mL). However, 0.8 M and 1.0 M salt eluate did

not have antiviral activity and FluA release detected was the same as the positive control treatment (10240 HAU/mL). Our results show that the antiviral protein(s) is 10-50 kDa with a strong positive charge and weak negative charge.

5) Discussion

Previously, our lab established that MC have an antiviral effect that restricts FluA release from MC[136]. Here, we have shown that MC also have an antiviral effect on FluA infection of EC (Figure 4.1). This helps substantiate that MC could play a beneficial role in viral infections by suppressing viral replication in host cells. However, the mechanism(s) underlying this antiviral effect was unknown.

It has been well-established that MC respond to FluA infections[131,135,136]. However, how MC affects FluA infections is unclear. In this study, we used human LAD2 and Calu-3 to represent MC and EC respectively. In a co-culture system, we enabled the growth of both Calu-3 and LAD2 in the same system, albeit with limited to no surface-to-surface contact. We were thus able to investigate the effects of MC on FluA replication and release in EC (refer to Table 4.1). We showed that FluA release was limited when MC were co-cultured with EC, when compared to EC alone. We also determined that MC selectively released Flt-3L and CCL-4 in co-culture when exposed to FluA. However, no study to our knowledge has shown that Flt-3L has antiviral activity, whereas CCL-4 was shown to suppress human immunodeficiency virus and herpes simplex virus[170,171]. When targeting CCL-4 for antiviral activity, anti-CCL4 antibody did not neutralize antiviral effect (Figure 4.6). However, we did not test the neutralization efficacy of the antibodies, but based our experiments on the supplier's recommended dosage of the antibody.

Taking a step back from this known candidate approach, we selected to begin to characterize the antiviral activity with various "fractionation strategies". When supernatant^{viral} (MC removed), from the co-culture system (3 days post-FluA infection), was introduced into a new co-culture system and EC were exposed to FluA, FluA release was still limited as with MC in co-culture (Figure 4.3). This supported our postulate that MC-derived antiviral activity involves

a soluble molecule(s) and does not require direct EC-MC contact for viral suppression in EC. Furthermore, we established that an active FluA infection of EC was necessary to elicit an antiviral response from MC as UVI-FluA in EC-MC co-culture could not (Figure 4.4).

Our studies to characterize the antiviral activity in supernatant^{viral} showed that the antiviral activity was sensitive to heat denaturation at 100°C (Figure 4.9). This most likely is due to mammalian proteins being sensitive to temperatures of ≥ 80°C, where irreversible misfolding and protein aggregation occurs, resulting in the loss of protein activity and complexes[172-174]. The antiviral activity in supernatant^{viral} was trypsin sensitive (Figure 4.10), collectively suggesting that the antiviral activity is dependent on a protein component. Size fractionation of supernatant^{viral} shows that antiviral activity was in the 10 kDa retentate and in the 50kDa filtrate (Figure 4.11 and 4.13). Cationic and anionic exchange columns showed that the antiviral activity had a relatively strong positive charge and a weak negative charge (Figure 4.14). These fractionation approaches collectively allowed successive fractionation to isolate and reduce protein content in supernatant^{viral}. Successive fractionation (10 kDa size fractionation → cationic exchange → anionic exchange) reduced the protein complexity in supernatant^{viral}, so that the identity of the antiviral moiety might be more easily established using protein mass spectrometry. However, our attempts with protein mass spectrometry did not elucidate possible protein candidates for the antiviral activity due to strong signals for keratins, normally thought to be contaminants in protein mass spectrometry. However, keratins are biomarkers of EC and interestingly, EC infected with FluA have enhanced keratin expression, possibly due to a host cells stress response[175-178]. However, the exact function of keratin(s) in EC responses to FluA infection, whether it is involved in the antiviral activity or not, will require further studies.

Our study provides new, important evidence about MC involvement during FluA infections. Previous studies focused on MC susceptibility to FluA infections, whereas our studies show that MC play a role in suppressing FluA infection of EC. Shirato *et al.* showed that RSV-infected EC induced MC degranulation, suggesting EC-MC interactions in the pathogenesis of pulmonary viruses. Graham *et al.* showed that MC were critical for local inflammation during FluA infection of mice, whereas MC-deficient mice had decreased inflammatory cytokine and chemokine release[131]. MC-deficient mice were also shown to have reduced lung damage suggesting a possible deleterious role that MC may play in FluA infections[131]. However, MC play a protective role in vaccinia virus infection by releasing cathelicidin antimicrobial peptides[179]. The varied MC responses to viral infections show the versatility of MC, whether as a secretory cell, an inflammatory effector cell or a protective, antiviral cell. Thus, MC are an important target for further study and to characterize their roles in various viral and other diseases.

Our study on MC interactions with EC during FluA infection clearly indicates a MC role in limiting FluA spread in EC. However, when MC are beneficial and when MC are harmful during viral infection remains unclear. Further studies will enhance our understanding of the role of MC in host defenses and viral pathogenesis.

6) Figures

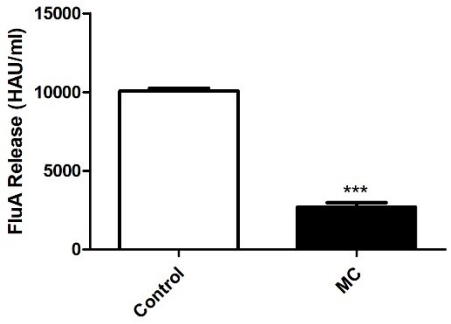

Figure 4.1 Mast Cells Limit Influenza A Release in Epithelial Cells. EC were seeded onto membranes of co-culture inserts and grown for 3 days. Control (white) was an EC only treatment group exposed to FluA (0.04 MOI) with the bottom chamber containing complete Stempro-34 media. MC (black) was a treatment group with EC in top chamber exposed to FluA (0.04 MOI) and MC in bottom chamber. FluA release measured 5 days post infection with hemagglutination assay. Paired *t*-test was used for statistical analysis (n=5; *** p <0.001).

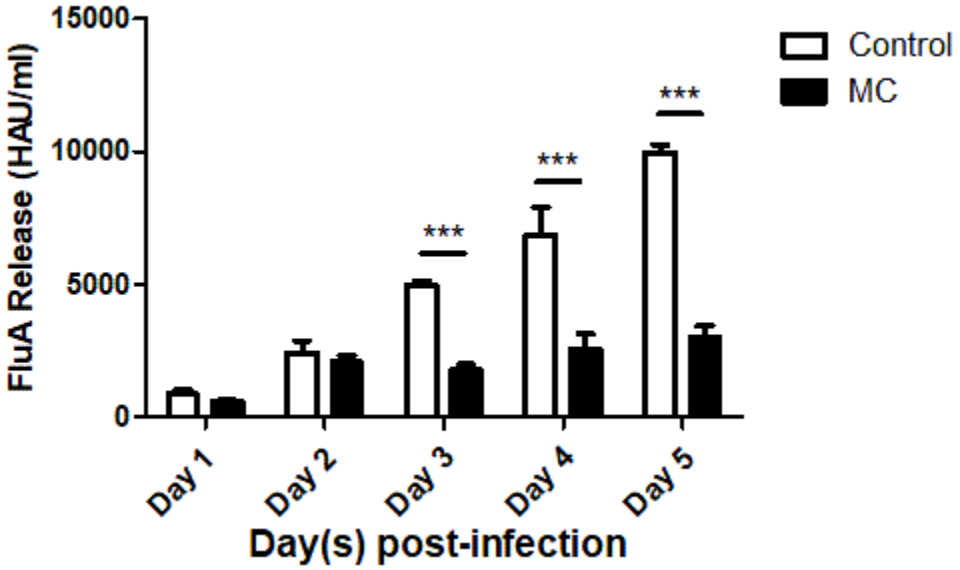

Figure 4.2 Time Course of Influenza A Release in Co-cultures. EC were seeded onto membranes of co-culture inserts and grown for 3 days. Control (white) was an EC only treatment group exposed to FluA (0.04 MOI) with bottom chamber containing complete Stempro-34 media. MC (black) was a treatment group with EC in top chamber and MC in bottom chamber exposed

to FluA (0.04 MOI) up to 5 days. Two-way ANOVA with Bonferroni post-test was used for statistical analysis (n=4; *** p <0.001).

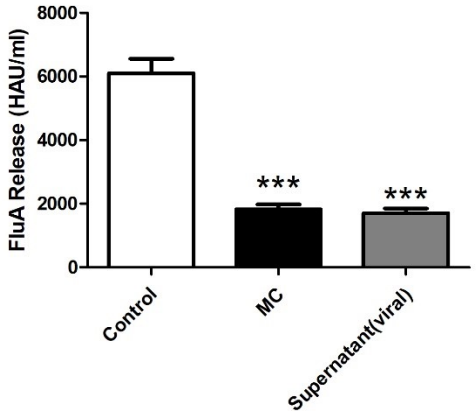

Figure 4.3 Supernatant^{viral} Retains Antiviral Effect Against Influenza A Virus Release in Epithelial Cells. MC supernatant^{viral} was prepared from 3 days post-infection bottom media in a co-culture system with EC, MC and FluA. EC were exposed to FluA in top chamber with MC media (white), MC (black) or MC supernatant^{viral} (grey) in bottom chamber. FluA (0.04 MOI) adsorption for 1 hour before removal with HBSS and media replenished. FluA release in EC was limited in the presence of MC and MC supernatant^{viral} compared to EC only (control), 3 days post-infection. One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=13; *** p <0.001 vs. control).

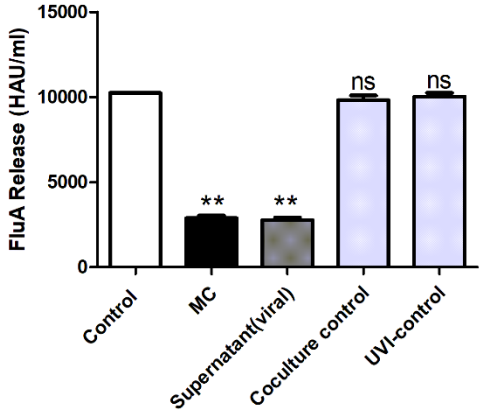

Figure 4.4 Mast Cell Antiviral Effect Requires Active Influenza A Infection. EC-MC co-cultures were exposed to PBS (co-culture control) or UVI-FluA (UVI-control) for 3 days. Bottom

supernatant from both co-culture controls were collected and tested for antiviral effect against FluA release in EC. No antiviral effect was seen in co-culture control and UVI-control. Co-culture control and UVI-control One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=4; ** p <0.01, ns p >0.05 vs. control).

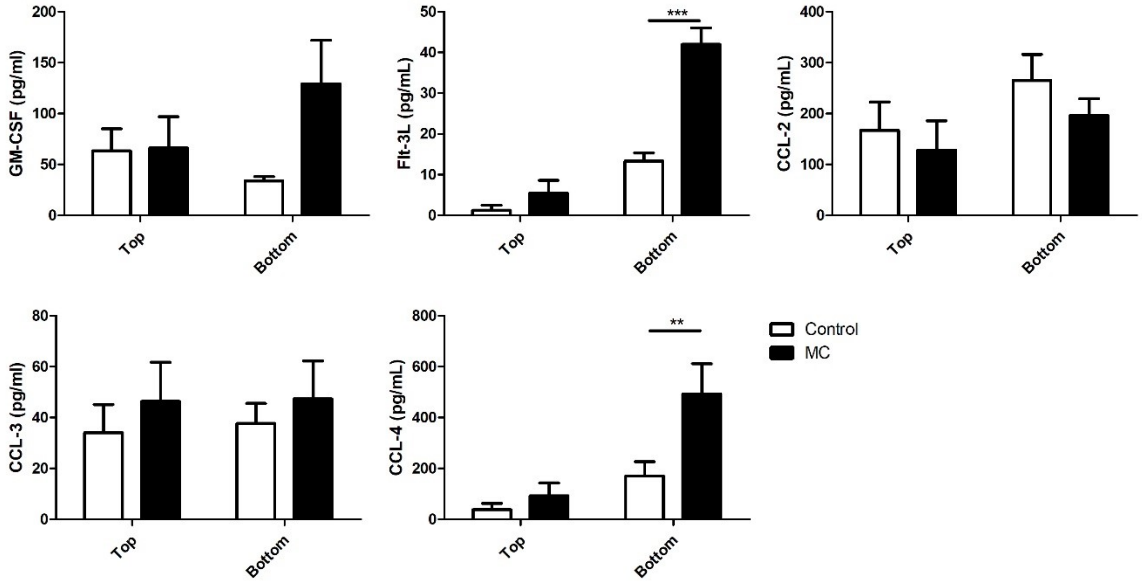

Figure 4.5 Selected Cytokine and Chemokine Release in Co-culture. Control (white) was an EC only treatment group exposed to FluA (0.04 MOI). MC (black) was a treatment group with EC in top chamber exposed to FluA (0.04 MOI) and MC in bottom chamber. Cytokine and Chemokine release in co-cultures was measured 5 days after FluA exposure. Two-way ANOVA with Bonferroni post-test was used for statistical analysis (n=6; ** p <0.01, *** p <0.001).

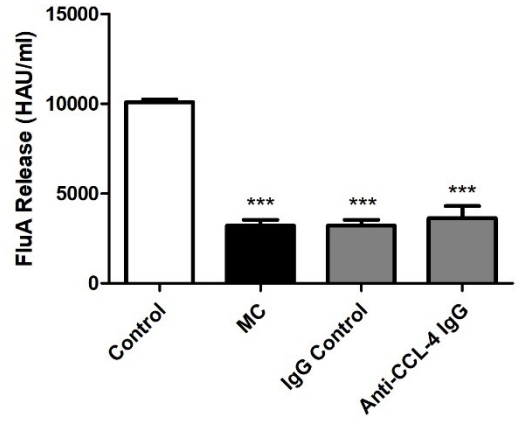

Figure 4.6 Blocking CCL-4 Did Not Affect Influenza A Release. EC were seeded onto membranes of co-culture inserts and grown for 3 days. Control (white) was an EC only treatment group exposed to FluA (0.04 MOI) with bottom chamber containing complete Stempro-34 media. MC (black) was a treatment group with EC in top chamber and MC in bottom chamber exposed to FluA (0.04 MOI). IgG control and anti-CCL-4 IgG were treatment groups with EC in top chamber and MC in bottom chamber exposed to FluA (0.04 MOI) with antibodies (20 µg/mL) added every 24 hours. Hemagglutination assay was performed 5 days post-FluA exposure. One-way ANOVA with Dunn's Multiple Comparison post-test was performed for statistical analysis (n=4; *** p <0.001).

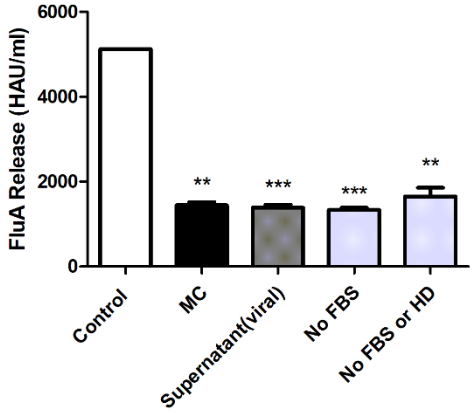

Figure 4.7 Effects of Reducing Protein Content in Media on Antiviral Effect of Supernatant^{viral}. When producing MC supernatant^{viral}, FBS were removed from EC media (green) to reduce source of confounding proteins that may affect identification of protein in mass spectrometry. No FBS (green) or hemi-depleted MC media treatment group (HD, blue) denotes the removal of FBS from EC media and the removal of hemi-depletion of MC media (by using only fresh MC media). Control (white) was EC only treatment group with Stempro-34 in bottom chamber. MC (black) was EC-MC co-culture treatment group. One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=4; *** p <0.001, ** p <0.01 vs. control).

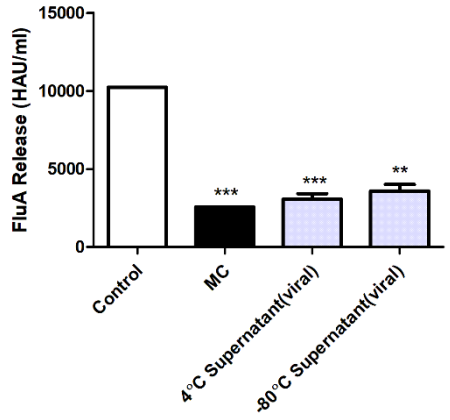

Figure 4.8 Storage Stability of Antiviral Activity in Supernatant^{viral} After Refrigeration and Freezing. MC supernatant harvested from post-FluA exposed co-cultures were stored for 48 hours in 4°C or 2-4 month in -80°C. Both treatment groups were warmed back to 37°C and used in the bottom chamber of the co-culture system. Control (white) was EC only treatment group with Stempro-34 in bottom chamber. MC (black) was EC-MC co-culture treatment group. One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=5; *** p <0.001, ** p <0.01 vs. control).

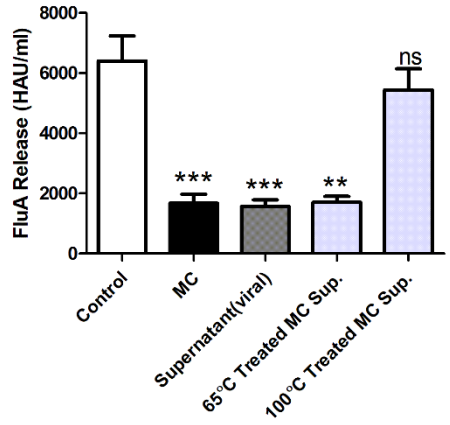

Figure 4.9 Mast Cell Supernatant^{viral} Antiviral Effect Was Sensitive to 100°C Heat Treatment. MC supernatant^{viral} was heated to 65°C and 100°C for 30 minutes before being used in the bottom chamber with EC in the top chamber of the co-culture system. Antiviral effect of MC supernatant^{viral} was not affected by 65°C heat treatment. Antiviral effect of MC supernatant^{viral} was lost when heat treated for 30 minutes at 100°C. One-way ANOVA with Dunn's Multiple

Comparison post-test was used for statistical analysis (n=4; *** p <0.001, ** p <0.01, ns p >0.05 vs. control).

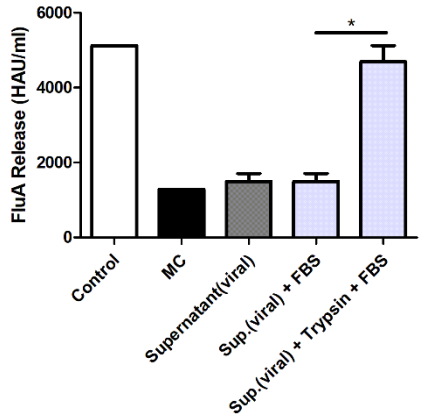

Figure 4.10 Mast Cell Supernatant^{viral} Loses Antiviral Effect After Trypsin Digestion. MC supernatant^{viral} was treated with trypsin for 2 hours prior to co-culture. FBS was used for trypsin neutralization and as a control. MC supernatant^{viral} was sensitive to serine protease digestion. One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=3; * p <0.05).

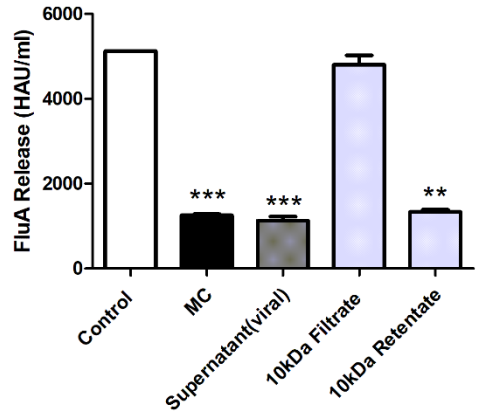

Figure 4.11 10 kDa Retentate Retains Antiviral Activity from Supernatant^{viral}. MC supernatant^{viral} was fractionated using a 10 kDa Centricon filter apparatus. Both filtrate and retentates were reconstituted back to original volumes before used in co-culture system. One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=6; *** p <0.001, ** p <0.01 vs. control).

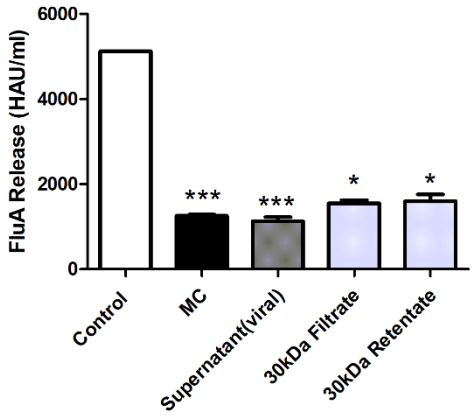

Figure 4.12 Antiviral Effect Was Retained in Both Filtrate and Retentate After 30 kDa Filtration. Supernatant^{viral} harvested from post-FluA exposed co-cultures were fractionated using a 30 kDa filter apparatus. Filtrate and retentate were reconstituted to original volumes and used in the bottom chamber of a co-culture system. Both filtrate and retentate showed antiviral activity. One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=4; *** p <0.001, * p <0.05 vs. control).

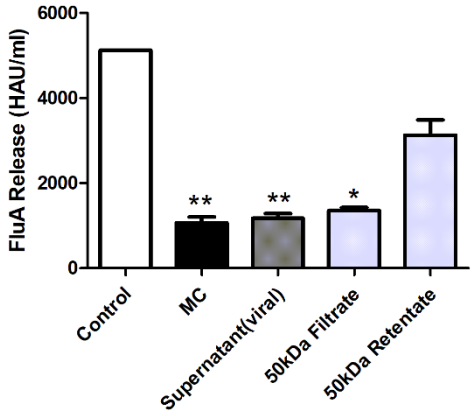

Figure 4.13 Antiviral Effect Was in the Filtrate After 50 kDa Filtration. MC supernatant^{viral} harvested from post-FluA exposed co-cultures were fractionated using a 50 kDa filter apparatus. Filtrate and retentate were reconstituted to original volumes and used in the bottom chamber of a co-culture system. Only the 50 kDa filtrate contained antiviral activity against FluA release in EC. One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=4; ** p <0.01, * p <0.05 vs. control).

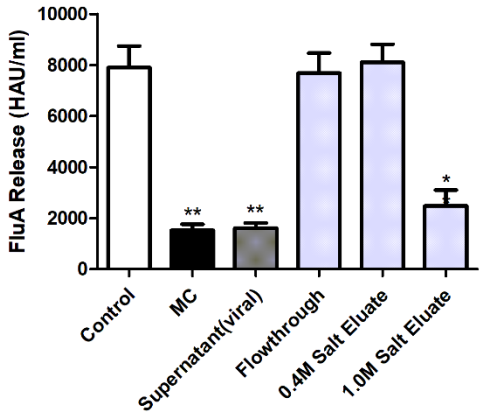

Figure 4.14 Mast Cell Supernatant^{viral} Antiviral Activity Had Relatively Strong Positive Charge. MC supernatant^{viral} were concentrated using 10 kDa filters before fractionated using cation exchange column. Flow through and 0.4 M salt eluate did not contain antiviral activity in co-culture. 1.0 M salt eluate retained antiviral activity against FluA in EC. One-way ANOVA with Dunn's Multiple Comparison post-test was used for statistical analysis (n=4; ** p <0.01, * p <0.05 vs. control).

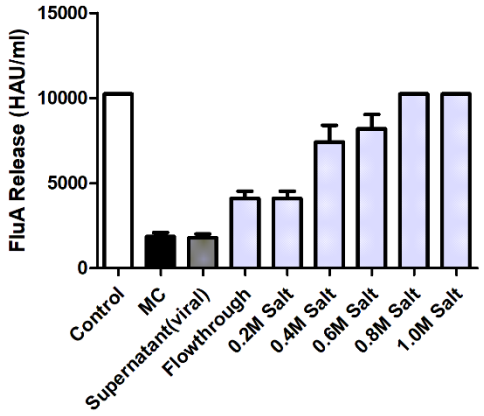

Figure 4.15 Antiviral Effect Was Retained in the Flow Through and Low Salt Fractions After Anionic Exchange Column. MC supernatant^{viral} was fractionated using an anionic exchange column. Increasing salt concentrations were used to elute different fractions from anionic exchange column. Flow through and 0.2 M salt eluate fractions showed possible antiviral activity in co-culture (n=2).

7. Tables

	EC-MC Co-culture
FluA Release	Low (Relative to EC only)
Cytokine/Chemokine Release	Increased Release of Flt-3L and CCL-4
Antiviral Mediator Properties:	
Heat Lability	Not Sensitive to 65°C Treatment
	Sensitive to 100°C Treatment
Protease Digestion	Sensitive to Trypsin Digestion
Size Filtration	Retained Between 10 kDa and 50 kDa Size Fractions
Ionic Exchange Column	Contains High Positive Charge (1.0 M Eluate) Contains Weak Negative Charge (0.2 M Eluate)

Table 4.1 Overview of Antiviral Activity Found in Epithelial Cell-Mast Cell Co-culture.
Characterization summary of antiviral activity found in supernatant[viral].

Chapter V: General Discussion and Conclusions

1) Summary of Findings

Treatment of MC with different strains of FluA elicits different mediator and antiviral gene response, showing that the host response to FluA differs between strains (refer to Table 3.4). We found that MC degranulated (histamine and β-hex) and released arachidonic acid metabolite (PGD_2) in a FluA strain-dependent manner. We also found that MC released CCL-4 (strain-dependent) but could not detect release of CCL-3, CCL-5, CXCL-10 or Flt-3L. EC release CCL-5 and CXCL-10 but not CCL-3, CCL-4 or Ftl-3L. We did detect low release of IFN-α in MC but not IFN-β or IFN-λ. Calu-3 did release IFN-λ (1, 2 and 3) but not IFN-α or IFN-β. MC upregulated RIG-I and MDA5 in a FluA strain-dependent manner but not Viperin or MAVS. EC upregulated RIG-I (strain-dependent), MDA5 (strain-dependent) and Viperin (all three strains)

With the knowledge that MC respond to FluA infection through the release of soluble mediators and upregulation of innate antiviral genes, we studied responses of EC and MC in co-culture against FluA infection (refer to Table 4.1). EC-MC co-culture has less FluA release when compared to EC only cultures. We found increased release of Flt-3L and CCL-4 in EC-MC co-cultures after FluA infection. We determined that the antiviral mediator(s) in supernatant^{viral} was sensitive to heat (100°C) and protease digestion. The antiviral mediator(s) in supernatant^{viral} was between 10-50 kDa and contained a high positive charge with weak negative charge. We attempted to identify the antiviral activity through mass spectrometry using a successive fractionation approach: 10 kDa retentate → strong cationic eluate → weak anionic eluate; to enrich mediator(s) in supernatant^{viral}. Our preliminary results from mass spectrometry detected significant amounts of keratins. Whether these are potential antiviral candidates, or merely methodological contamination requires further investigation.

2) Conceptual Model

Pulmonary MC are abundant beneath airway EC, allowing it to detect and respond to pathogens or other insults to the epithelium. When FluA infects EC, viral replication occurs within the cell and new viral particles are released after assembly (Figure 5.1). EC will also release various cytokines and chemokines to signal surrounding cells. MC will be exposed to FluA along with the EC cytokines and chemokines. FluA infects MC, but MC have antiviral responses that suppress viral infection, limiting the release of viral progenies from MC. MC respond to viral infection and the EC derived cytokines and chemokines by developing their own responses and releasing selected mediators. These mediators will contribute to host innate immune response and some likely act upon FluA viral replication in EC, limiting FluA release and its spread in EC layer.

3) Results and Their Relevance to Initial Objectives

1. To characterize FluA-induced mediator secretion and antiviral gene expression in MC and EC.

Since FluA can infect MC, we investigated MC response to different strains of FluA[136]. The MC response during FluA infection was strain specific. A/HK/8/68 induced release of both β-hex and histamine in MC after FluA infection, but A/PR/8/34 and A/WS/33 did not. Both A/WS/33 and A/HK/8/68 induced the release of arachidonic acid metabolite, PGD_2, whilst A/PR/8/34 did not. FluA exposure induced CCL-4 release from MC but not from Calu-3. Calu-3 released CCL-5, CXCL-10, IFN-λ1, IFN-λ2 and IFN-λ3. We also looked at release of CCL-3, GM-CSF, Flt-3L, IFN-β and IFN-γ but did not detect any reproducible release from either MC or EC. After consideration of existing literature and our pilot studies, we investigated four anti-viral genes and found that MC upregulated mRNA expression of RIG-I and MDA5 but not MAVS or Viperin after FluA infection. In EC, Viperin, MDA5 and RIG-I mRNA were upregulated but not MAVS after FluA infection. The responses of MC and EC differed depending on FluA strain used for infection, which highlights the complexity of FluA infections, host responses and pathogenesis in humans.

2. To compare cytokine and chemokine release in EC-MC FluA co-culture systems.

We found that EC-MC FluA co-culture systems limits FluA release in EC. To investigate the mechanism(s) underlying this observation, we first focused on examining cytokine and chemokine release in the co-culture system. We hypothesized that the antiviral activity was caused by mediator derived from MC. To this end, we investigated cytokines and chemokines that showed enhanced release in co-cultures compared to cultures with EC alone. We used a 41 cytokine/chemokine multiplex assay service from Eve Technologies and found elevated levels of

GM-CSF, Flt-3L, CCL-2, CCL-3 and CCL-4 release after FluA exposure in co-cultures. However, only Flt-3L and CCL-4 were consistently detected by ELISA in co-cultures after FluA exposure (Figure 5.1). When investigating whether CCL-4 was involved with limited FluA release in EC, we found that blocking CCL-4 with antibodies did not affect FluA release. This indicates that the antiviral activity seen in the co-culture system was not dependant on CCL-4. However, it will be necessary in future experiments to validate that the anti-CCL-4 antibodies used neutralize the effects of CCL-4 in a positive control system.

3. To characterize the antiviral activity in EC-MC co-cultures.

Given our goal to identify the molecule(s) responsible for the antiviral activity, and our limited success in using a candidate approach, e.g. CCL-4., we decided to use an unknown candidate approach to characterize the antiviral activity. We found that the supernatant^{viral} from FluA-exposed EC-MC co-cultures retained antiviral activity after MC removal, indicating that a soluble mediator was involved with antiviral activity. We employed high heat treatment and trypsin exposure and developed evidence that the antiviral activity was dependent on a protein.

After determining that a protein was involved, we decided to identify candidate proteins within the supernatant^{viral} through using mass spectrometry. However, we had to reduce and remove possible confounding proteins from our co-cultures. First, we removed FBS from Calu-3 media and hemi-depletion of LAD2 media from our co-cultures, to reduce protein content within media composition. We found that removal of these two sources of proteins did not affect the antiviral activity in the subsequently made supernatant^{viral} (Figure 5.4). Size fractionation approaches were used to estimate the molecular size of the antiviral activity. We found that the

antiviral activity has a molecular size between 10 – 50 kDa. However, despite attempts to fractionate the supernatant^{viral} using a 30 kDa filter, we were unable to isolate the antiviral activity into one fraction. This may suggest that the protein is involved in a complex that may result in different molecular sizes seen in our experiments. This complicates the isolation of the antiviral activity in supernatant^{viral}, leading us to remove the smaller fraction (less than 10 kDa) to ensure that full antiviral activity was retained in the supernatant^{viral}. We then used ionic exchange columns to fractionate and assess relative molecular charge of the antiviral activity. Using cationic exchange columns, we established that the antiviral activity has relatively strong positive charge (1.0 M salt eluate). Using anionic exchange column, the antiviral activity was retained in the low negative charge eluate (0.2 M salt). All of these processes helped identify several characteristics of the antiviral activity, and collectively we used methods to process our supernatant^{viral}. We then attempted to identify different protein candidates in our processed supernatant^{viral}. Our first few attempts were unsuccessful in determining possible candidates due to strong readings of keratin in our samples, which may be a result of either methodological contamination or EC keratin as a host cell stress response due to FluA[175-178].

4) Significance of Findings

We demonstrated that MC response to FluA infection is virus strain-dependent. The differences in mediator release between strains indicate that the strain-dependent dynamics of host responses and pathogenesis of FluA infections warrants further investigation about the underlying causes. By understanding the molecular bases of such differences, it may be possible to develop targeted therapeutic approaches against FluA outbreaks, especially for highly virulent strains. As part of our investigation in MC response to FluA infection, we found that MC upregulates RIG-I and MDA5 mRNA suggesting that they are important in viral detection of FluA. However, it is important to perform further studies to investigate the roles of MDA5 and RIG-I, such as using siRNA knockdowns to determine whether they are involved with the ability of MC to limit viral progenies in MC.

Our findings on MC antiviral activity the limits FluA release in EC demonstrates that MC can suppress viral infections in EC. Further studies are necessary to investigate whether similar interactions occur in different viral infections and whether those interactions lead to beneficial outcomes. It would also be interesting to examine whether MC affects EC expression of various antiviral genes in EC. Identifying the mechanism in EC will help elucidate the process in which MC affects EC antiviral responses. Whether other cells also interact with EC to limit viral replication and production is likely, and whether these interactions use mechanisms similar to EC-MC interactions remains to be studied. Such new knowledge could lead to the development of new drug targets for antiviral treatments against FluA and other viruses.

Our investigation on characteristics of the antiviral activity in supernatant^{viral} also determined that the antiviral mediators are stable in frozen storage. This may lead to development of new vaccination strategies. Current FluA vaccination uses either inactivated or attenuated

viruses. Use of the antiviral mediators as an adjuvant during vaccination with attenuated viruses may prove to be beneficial by further enhancing the beneficial effects of the vaccine. This might also reduce the risk of reversion to virulence, increasing the safety of such vaccine. This could be developed to affect not just FluA vaccines, but also other viral vaccines.

5) Future Directions

We have shown that MC upregulates RIG-1 and MDA5 mRNA expression as part of their antiviral response. These MC responses varied depending on the FluA strain used. Our investigation on antiviral gene expression also examined mRNA expression of Viperin and MAVS, both of which were not significantly affected by FluA exposure in MC. MAVS is an adaptor protein involved in RIG-I and MDA5 signalling pathways. These results suggest that MC upregulation of RIG-I and MDA5 may involve a MAVS-independent pathway, a postulate that warrants further investigation. Viperin is an antiviral protein that disrupts lipid raft formation during FluA infection, which in turn will inhibit the release of viral progeny. Given that our previous study showed that in the MC FluA begins to replicate and produces viral proteins, we postulated that Viperin may be a possible antiviral gene associated with few viral progenies being released in MC. Instead we found that Viperin was not upregulated in MC, suggesting that MC uses an alternative antiviral mechanism to limit FluA release. This also warrants further investigation.

Our co-culture experiments showed that EC-MC co-cultures had limited FluA release when compared to EC alone. However, we did not investigate how MC detect FluA infection of EC. We postulate that MC virus recognition receptors, such as TLR 3 and RIG-I, will not play a major role in detecting FluA infection in co-culture. This is due to previous study showing that FluA was not detectable in the bottom chamber (MC)[137]. Hence, it is likely that FluA-infected EC released mediators that were responsible for signalling MC of active viral infection. Therefore, it would be of interest to determine how EC-FluA activated MC response.

We determined that the MC antiviral activity was dependent on a protein component. Our attempt to isolate and identify said protein found strong keratin readings. It is unknown as whether

the keratin originated from methodological contamination or from the co-culture system. It was shown that EC infected with FluA has been shown to upregulate keratin[175-178]. However, it is unknown how the keratin can be found in the supernatant if the source is epithelial cells. There was a recent study showed macrophages released extracellular exosome containing various proteins, including keratin, after FluA exposure[180]. Furthermore, EC was also found to release extracellular vesicle during FluA infection with antiviral activity[181,182]. However, though this may explain keratin presence in co-culture, it does not elucidate the identity of MC mediator with antiviral activity. Hence, it would be pertinent to remove extracellular vesicles from supernatant^{viral} through ultracentrifugation, thus cleaning up the supernatant for subsequent mass spectrometry studies. If a potential antiviral candidate(s) is identified, it would be necessary to confirm and establish that the antiviral activity was derived from the candidate(s). First, investigating whether exogenous addition of the candidate(s) affect FluA release from EC after infection would support antiviral activity of the candidate(s). Use of inhibitory antibodies against the candidate(s) in EC-MC co-cultures would show that the MC-derived antiviral activity was dependent on the candidate. Finally, using short-interfering RNA to prevent the translation of the candidate(s) mRNA, would further support the MC-derived antiviral activity of the candidate. Ultimately, these procedures would help determine whether the candidate(s) has antiviral activity and that in EC-MC co-cultures, the antiviral activity was dependent on said candidate(s).

In addition to mediator interaction between EC and MC during FluA infection, it would be valuable to test whether MC exert antiviral effects on EC through physical interactions. *In vivo*, MC and EC can be in direct contact and have binding interactions. It may be beneficial to determine the importance of MC adherence to EC during FluA infection and whether different antiviral pathways are affected by such interaction.

6) Figures

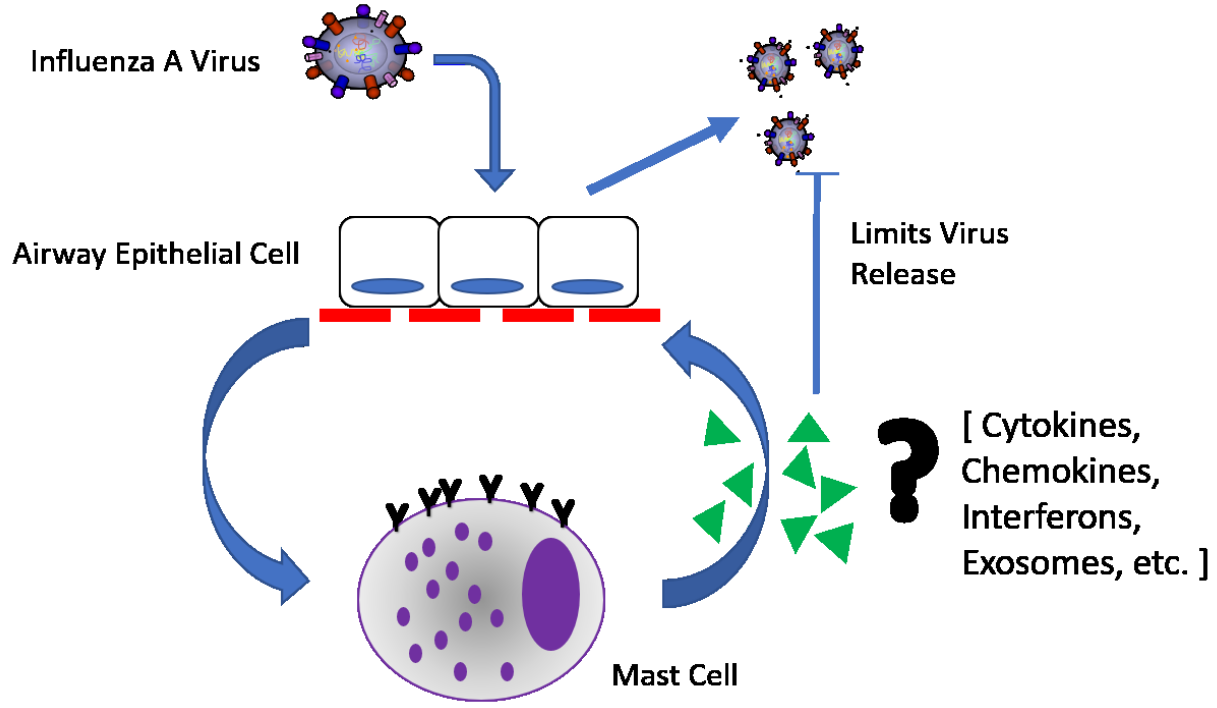

Figure 5.1 Conceptual Model of Influenza A Virus Infecting Epithelial Cell-Mast Cell Co-culture. Influenza A (FluA) infects epithelial cells (EC) leading to the release of FluA-induced mediators. Mast cells (MC) detect FluA infected EC. MC responds to viral infection by release of MC mediators. One or more mediators has an antiviral effect, limiting FluA release in EC.

References

1. Villalon-Letelier F, Brooks AG, Saunders PM, Londrigan SL, Reading PC. Host cell restriction factors that limit influenza A infection. *Viruses*. 2017;9(12):10.3390/v9120376. doi: E376 [pii].

2. Connor RJ, Kawaoka Y, Webster RG, Paulson JC. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology*. 1994;205(1):17-23. http://www.sciencedirect.com.login.ezproxy.library.ualberta.ca/science/article/pii/S0042682284716151. doi: //doi-org.login.ezproxy.library.ualberta.ca/10.1006/viro.1994.1615.

3. Palese P, Compans RW. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): Mechanism of action. *J Gen Virol*. 1976;33(1):159-163. doi: 10.1099/0022-1317-33-1-159 [doi].

4. Palese P, Tobita K, Ueda M, Compans RW. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology*. 1974;61(2):397-410.

5. Bui M, Whittaker G, Helenius A. Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. *J Virol*. 1996;70(12):8391-8401.

6. Martin K, Helenius A. Nuclear transport of influenza virus ribonucleoproteins: The viral matrix protein (M1) promotes export and inhibits import. *Cell*. 1991;67(1):117-130. doi: 0092-8674(91)90576-K [pii].

7. Huang X, Liu T, Muller J, Levandowski RA, Ye Z. Effect of influenza virus matrix protein and viral RNA on ribonucleoprotein formation and nuclear export. *Virology*. 2001;287(2):405-416. doi: 10.1006/viro.2001.1067 [doi].

8. Ye Z, Liu T, Offringa DP, McInnis J, Levandowski RA. Association of influenza virus matrix protein with ribonucleoproteins. *J Virol*. 1999;73(9):7467-7473.

9. Huang TS, Palese P, Krystal M. Determination of influenza virus proteins required for genome replication. *J Virol*. 1990;64(11):5669-5673.

10. Perales B, Ortin J. The influenza A virus PB2 polymerase subunit is required for the replication of viral RNA. *J Virol*. 1997;71(2):1381-1385.

11. Hatada E, Fukuda R. Binding of influenza A virus NS1 protein to dsRNA in vitro. *J Gen Virol*. 1992;73 (Pt 12)(Pt 12):3325-3329. doi: 10.1099/0022-1317-73-12-3325 [doi].

12. Hatada E, Takizawa T, Fukuda R. Specific binding of influenza A virus NS1 protein to the virus minus-sense RNA in vitro. *J Gen Virol*. 1992;73 (Pt 1)(Pt 1):17-25. doi: 10.1099/0022-1317-73-1-17 [doi].

13. Marion RM, Zurcher T, de la Luna S, Ortin J. Influenza virus NS1 protein interacts with viral transcription-replication complexes in vivo. *J Gen Virol*. 1997;78 (Pt 10)(Pt 10):2447-2451. doi: 10.1099/0022-1317-78-10-2447 [doi].

14. Qiu Y, Nemeroff M, Krug RM. The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing. *RNA*. 1995;1(3):304-316.

15. Fortes P, Beloso A, Ortin J. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. *EMBO J*. 1994;13(3):704-712.

16. Lu Y, Qian XY, Krug RM. The influenza virus NS1 protein: A novel inhibitor of pre-mRNA splicing. *Genes Dev*. 1994;8(15):1817-1828.

17. Alonso-Caplen FV, Nemeroff ME, Qiu Y, Krug RM. Nucleocytoplasmic transport: The influenza virus NS1 protein regulates the transport of spliced NS2 mRNA and its precursor NS1 mRNA. *Genes Dev*. 1992;6(2):255-267.

18. Marion RM, Aragon T, Beloso A, Nieto A, Ortin J. The N-terminal half of the influenza virus NS1 protein is sufficient for nuclear retention of mRNA and enhancement of viral mRNA translation. *Nucleic Acids Res*. 1997;25(21):4271-4277. doi: gka694 [pii].

19. Qian XY, Alonso-Caplen F, Krug RM. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. *J Virol*. 1994;68(4):2433-2441.

20. Nemeroff ME, Barabino SM, Li Y, Keller W, Krug RM. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3'end formation of cellular pre-mRNAs. *Mol Cell*. 1998;1(7):991-1000. doi: S1097-2765(00)80099-4 [pii].

21. de la Luna S, Fortes P, Beloso A, Ortin J. Influenza virus NS1 protein enhances the rate of translation initiation of viral mRNAs. *J Virol*. 1995;69(4):2427-2433.

22. Enami K, Sato TA, Nakada S, Enami M. Influenza virus NS1 protein stimulates translation of the M1 protein. *J Virol*. 1994;68(3):1432-1437.

23. Cheung CY, Poon LL, Lau AS, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: A mechanism for the unusual severity of human disease? *Lancet*. 2002;360(9348):1831-1837. doi: S0140673602117727 [pii].

24. Neumann G, Hughes MT, Kawaoka Y. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *EMBO J*. 2000;19(24):6751-6758. doi: 10.1093/emboj/19.24.6751 [doi].

25. Ward AC, Castelli LA, Lucantoni AC, White JF, Azad AA, Macreadie IG. Expression and analysis of the NS2 protein of influenza A virus. *Arch Virol*. 1995;140(11):2067-2073.

26. Digard P, Blok VC, Inglis SC. Complex formation between influenza virus polymerase proteins expressed in xenopus oocytes. *Virology*. 1989;171(1):162-169.

27. Nath ST, Nayak DP. Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1 N1). *Mol Cell Biol*. 1990;10(8):4139-4145.

28. Biswas SK, Nayak DP. Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1. *J Virol*. 1994;68(3):1819-1826.

29. Kobayashi M, Toyoda T, Ishihama A. Influenza virus PB1 protein is the minimal and essential subunit of RNA polymerase. *Arch Virol*. 1996;141(3-4):525-539.

30. Poch O, Sauvaget I, Delarue M, Tordo N. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J*. 1989;8(12):3867-3874.

31. Vasin AV, Temkina OA, Egorov VV, Klotchenko SA, Plotnikova MA, Kiselev OI. Molecular mechanisms enhancing the proteome of influenza A viruses: An overview of recently discovered proteins. *Virus Res*. 2014;185:53-63. doi: 10.1016/j.virusres.2014.03.015 [doi].

32. Chen W, Calvo PA, Malide D, et al. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med*. 2001;7(12):1306-1312. doi: 10.1038/nm1201-1306 [doi].

33. Wise HM, Foeglein A, Sun J, et al. A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *J Virol*. 2009;83(16):8021-8031. doi: 10.1128/JVI.00826-09 [doi].

34. Blaas D, Patzelt E, Kuechler E. Identification of the cap binding protein of influenza virus. *Nucleic Acids Res*. 1982;10(15):4803-4812.

35. Braam J, Ulmanen I, Krug RM. Molecular model of a eucaryotic transcription complex: Functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell*. 1983;34(2):609-618. doi: 0092-8674(83)90393-8 [pii].

36. Ulmanen I, Broni BA, Krug RM. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription. *Proc Natl Acad Sci U S A*. 1981;78(12):7355-7359.

37. Ulmanen I, Broni B, Krug RM. Influenza virus temperature-sensitive cap (m7GpppNm)-dependent endonuclease. *J Virol*. 1983;45(1):27-35.

38. Mukaigawa J, Nayak DP. Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. *J Virol*. 1991;65(1):245-253.

39. Poole E, Elton D, Medcalf L, Digard P. Functional domains of the influenza A virus PB2 protein: Identification of NP- and PB1-binding sites. *Virology*. 2004;321(1):120-133. doi: 10.1016/j.virol.2003.12.022 [doi].

40. Perales B, de la Luna S, Palacios I, Ortin J. Mutational analysis identifies functional domains in the influenza A virus PB2 polymerase subunit. *J Virol*. 1996;70(3):1678-1686.

41. Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of hong kong H5N1 influenza A viruses. *Science*. 2001;293(5536):1840-1842. doi: 10.1126/science.1062882 [doi].

42. Honda A, Mizumoto K, Ishihama A. Minimum molecular architectures for transcription and replication of the influenza virus. *Proc Natl Acad Sci U S A*. 2002;99(20):13166-13171. doi: 10.1073/pnas.152456799 [doi].

43. Dias A, Bouvier D, Crepin T, et al. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature*. 2009;458(7240):914-918. doi: 10.1038/nature07745 [doi].

44. Hara K, Schmidt FI, Crow M, Brownlee GG. Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. *J Virol*. 2006;80(16):7789-7798. doi: 80/16/7789 [pii].

45. Jagger BW, Wise HM, Kash JC, et al. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science*. 2012;337(6091):199-204. doi: 10.1126/science.1222213 [doi].

46. Muramoto Y, Noda T, Kawakami E, Akkina R, Kawaoka Y. Identification of novel influenza A virus proteins translated from PA mRNA. *J Virol*. 2013;87(5):2455-2462. doi: 10.1128/JVI.02656-12 [doi].

47. Stephenson I, Zambon M. The epidemiology of influenza. *Occup Med (Lond)*. 2002;52(5):241-247.

48. Zhu W, Wang C, Wang BZ. From variation of influenza viral proteins to vaccine development. *Int J Mol Sci*. 2017;18(7):10.3390/ijms18071554. doi: E1554 [pii].

49. Chen J, Deng YM. Influenza virus antigenic variation, host antibody production and new approach to control epidemics. *Virol J*. 2009;6:30. doi: 10.1186/1743-422X-6-30 [doi].

50. Weber TP, Stilianakis NI. Inactivation of influenza A viruses in the environment and modes of transmission: A critical review. *J Infect*. 2008;57(5):361-373. doi: 10.1016/j.jinf.2008.08.013 [doi].

51. Shao W, Li X, Goraya MU, Wang S, Chen JL. Evolution of influenza A virus by mutation and re-assortment. *Int J Mol Sci*. 2017;18(8):10.3390/ijms18081650. doi: E1650 [pii].

52. Yoo SJ, Kwon T, Lyoo YS. Challenges of influenza A viruses in humans and animals and current animal vaccines as an effective control measure. *Clin Exp Vaccine Res*. 2018;7(1):1-15. doi: 10.7774/cevr.2018.7.1.1 [doi].

53. Fournie G, Guitian J, Desvaux S, et al. Interventions for avian influenza A (H5N1) risk management in live bird market networks. *Proc Natl Acad Sci U S A*. 2013;110(22):9177-9182. doi: 10.1073/pnas.1220815110 [doi].

54. Public Health Agency of Canada. *An advisory committee statement (ACS) national advisory committee on immunization (NACI).* Public Health Agency of Canada; 2017. http://deslibris.ca/ID/10091368.

55. Aoki FY, Allen UD, Stiver HG, Laverdière M, Evans GA. The use of antiviral drugs for influenza: A foundation document for practitioners. *Can J Infect Dis Med Microbiol*. 2013;24(suppl c):15C. https://doaj.org/article/9aa3ffdd1e114b808ed5a2d1103f3c95. doi: 10.1155/2013/130913.

56. Allen UP, Aoki FY, Evans GA, Laverdiere M, Skowronski DM, Stiver HG. Guidance on use of antiviral drugs given potential low vaccine effectiveness for the 2017-18 influenza season. 2017.

57. Samji T. Influenza A: Understanding the viral life cycle. *Yale J Biol Med*. 2009;82(4):153-159.

58. Di Domizio J, Blum A, Gallagher-Gambarelli M, Molens JP, Chaperot L, Plumas J. TLR7 stimulation in human plasmacytoid dendritic cells leads to the induction of early IFN-inducible genes in the absence of type I IFN. *Blood*. 2009;114(9):1794-1802. doi: 10.1182/blood-2009-04-216770 [doi].

59. Lund JM, Alexopoulou L, Sato A, et al. Recognition of single-stranded RNA viruses by toll-like receptor 7. *Proc Natl Acad Sci U S A*. 2004;101(15):5598-5603. doi: 10.1073/pnas.0400937101 [doi].

60. Wang JP, Bowen GN, Padden C, et al. Toll-like receptor-mediated activation of neutrophils by influenza A virus. *Blood*. 2008;112(5):2028-2034. doi: 10.1182/blood-2008-01-132860 [doi].

61. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by toll-like receptor 3. *Nature*. 2001;413(6857):732-738. doi: 10.1038/35099560 [doi].

62. Desselberger U, Racaniello VR, Zazra JJ, Palese P. The 3' and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. *Gene*. 1980;8(3):315-328. doi: 0378-1119(80)90007-4 [pii].

63. Rehwinkel J, Tan CP, Goubau D, et al. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell*. 2010;140(3):397-408. doi: 10.1016/j.cell.2010.01.020 [doi].

64. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*. 2006;441(7089):101-105. doi: nature04734 [pii].

65. Le Goffic R, Pothlichet J, Vitour D, et al. Cutting edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *J Immunol*. 2007;178(6):3368-3372. doi: 178/6/3368 [pii].

66. Siren J, Imaizumi T, Sarkar D, et al. Retinoic acid inducible gene-I and mda-5 are involved in influenza A virus-induced expression of antiviral cytokines. *Microbes Infect*. 2006;8(8):2013-2020. doi: S1286-4579(06)00155-9 [pii].

67. Ohman T, Rintahaka J, Kalkkinen N, Matikainen S, Nyman TA. Actin and RIG-I/MAVS signaling components translocate to mitochondria upon influenza A virus infection of human primary macrophages. *J Immunol*. 2009;182(9):5682-5692. doi: 10.4049/jimmunol.0803093 [doi].

68. de Jong MD, Simmons CP, Thanh TT, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med*. 2006;12(10):1203-1207. doi: nm1477 [pii].

69. Guo XJ, Thomas PG. New fronts emerge in the influenza cytokine storm. *Semin Immunopathol*. 2017;39(5):541-550. doi: 10.1007/s00281-017-0636-y [doi].

70. Matsukura S, Kokubu F, Noda H, Tokunaga H, Adachi M. Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A. *J Allergy Clin Immunol*. 1996;98(6 Pt 1):1080-1087. doi: S0091674996003533 [pii].

71. Matsukura S, Kokubu F, Kubo H, et al. Expression of RANTES by normal airway epithelial cells after influenza virus A infection. *Am J Respir Cell Mol Biol*. 1998;18(2):255-264. doi: 10.1165/ajrcmb.18.2.2822 [doi].

72. Bussfeld D, Kaufmann A, Meyer RG, Gemsa D, Sprenger H. Differential mononuclear leukocyte attracting chemokine production after stimulation with active and inactivated influenza A virus. *Cell Immunol*. 1998;186(1):1-7. doi: S0008-8749(98)91295-3 [pii].

73. Hao X, Kim TS, Braciale TJ. Differential response of respiratory dendritic cell subsets to influenza virus infection. *J Virol*. 2008;82(10):4908-4919. doi: 10.1128/JVI.02367-07 [doi].

74. Garcia-Sastre A, Durbin RK, Zheng H, et al. The role of interferon in influenza virus tissue tropism. *J Virol*. 1998;72(11):8550-8558.

75. Kim MJ, Latham AG, Krug RM. Human influenza viruses activate an interferon-independent transcription of cellular antiviral genes: Outcome with influenza A virus is unique. *Proc Natl Acad Sci U S A*. 2002;99(15):10096-10101. doi: 10.1073/pnas.152327499 [doi].

76. Lee SB, Green SR, Mathews MB, Esteban M. Activation of the double-stranded RNA (dsRNA)-activated human protein kinase in vivo in the absence of its dsRNA binding domain. *Proc Natl Acad Sci U S A*. 1994;91(22):10551-10555.

77. Garcia MA, Meurs EF, Esteban M. The dsRNA protein kinase PKR: Virus and cell control. *Biochimie*. 2007;89(6-7):799-811. doi: S0300-9084(07)00051-X [pii].

78. Balachandran S, Roberts PC, Brown LE, et al. Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity*. 2000;13(1):129-141. doi: S1074-7613(00)00014-5 [pii].

79. Hatada E, Saito S, Fukuda R. Mutant influenza viruses with a defective NS1 protein cannot block the activation of PKR in infected cells. *J Virol*. 1999;73(3):2425-2433.

80. Zheng W, Cao S, Chen C, et al. Threonine 80 phosphorylation of non-structural protein 1 regulates the replication of influenza A virus by reducing the binding affinity with RIG-I. *Cell Microbiol*. 2017;19(2):10.1111/cmi.12643. Epub 2016 Aug 4. doi: 10.1111/cmi.12643 [doi].

81. Bahadoran A, Lee SH, Wang SM, et al. Immune responses to influenza virus and its correlation to age and inherited factors. *Front Microbiol*. 2016;7:1841. doi: 10.3389/fmicb.2016.01841 [doi].

82. Warner JA, Kroegel C. Pulmonary immune cells in health and disease: Mast cells and basophils. *Eur Respir J*. 1994;7(7):1326-1341.

83. Costa JJ, Demetri GD, Harrist TJ, et al. Recombinant human stem cell factor (kit ligand) promotes human mast cell and melanocyte hyperplasia and functional activation in vivo. *J Exp Med*. 1996;183(6):2681-2686.

84. Metcalfe DD. Mast cells and mastocytosis. *Blood*. 2008;112(4):946-956. doi: 10.1182/blood-2007-11-078097 [doi].

85. Moon TC, St Laurent CD, Morris KE, et al. Advances in mast cell biology: New understanding of heterogeneity and function. *Mucosal Immunol*. 2010;3(2):111-128. doi: 10.1038/mi.2009.136 [doi].

86. Ryan JJ, Fernando JF. Mast cell modulation of the immune response. *Curr Allergy Asthma Rep*. 2009;9(5):353-359.

87. Vliagoftis H, Befus AD. Rapidly changing perspectives about mast cells at mucosal surfaces. *Immunol Rev*. 2005;206:190-203. doi: IMR279 [pii].

88. Kitamura Y. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annu Rev Immunol*. 1989;7:59-76. doi: 10.1146/annurev.iy.07.040189.000423 [doi].

89. Aldenborg F, Enerback L. Histochemical heterogeneity of dermal mast cells in athymic and normal rats. *Histochem J*. 1988;20(1):19-28.

90. Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proc Natl Acad Sci U S A*. 1986;83(12):4464-4468.

91. Schwartz LB, Irani AM, Roller K, Castells MC, Schechter NM. Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. *J Immunol*. 1987;138(8):2611-2615.

92. Weidner N, Austen KF. Ultrastructural and immunohistochemical characterization of normal mast cells at multiple body sites. *J Invest Dermatol*. 1991;96(3):31S.

93. Andersson CK, Mori M, Bjermer L, Lofdahl CG, Erjefalt JS. Novel site-specific mast cell subpopulations in the human lung. *Thorax*. 2009;64(4):297-305. doi: 10.1136/thx.2008.101683 [doi].

94. Balzar S, Chu HW, Strand M, Wenzel S. Relationship of small airway chymase-positive mast cells and lung function in severe asthma. *Am J Respir Crit Care Med*. 2005;171(5):431-439. doi: 200407-949OC [pii].

95. Turner H, Kinet JP. Signalling through the high-affinity IgE receptor fc epsilonRI. *Nature*. 1999;402(6760 Suppl):24.

96. Galli SJ, Tsai M. Mast cells: Versatile regulators of inflammation, tissue remodeling, host defense and homeostasis. *J Dermatol Sci*. 2008;49(1):7-19. doi: S0923-1811(07)00323-4 [pii].

97. Lowman MA, Rees PH, Benyon RC, Church MK. Human mast cell heterogeneity: Histamine release from mast cells dispersed from skin, lung, adenoids, tonsils, and colon in response to IgE-dependent and nonimmunologic stimuli. *J Allergy Clin Immunol*. 1988;81(3):590-597. doi: 0091-6749(88)90199-6 [pii].

98. Rao KN, Brown MA. Mast cells: Multifaceted immune cells with diverse roles in health and disease. *Ann N Y Acad Sci*. 2008;1143:83-104. doi: 10.1196/annals.1443.023 [doi].

99. Iba Y, Shibata A, Kato M, Masukawa T. Possible involvement of mast cells in collagen remodeling in the late phase of cutaneous wound healing in mice. *Int Immunopharmacol*. 2004;4(14):1873-1880. doi: S1567-5769(04)00274-7 [pii].

100. Weller K, Foitzik K, Paus R, Syska W, Maurer M. Mast cells are required for normal healing of skin wounds in mice. *FASEB J*. 2006;20(13):2366-2368. doi: fj.06-5837fje [pii].

101. Noli C, Miolo A. The mast cell in wound healing. *Vet Dermatol*. 2001;12(6):303-313. doi: 272 [pii].

102. Jacob C, Yang PC, Darmoul D, et al. Mast cell tryptase controls paracellular permeability of the intestine. role of protease-activated receptor 2 and beta-arrestins. *J Biol Chem*. 2005;280(36):31936-31948. doi: M506338200 [pii].

103. Nakamura E, Kataoka T, Furutani K, et al. Lack of histamine alters gastric mucosal morphology: Comparison of histidine decarboxylase-deficient and mast cell-deficient mice. *Am J Physiol Gastrointest Liver Physiol*. 2004;287(5):1053. doi: 10.1152/ajpgi.00353.2003 [doi].

104. Tanaka S, Hamada K, Yamada N, et al. Gastric acid secretion in L-histidine decarboxylase-deficient mice. *Gastroenterology*. 2002;122(1):145-155. doi: S0016508502995703 [pii].

105. Varadaradjalou S, Feger F, Thieblemont N, et al. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. *Eur J Immunol*. 2003;33(4):899-906. doi: 10.1002/eji.200323830 [doi].

106. Underhill DM, Ozinsky A. Toll-like receptors: Key mediators of microbe detection. *Curr Opin Immunol*. 2002;14(1):103-110. doi: S0952791501003041 [pii].

107. Ozinsky A, Underhill DM, Fontenot JD, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A*. 2000;97(25):13766-13771. doi: 10.1073/pnas.250476497 [doi].

108. McCurdy JD, Olynych TJ, Maher LH, Marshall JS. Cutting edge: Distinct toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *J Immunol*. 2003;170(4):1625-1629.

109. Leal-Berumen I, Conlon P, Marshall JS. IL-6 production by rat peritoneal mast cells is not necessarily preceded by histamine release and can be induced by bacterial lipopolysaccharide. *J Immunol*. 1994;152(11):5468-5476.

110. Supajatura V, Ushio H, Nakao A, Okumura K, Ra C, Ogawa H. Protective roles of mast cells against enterobacterial infection are mediated by toll-like receptor 4. *J Immunol*. 2001;167(4):2250-2256.

111. McCurdy JD, Lin TJ, Marshall JS. Toll-like receptor 4-mediated activation of murine mast cells. *J Leukoc Biol*. 2001;70(6):977-984.

112. Niyonsaba F, Ushio H, Hara M, et al. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. *J Immunol*. 2010;184(7):3526-3534. doi: 10.4049/jimmunol.0900712 [doi].

113. Niyonsaba F, Hirata M, Ogawa H, Nagaoka I. Epithelial cell-derived antibacterial peptides human beta-defensins and cathelicidin: Multifunctional activities on mast cells. *Curr Drug Targets Inflamm Allergy*. 2003;2(3):224-231.

114. Orinska Z, Bulanova E, Budagian V, Metz M, Maurer M, Bulfone-Paus S. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. *Blood*. 2005;106(3):978-987. doi: 2004-07-2656 [pii].

115. Razin E, Ihle JN, Seldin D, et al. Interleukin 3: A differentiation and growth factor for the mouse mast cell that contains chondroitin sulfate E proteoglycan. *J Immunol*. 1984;132(3):1479-1486.

116. Butterfield JH, Weiler D, Dewald G, Gleich GJ. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res*. 1988;12(4):345-355.

117. Radinger M, Jensen BM, Kuehn HS, Kirshenbaum A, Gilfillan AM. Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. *Curr Protoc Immunol*. 2010;Chapter 7:Unit 7.37. doi: 10.1002/0471142735.im0737s90 [doi].

118. Kirshenbaum A. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leuk Res*. 2003;27(8):677-682. doi: 10.1016/S0145-2126(02)00343-0.

119. Metcalfe DD. Isolation of tissue mast cells. *Curr Protoc Immunol*. 2001;Chapter 7:Unit 7.25. http://www.ncbi.nlm.nih.gov/pubmed/18432836.

120. Kulka M, Alexopoulou L, Flavell RA, Metcalfe DD. Activation of mast cells by double-stranded RNA: Evidence for activation through toll-like receptor 3. *J Allergy Clin Immunol*. 2004;114(1):174-182. doi: 10.1016/j.jaci.2004.03.049 [doi].

121. Marshall JS, Jawdat DM. Mast cells in innate immunity. *J Allergy Clin Immunol*. 2004;114(1):21-27. doi: 10.1016/j.jaci.2004.04.045 [doi].

122. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science*. 2004;303(5663):1529-1531. doi: 10.1126/science.1093616 [doi].

123. Redegeld FA, Yu Y, Kumari S, Charles N, Blank U. Non-IgE mediated mast cell activation. *Immunol Rev*. 2018;282(1):87-113. doi: 10.1111/imr.12629 [doi].

124. Matsushima H, Yamada N, Matsue H, Shimada S. TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells. *J Immunol*. 2004;173(1):531-541.

125. Li Y, Li L, Wadley R, et al. Mast cells/basophils in the peripheral blood of allergic individuals who are HIV-1 susceptible due to their surface expression of CD4 and the chemokine receptors CCR3, CCR5, and CXCR4. *Blood*. 2001;97(11):3484-3490.

126. Taub DD, Mikovits JA, Nilsson G, et al. Alterations in mast cell function and survival following in vitro infection with human immunodeficiency viruses-1 through CXCR4. *Cell Immunol*. 2004;230(2):65-80. doi: S0008-8749(04)00136-4 [pii].

127. King CA, Marshall JS, Alshurafa H, Anderson R. Release of vasoactive cytokines by antibody-enhanced dengue virus infection of a human mast cell/basophil line. *J Virol*. 2000;74(15):7146-7150.

128. King CA, Anderson R, Marshall JS. Dengue virus selectively induces human mast cell chemokine production. *J Virol*. 2002;76(16):8408-8419.

129. Furuta T, Murao LA, Lan NT, et al. Association of mast cell-derived VEGF and proteases in dengue shock syndrome. *PLoS Negl Trop Dis*. 2012;6(2):e1505. doi: 10.1371/journal.pntd.0001505 [doi].

130. Lappalainen J, Rintahaka J, Kovanen PT, Matikainen S, Eklund KK. Intracellular RNA recognition pathway activates strong anti-viral response in human mast cells. *Clin Exp Immunol*. 2013;172(1):121-128. doi: 10.1111/cei.12042 [doi].

131. Graham AC, Hilmer KM, Zickovich JM, Obar JJ. Inflammatory response of mast cells during influenza A virus infection is mediated by active infection and RIG-I signaling. *J Immunol*. 2013;190(9):4676-4684. http://www.ncbi.nlm.nih.gov/pubmed/23526820. doi: 10.4049/jimmunol.1202096.

132. Meng D, Huo C, Wang M, et al. Influenza A viruses replicate productively in mouse mastocytoma cells (P815) and trigger pro-inflammatory cytokine and chemokine production through TLR3 signaling pathway. *Front Microbiol*. 2017;7:2130. doi: 10.3389/fmicb.2016.02130 [doi].

133. Hu Y, Jin Y, Han D, et al. Mast cell-induced lung injury in mice infected with H5N1 influenza virus. *J Virol*. 2012;86(6):3347-3356. doi: 10.1128/JVI.06053-11 [doi].

134. Liu B, Meng D, Wei T, Zhang S, Hu Y, Wang M. Apoptosis and pro-inflammatory cytokine response of mast cells induced by influenza A viruses. *PLoS One*. 2014;9(6):e100109. doi: 10.1371/journal.pone.0100109 [doi].

135. Lee IH, Kim HS, Seo SH. Porcine mast cells infected with H1N1 influenza virus release histamine and inflammatory cytokines and chemokines. *Arch Virol*. 2017;162(4):1067-1071. doi: 10.1007/s00705-016-3216-x [doi].

136. Marcet C, St. Laurent C, Moon T, Singh N, Befus A. Limited replication of influenza A virus in human mast cells. *Immunol Res*. 2013;56(1):32-43. http://www.ncbi.nlm.nih.gov/pubmed/23055084. doi: 10.1007/s12026-012-8377-4.

137. Marcet C. *Antiviral and cytokine responses of human mast cells to influenza A virus infection. [PhD thesis]*. Edmonton, AB: University of Alberta; 2010.

138. Hsieh FH, Sharma P, Gibbons A, Goggans T, Erzurum SC, Haque SJ. Human airway epithelial cell determinants of survival and functional phenotype for primary human mast cells. *Proc Natl Acad Sci U S A*. 2005;102(40):14380-14385. doi: 0503948102 [pii].

139. Shirato K, Taguchi F. Mast cell degranulation is induced by A549 airway epithelial cell infected with respiratory syncytial virus. *Virology*. 2009;386(1):88-93. doi: 10.1016/j.virol.2009.01.011 [doi].

140. Martin N, Ruddick A, Arthur GK, et al. Primary human airway epithelial cell-dependent inhibition of human lung mast cell degranulation. *PLoS One*. 2012;7(8):e43545. doi: 10.1371/journal.pone.0043545 [doi].

141. Hollander C, Nystrom M, Janciauskiene S, Westin U. Human mast cells decrease SLPI levels in type II - like alveolar cell model, in vitro. *Cancer Cell Int*. 2003;3(1):14. doi: 10.1186/1475-2867-3-14 [doi].

142. Muir A, Soong G, Sokol S, et al. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol*. 2004;30(6):777-783. doi: 10.1165/rcmb.2003-0329OC [doi].

143. Guillot L, Le Goffic R, Bloch S, et al. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem*. 2005;280(7):5571-5580. doi: M410592200 [pii].

144. Li H, Zhang J, Kumar A, Zheng M, Atherton SS, Yu FS. Herpes simplex virus 1 infection induces the expression of proinflammatory cytokines, interferons and TLR7 in human corneal epithelial cells. *Immunology*. 2006;117(2):167-176. doi: IMM2275 [pii].

145. Andersen JM, Al-Khairy D, Ingalls RR. Innate immunity at the mucosal surface: Role of toll-like receptor 3 and toll-like receptor 9 in cervical epithelial cell responses to microbial pathogens. *Biol Reprod*. 2006;74(5):824-831. doi: biolreprod.105.048629 [pii].

146. Bissonnette EY, Chin B, Befus AD. Interferons differentially regulate histamine and TNF-alpha in rat intestinal mucosal mast cells. *Immunology*. 1995;86(1):12-17.

147. Fujita T, Kambe N, Uchiyama T, Hori T. Type I interferons attenuate T cell activating functions of human mast cells by decreasing TNF-alpha production and OX40 ligand expression while increasing IL-10 production. *J Clin Immunol*. 2006;26(6):512-518. doi: 10.1007/s10875-006-9043-1 [doi].

148. Schoeler D, Grutzkau A, Henz BM, Kuchler J, Kruger-Krasagakis S. Interleukin-6 enhances whereas tumor necrosis factor alpha and interferons inhibit integrin expression and adhesion

of human mast cells to extracellular matrix proteins. *J Invest Dermatol*. 2003;120(5):795-801. doi: S0022-202X(15)30252-9 [pii].

149. Sekar Y, Moon TC, Munoz S, Befus AD. Role of nitric oxide in mast cells: Controversies, current knowledge, and future applications. *Immunol Res*. 2005;33(3):223-239. doi: IR:33:3:223 [pii].

150. Rastogi P, Rickard A, Dorokhov N, Klumpp DJ, McHowat J. Loss of prostaglandin E2 release from immortalized urothelial cells obtained from interstitial cystitis patient bladders. *Am J Physiol Renal Physiol*. 2008;294(5):1129. doi: 10.1152/ajprenal.00572.2007 [doi].

151. Wang H, Zheng Y, He S. Induction of release and up-regulated gene expression of interleukin (IL)-8 in A549 cells by serine proteinases. *BMC Cell Biol*. 2006;7:22. doi: 1471-2121-7-22 [pii].

152. Wang H, Yi T, Zheng Y, He S. Induction of monocyte chemoattractant protein-1 release from A549 cells by agonists of protease-activated receptor-1 and -2. *Eur J Cell Biol*. 2007;86(4):233-242. doi: S0171-9335(06)00192-0 [pii].

153. Vliagoftis H, Schwingshackl A, Milne CD, et al. Proteinase-activated receptor-2-mediated matrix metalloproteinase-9 release from airway epithelial cells. *J Allergy Clin Immunol*. 2000;106(3):537-545. doi: S0091-6749(00)31092-2 [pii].

154. Vliagoftis H, Befus AD, Hollenberg MD, Moqbel R. Airway epithelial cells release eosinophil survival-promoting factors (GM-CSF) after stimulation of proteinase-activated

receptor 2. *J Allergy Clin Immunol*. 2001;107(4):679-685. doi: S0091-6749(01)52209-5 [pii].

155. Hou YF, Zhou YC, Zheng XX, et al. Modulation of expression and function of toll-like receptor 3 in A549 and H292 cells by histamine. *Mol Immunol*. 2006;43(12):1982-1992. doi: S0161-5890(05)00411-6 [pii].

156. Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J Biol Chem*. 1995;270(9):4689-4696.

157. Cottey R, Rowe CA, Bender BS. Influenza virus. *Curr Protoc Immunol*. 2001;Chapter 19:Unit 19.11. doi: 10.1002/0471142735.im1911s42 [doi].

158. Galli SJ, Maurer M, Lantz CS. Mast cells as sentinels of innate immunity. *Curr Opin Immunol*. 1999;11(1):53-59. doi: S0952-7915(99)80010-7 [pii].

159. Husser L, Alves MP, Ruggli N, Summerfield A. Identification of the role of RIG-I, MDA-5 and TLR3 in sensing RNA viruses in porcine epithelial cells using lentivirus-driven RNA interference. *Virus Res*. 2011;159(1):9-16. doi: 10.1016/j.virusres.2011.04.005 [doi].

160. Tan KS, Olfat F, Phoon MC, et al. In vivo and in vitro studies on the antiviral activities of viperin against influenza H1N1 virus infection. *J Gen Virol*. 2012;93(Pt 6):1269-1277. doi: 10.1099/vir.0.040824-0 [doi].

161. Wang X, Hinson ER, Cresswell P. The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. *Cell Host Microbe*. 2007;2(2):96-105. doi: S1931-3128(07)00135-7 [pii].

162. Arizmendi-Puga NG, Enciso JA, Ortega-Pierres G, et al. Trichinella spiralis: Histamine secretion induced by TSL-1 antigens from unsensitized mast cells. *Exp Parasitol*. 2006;114(2):67-76. doi: S0014-4894(06)00040-3 [pii].

163. Duta F, Ulanova M, Seidel D, et al. Differential expression of spleen tyrosine kinase syk isoforms in tissues: Effects of the microbial flora. *Histochem Cell Biol*. 2006;126(4):495-505. doi: 10.1007/s00418-006-0188-z [doi].

164. Gilchrist M, Befus AD. Interferon-gamma regulates chemokine expression and release in the human mast cell line HMC1: Role of nitric oxide. *Immunology*. 2008;123(2):209-217. doi: IMM2688 [pii].

165. Hofmann P, Sprenger H, Kaufmann A, et al. Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. *J Leukoc Biol*. 1997;61(4):408-414.

166. Ronni T, Matikainen S, Sareneva T, et al. Regulation of IFN-alpha/beta, MxA, 2',5'-oligoadenylate synthetase, and HLA gene expression in influenza A-infected human lung epithelial cells. *J Immunol*. 1997;158(5):2363-2374.

167. Al-Afif A, MSc, Alyazidi R, MD, Oldford SA, PhD, et al. Respiratory syncytial virus infection of primary human mast cells induces the selective production of type I interferons,

CXCL10, and CCL4. *J Allergy Clin Immunol*. 2015;136(5):1354.e1. https://www.clinicalkey.es/playcontent/1-s2.0-S0091674915001876. doi: 10.1016/j.jaci.2015.01.042.

168. Hosoda M, Yamaya M, Suzuki T, et al. Effects of rhinovirus infection on histamine and cytokine production by cell lines from human mast cells and basophils. *J Immunol*. 2002;169(3):1482-1491. http://www.jimmunol.org/cgi/content/abstract/169/3/1482.

169. Akoto C, Davies DE, Swindle EJ. Mast cells are permissive for rhinovirus replication: Potential implications for asthma exacerbations. *Clin Exp Allergy*. 2017;47(3):351-360. http://onlinelibrary.wiley.com/doi/10.1111/cea.12879/abstract. doi: 10.1111/cea.12879.

170. Nakayama T, Shirane J, Hieshima K, et al. Novel antiviral activity of chemokines. *Virology*. 2006;350(2):484-492. doi: S0042-6822(06)00165-6 [pii].

171. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science*. 1995;270(5243):1811-1815.

172. Hester K, Liu J, Flynn N, et al. Polyionic complexes of butyrylcholinesterase and poly-l-lysine-g-poly(ethylene glycol): Comparative kinetics of catalysis and inhibition and in vitro inactivation by proteases and heat. *Chem Biol Interact*. 2017;275:86-94. doi: S0009-2797(17)30483-0 [pii].

173. Lepock JR. How do cells respond to their thermal environment? *Int J Hyperthermia*. 2005;21(8):681-687.

http://www.ingentaconnect.com/content/apl/thth/2005/00000021/00000008/art00003. doi: 10.1080/02656730500307298.

174. Lepock JR. Measurement of protein stability and protein denaturation in cells using differential scanning calorimetry. *Methods*. 2005;35(2):117-125. https://www.sciencedirect.com/science/article/pii/S104620230400177X. doi: 10.1016/j.ymeth.2004.08.002.

175. Arcangeletti MC, Pinardi F, Missorini S, et al. Modification of cytoskeleton and prosome networks in relation to protein synthesis in influenza A virus-infected LLC-MK2 cells. *Virus Res*. 1997;51(1):19-34. doi: S0168170297000749 [pii].

176. Liu N, Song W, Wang P, et al. Proteomics analysis of differential expression of cellular proteins in response to avian H9N2 virus infection in human cells. *Proteomics*. 2008;8(9):1851-1858. doi: 10.1002/pmic.200700757 [doi].

177. Vester D, Rapp E, Gade D, Genzel Y, Reichl U. Quantitative analysis of cellular proteome alterations in human influenza A virus-infected mammalian cell lines. *Proteomics*. 2009;9(12):3316-3327. doi: 10.1002/pmic.200800893 [doi].

178. Yu G, Liang W, Liu J, et al. Proteomic analysis of differential expression of cellular proteins in response to avian H9N2 virus infection of A549 cells. *Front Microbiol*. 2016;7:1962. doi: 10.3389/fmicb.2016.01962 [doi].

179. Wang Z, Lai Y, Bernard JJ, et al. Skin mast cells protect mice against vaccinia virus by triggering mast cell receptor S1PR2 and releasing antimicrobial peptides. *J Immunol*. 2012;188(1):345-357. doi: 10.4049/jimmunol.1101703 [doi].

180. Cypryk W, Lorey M, Puustinen A, Nyman TA, Matikainen S. Proteomic and bioinformatic characterization of extracellular vesicles released from human macrophages upon influenza A virus infection. *J Proteome Res*. 2017;16(1):217-227. doi: 10.1021/acs.jproteome.6b00596 [doi].

181. Kesimer M, Scull M, Brighton B, et al. Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: A possible role in innate defense. *FASEB J*. 2009;23(6):1858-1868. doi: 10.1096/fj.08-119131 [doi].

182. Suptawiwat O, Ruangrung K, Boonarkart C, et al. Microparticle and anti-influenza activity in human respiratory secretion. *PLoS One*. 2017;12(8):e0183717. doi: 10.1371/journal.pone.0183717 [doi].

Appendix A

Results of 41 Cytokine/Chemokine Multiplex Assay from EVE Technologies (Calgary, AB) were obtained from experiments performed by Tae Chul Moon. The following cytokine/chemokine release was measured in single cultures of MC (PBMC, LAD2) and EC (Calu-3):

EGF, FGF2, Eotaxin, TGF-α, G-CSF, Flt-3L, GM-CSF, Fractalkine, IFNα2, IFNγ, GRO pan, IL-10, MCP-3, IL-12, MDC, IL-12 P70, PDGF-AA, IL-13, PDGF-BB, IL-15, sCD40L, IL-17A, IL-1RA, IL-1a, IL-9, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, CXCL-10, CCL-2, CCL-3, CCL-4, CCL-5, TNF, TNFβ, VEGF-A.

Figures A.1-A.4 contains experiments using peripheral blood-derived human mast cells (PBMC) which were isolated as described[117].

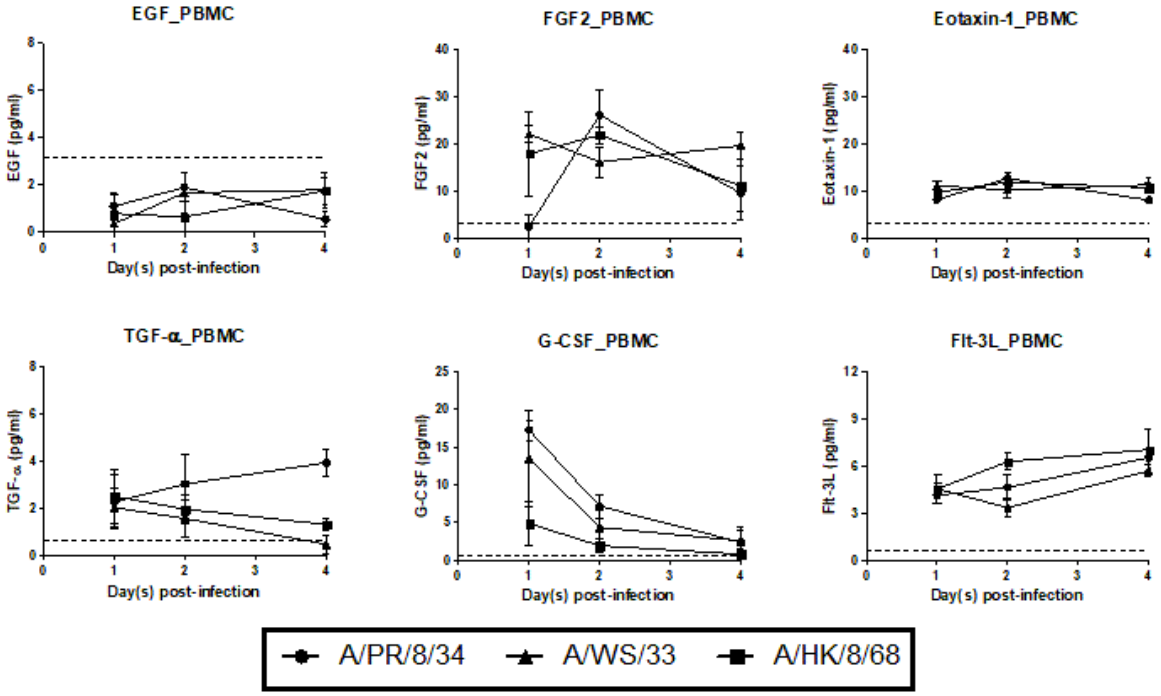

Figure A.1 Cytokine/Chemokine Release in PBMC. EGF, FGF2, Eotaxin, TGF-α, G-CSF and Flt-3L release in PBMC. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68; 1.0 MOI) were used to infect PBMC. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

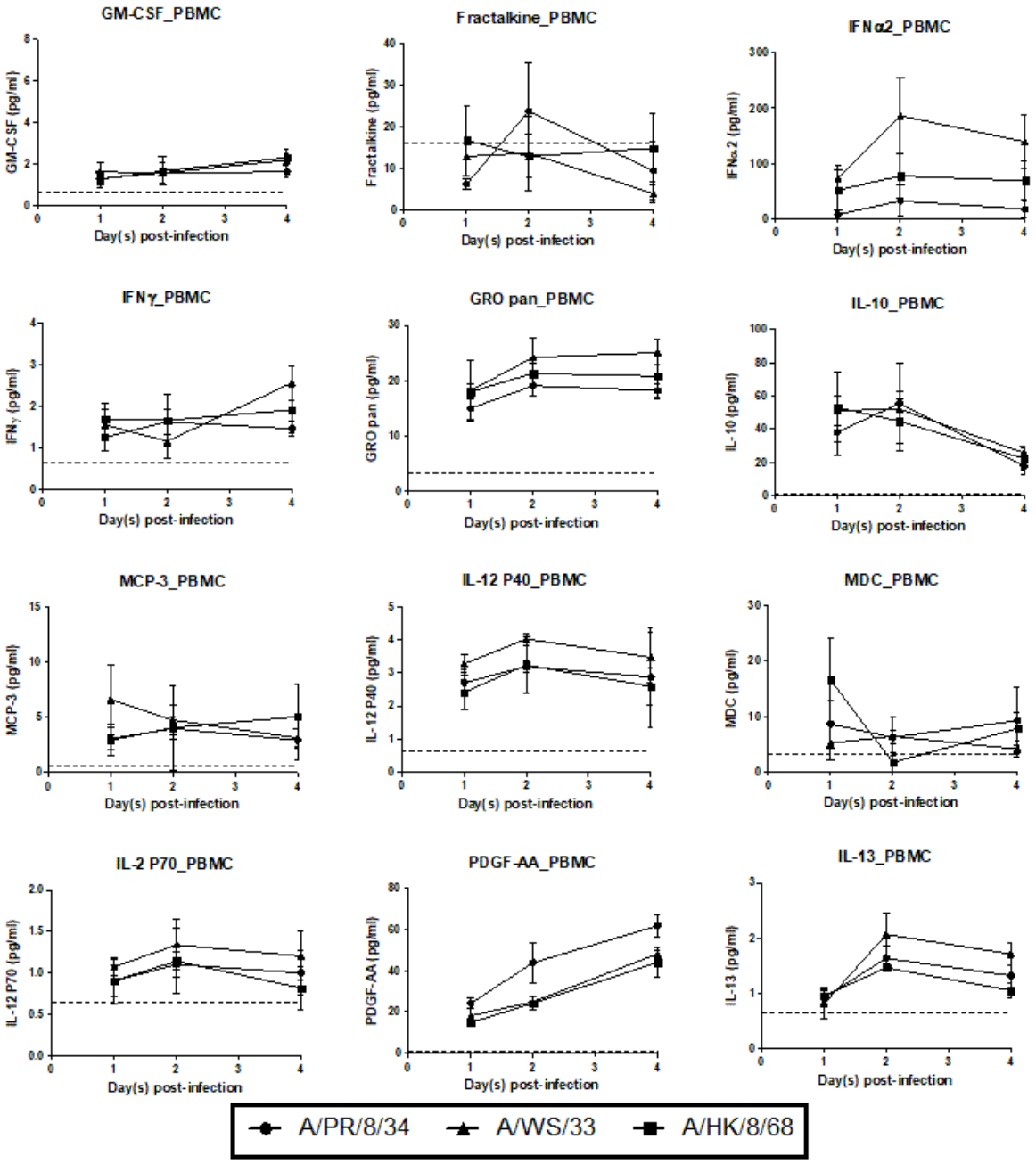

Figure A.2 Cytokine/Chemokine Release in PBMC. GM-CSF, Fractalkine, IFNα2, IFNγ, GRO pan, IL-10, MCP-3, IL-12, MDC, IL-12 P70, PDGF-AA and IL-13 release in PBMC. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68; 1.0 MOI) were used to infect PBMC. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

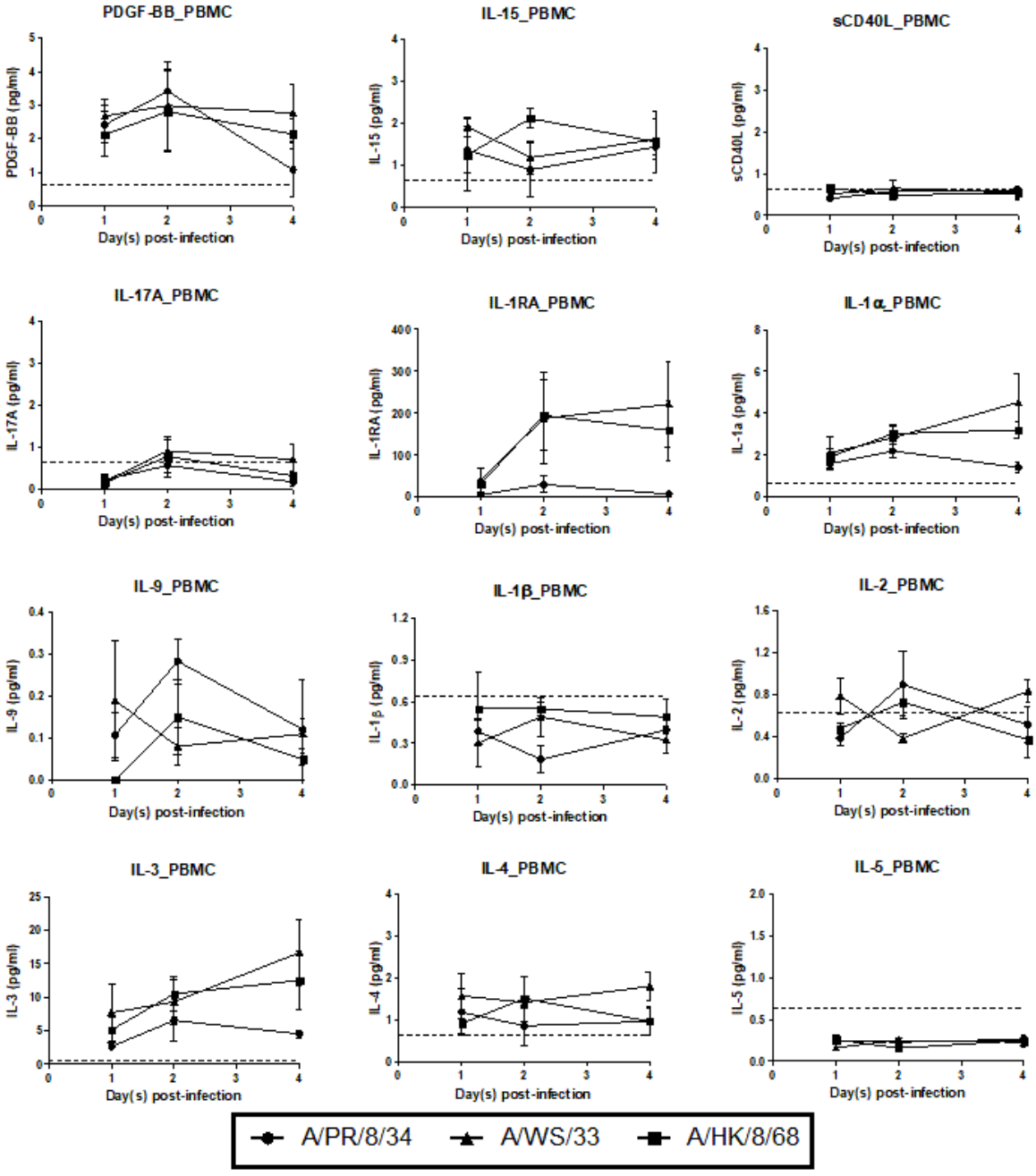

Figure A.3 Cytokine/Chemokine Release in PBMC. PDGF-BB, IL-15, sCD40L, IL-17A, IL-1RA, IL-1a, IL-9, IL-1b, IL-2, IL-3, IL-4 and IL-5 release in PBMC. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68; 1.0 MOI) were used to infect PBMC. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

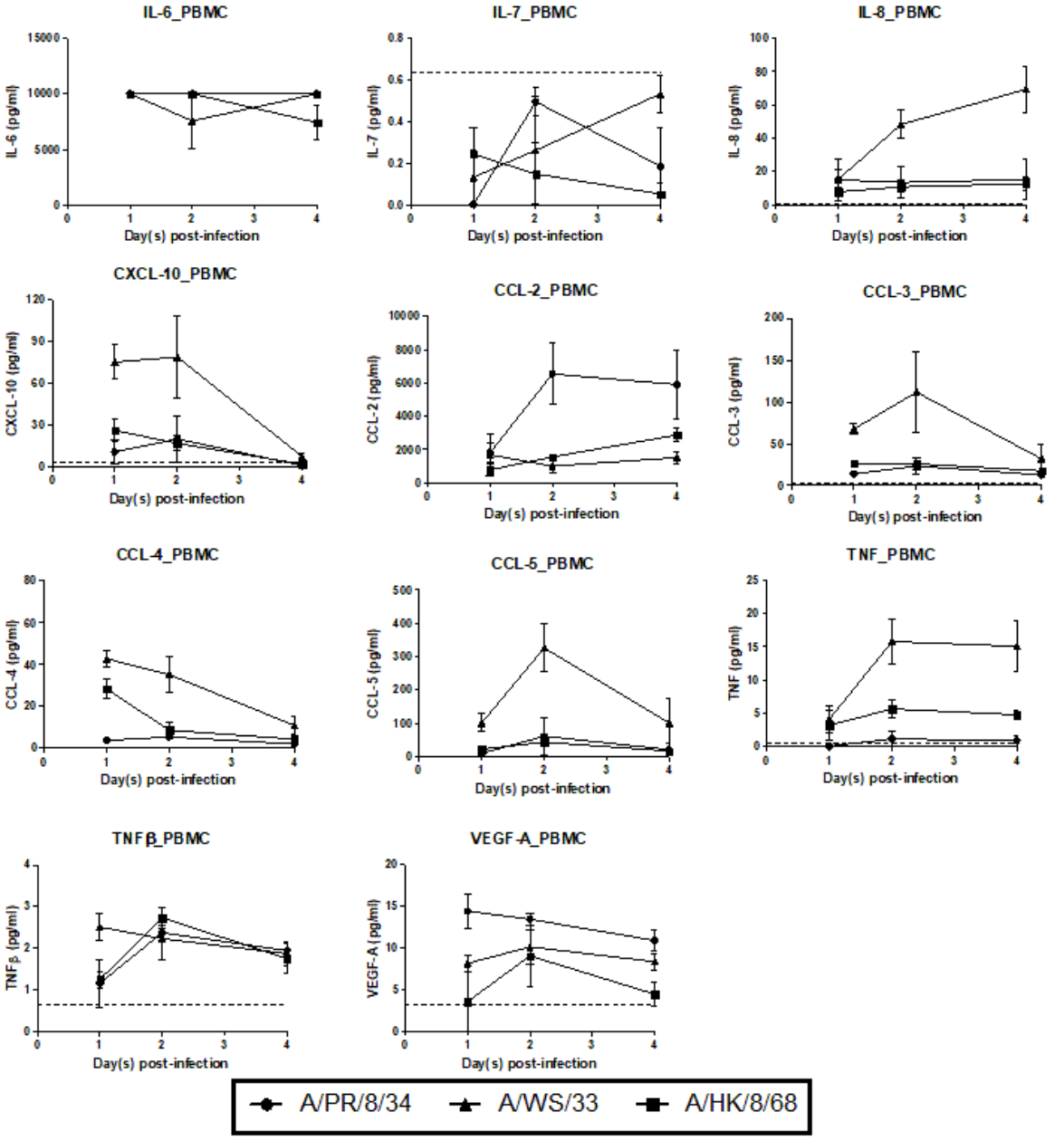

Figure A.4 Cytokine/Chemokine Release in PBMC. IL-6, IL-7, IL-8, CXCL-10, CCL-2, CCL-3, CCL-4, CCL-5, TNF, TNFβ and VEGF-A release in PBMC. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68; 1.0 MOI) were used to infect PBMC. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

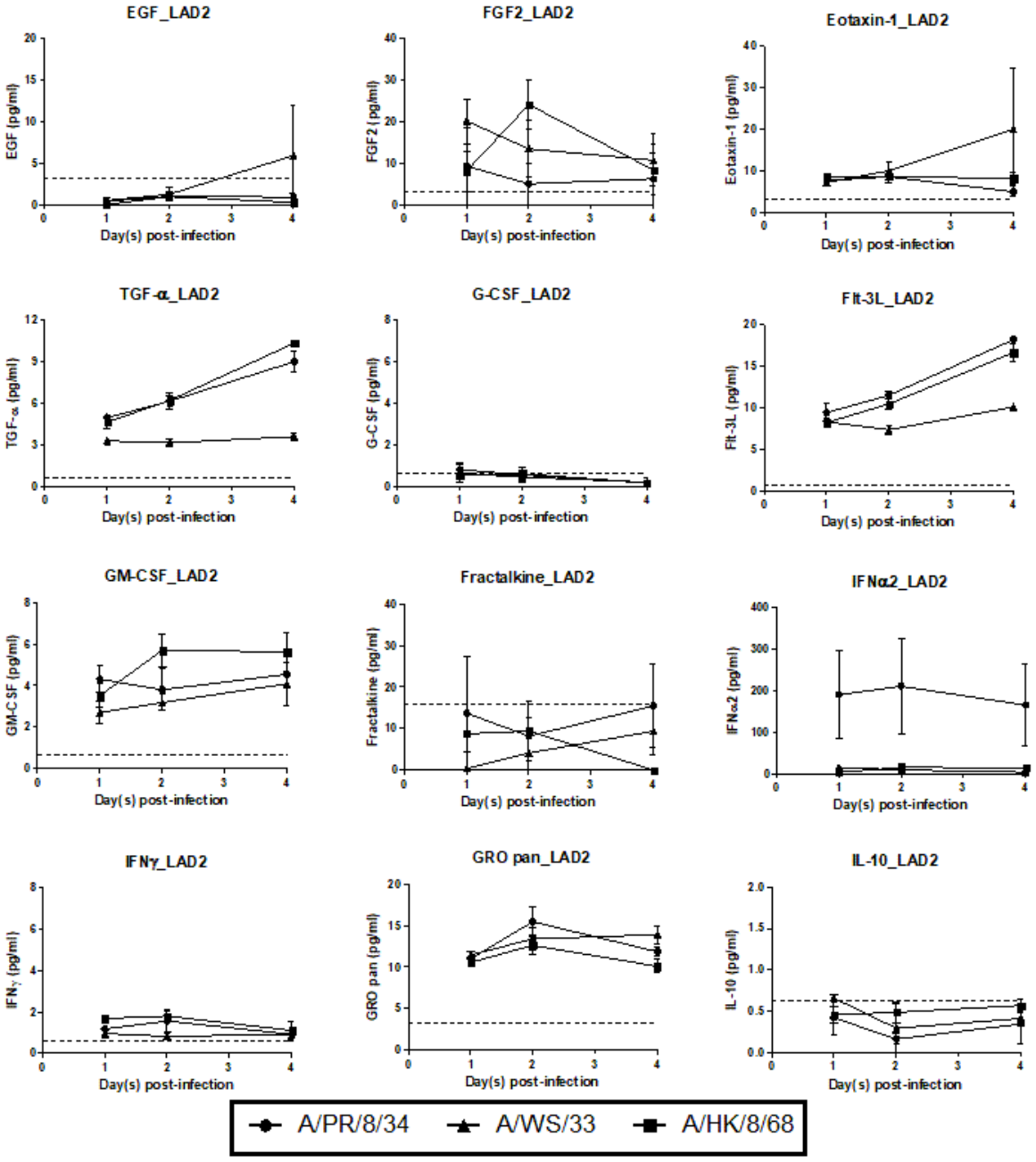

Figure A.5 Cytokine/Chemokine Release in LAD2. EGF, FGF2, Eotaxin, TGF-α, G-CSF, Flt-3L, GM-CSF, Fractalkine, IFNα2, IFNγ, GRO pan and IL-10 release in LAD2. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68) were used to infect LAD2. Supernatants were harvested on1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

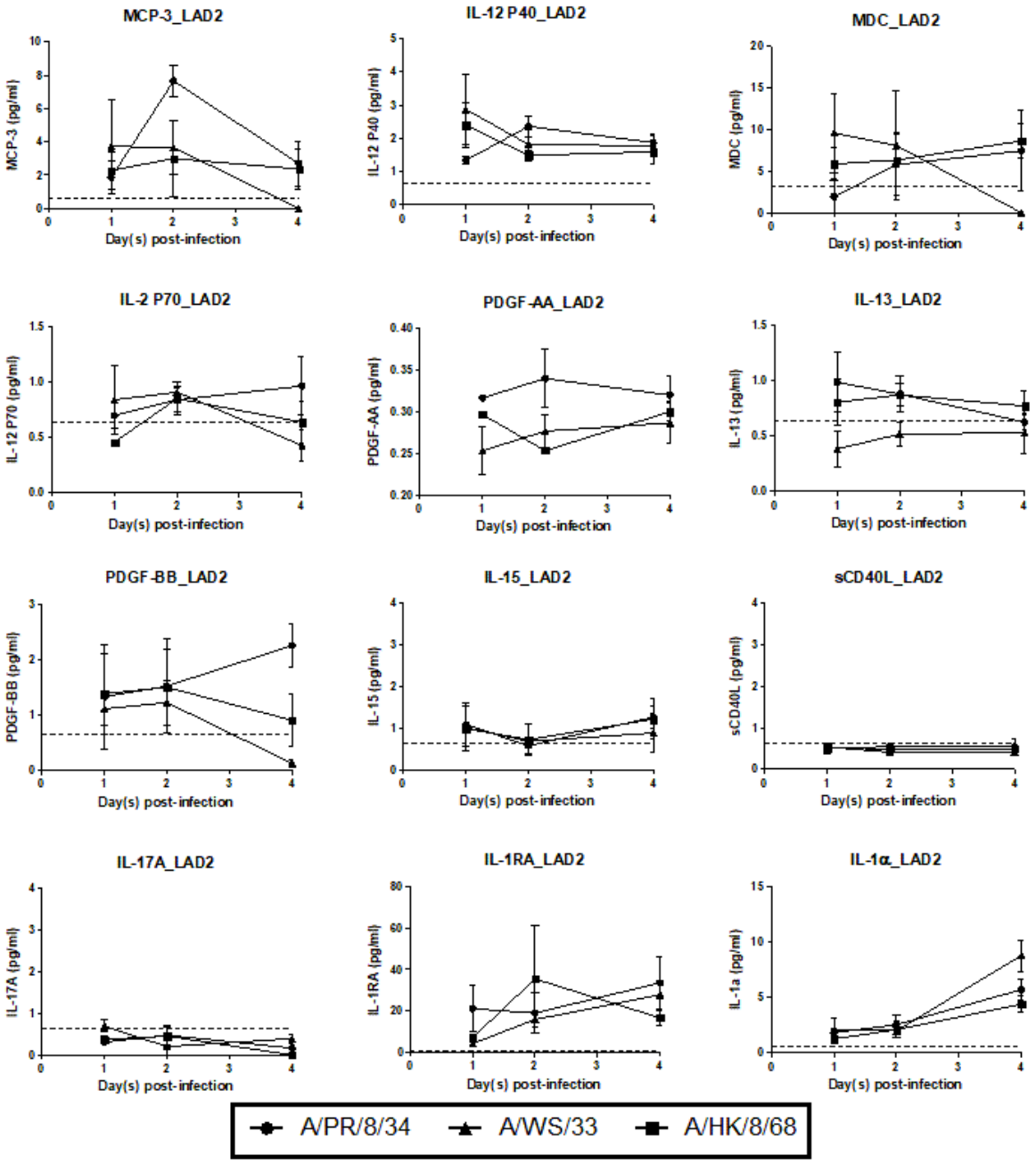

Figure A.6 Cytokine/Chemokine Release in LAD2. MCP-3, IL-12, MDC, IL-12 P70, PDGF-AA, IL-13, PDGF-BB, IL-15, sCD40L, IL-17A, IL-1RA and IL-1a release in LAD2. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68) were used to infect LAD2. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

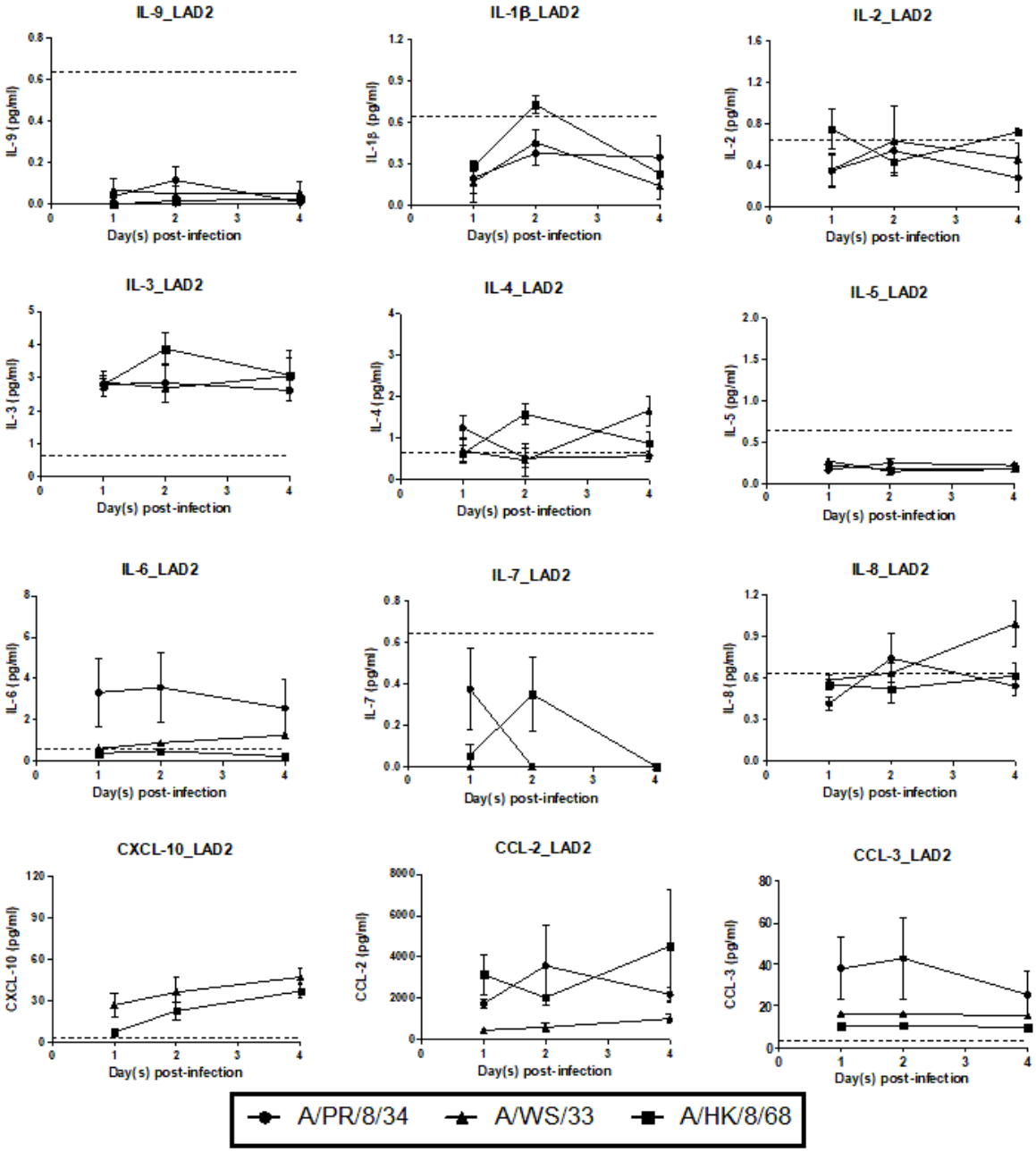

Figure A.7 Cytokine/Chemokine Release in LAD2. IL-9, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, CXCL-10, CCL-2 and CCL-3 release in LAD2. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68) were used to infect LAD2. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

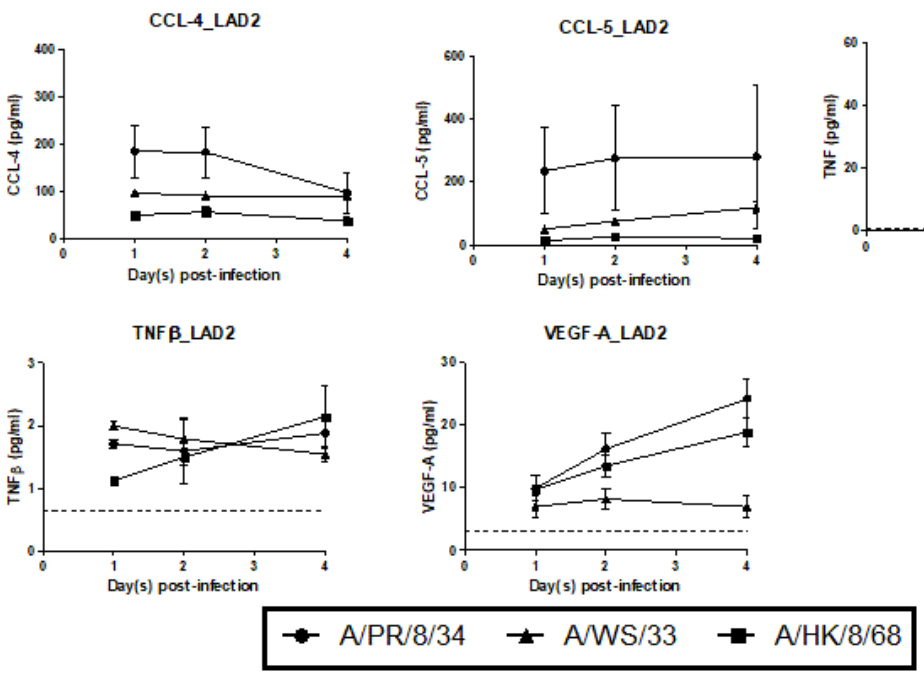

Figure A.8 Cytokine/Chemokine Release in LAD2. CCL-4, CCL-5, TNF, TNFβ and VEGF-A release in LAD2. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68) were used to infect LAD2. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

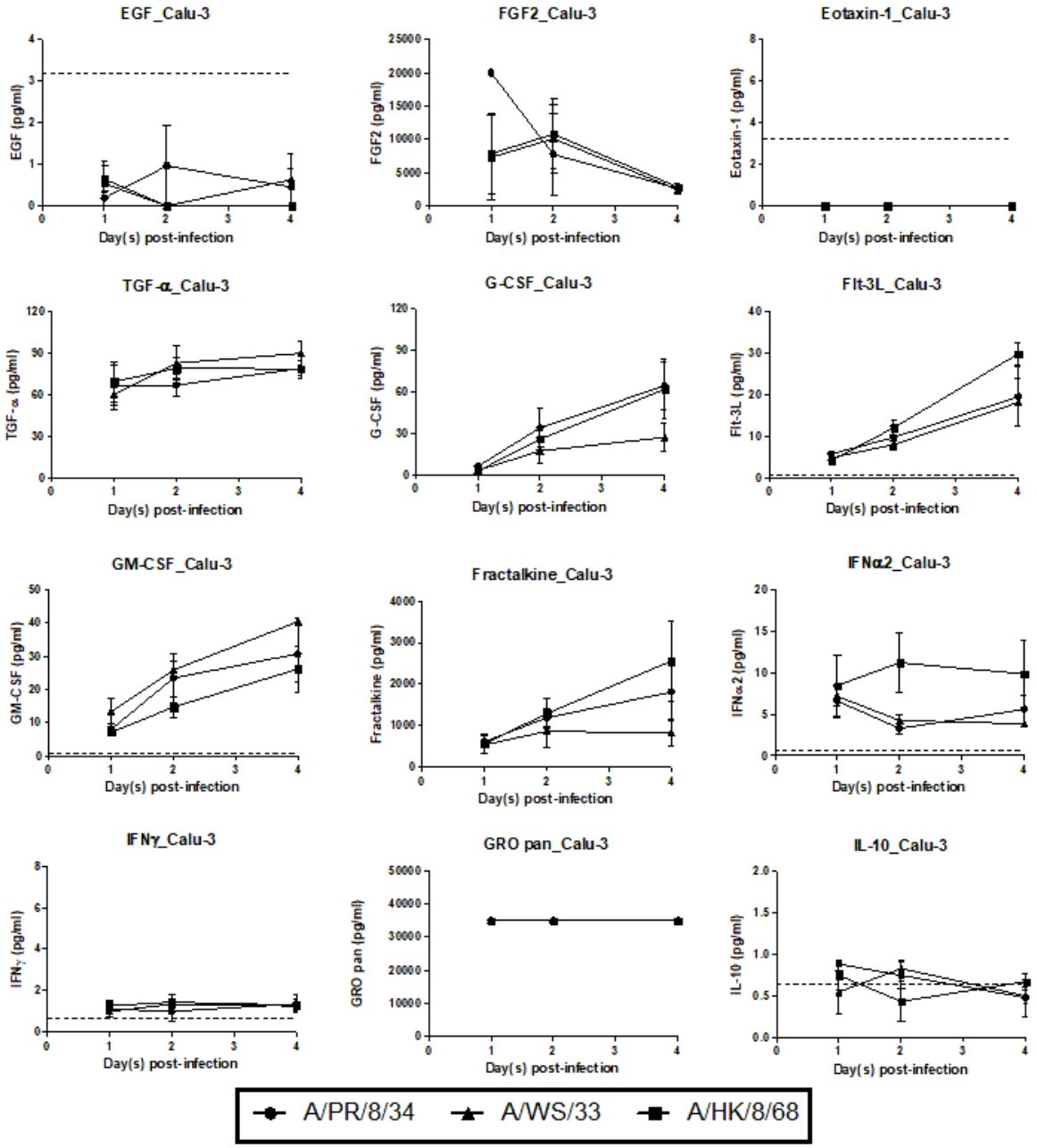

Figure A.9 Cytokine/Chemokine Release in Calu-3. EGF, FGF2, Eotaxin, TGF-α, G-CSF, Flt-3L, GM-CSF, Fractalkine, IFNα2, IFNγ, GRO pan and IL-10 release in Calu-3. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68) were used to infect Calu-3. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

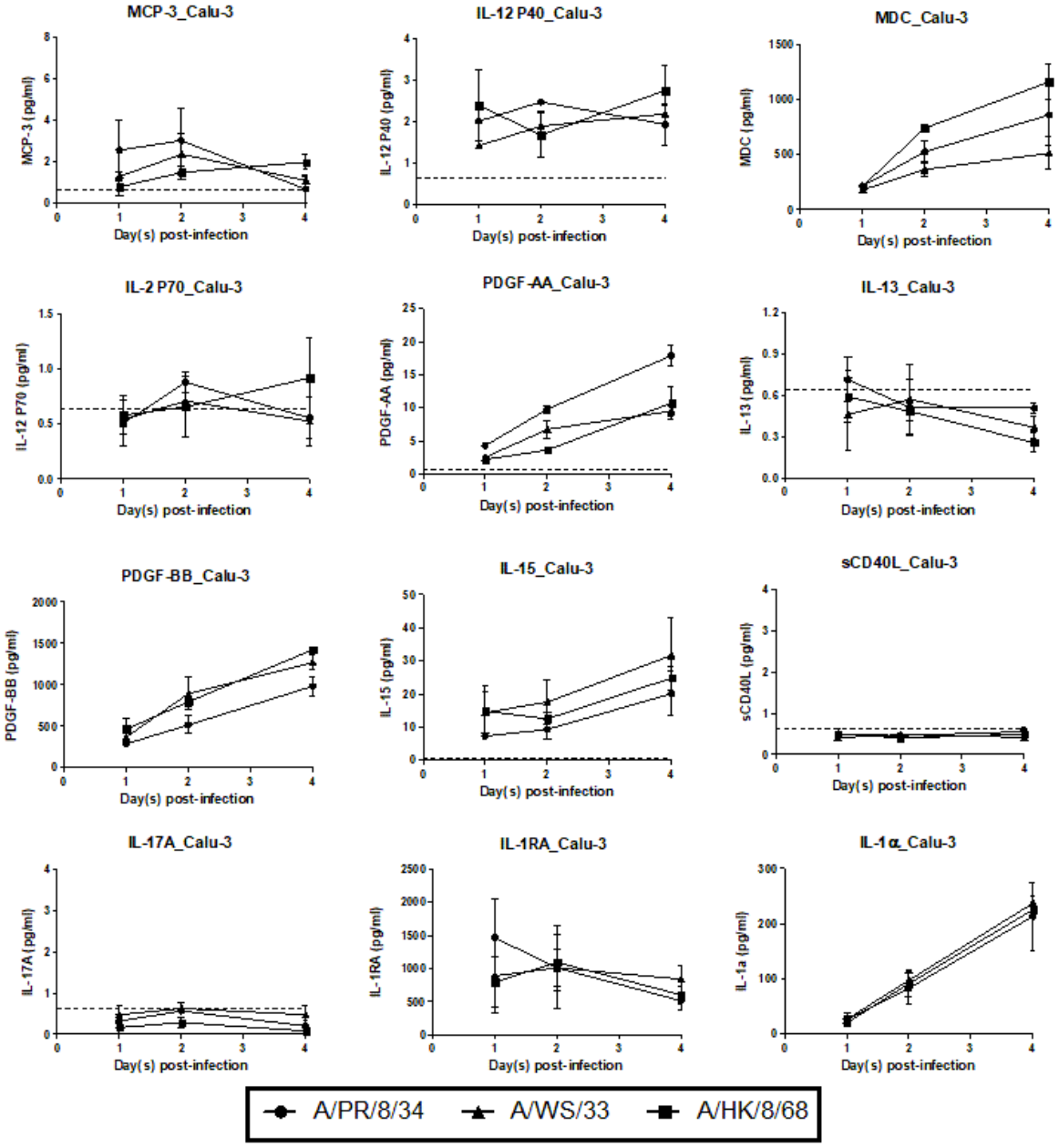

Figure A.10 Cytokine/Chemokine Release in Calu-3. MCP-3, IL-12, MDC, IL-12 P70, PDGF-AA, IL-13, PDGF-BB, IL-15, sCD40L, IL-17A, IL-1RA and IL-1a release in Calu-3. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68) were used to infect Calu-3. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

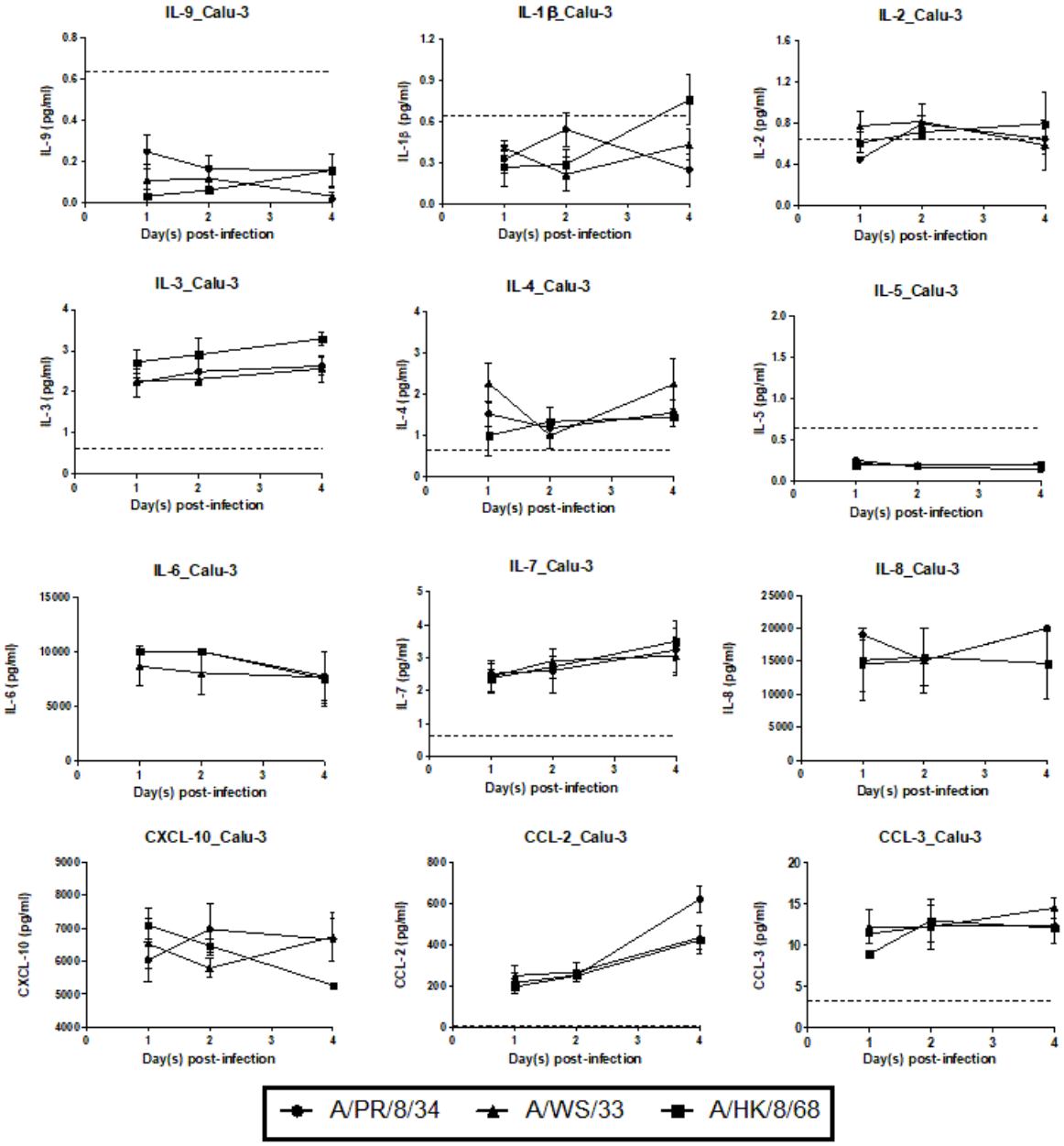

Figure A.11 Cytokine/Chemokine Release in Calu-3. IL-9, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, CXCL-10, CCL-2 and CCL-3 release in Calu-3. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68) were used to infect Calu-3. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

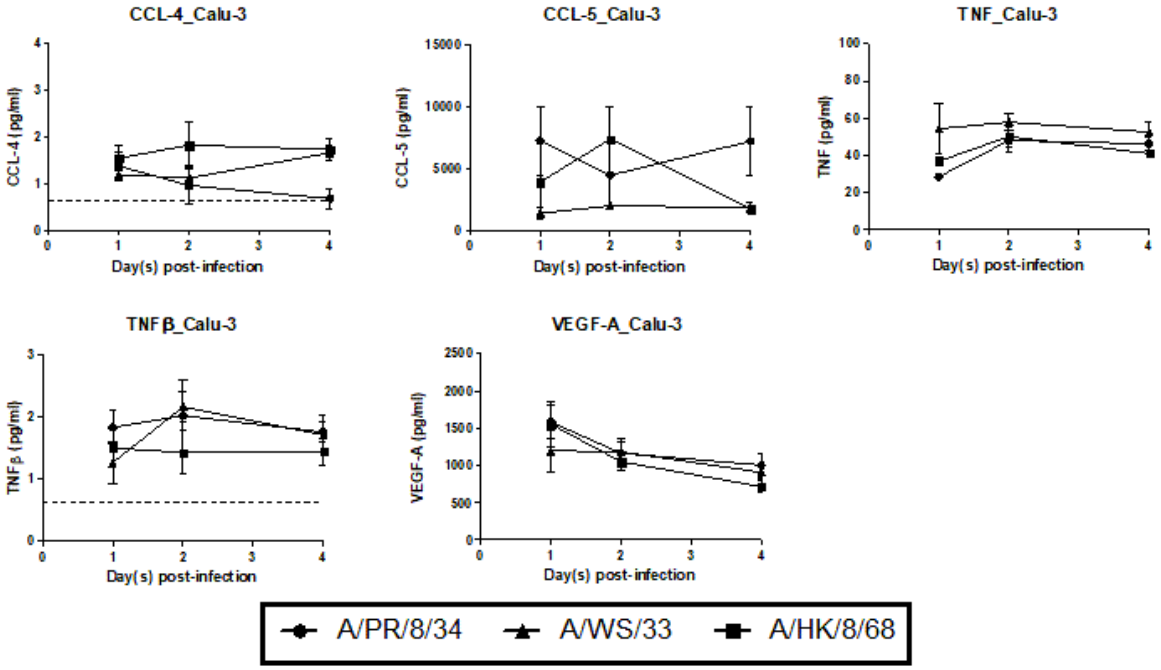

Figure A.12 Cytokine/Chemokine Release in Calu-3. CCL-4, CCL-5, TNF, TNFβ and VEGF-A release in Calu-3. Three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68) were used to infect Calu-3. Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

Appendix B

Supplementary data to Chapter III: Responses of Mast Cells and Epithelial Cells Following Exposure to Influenza A Virus.

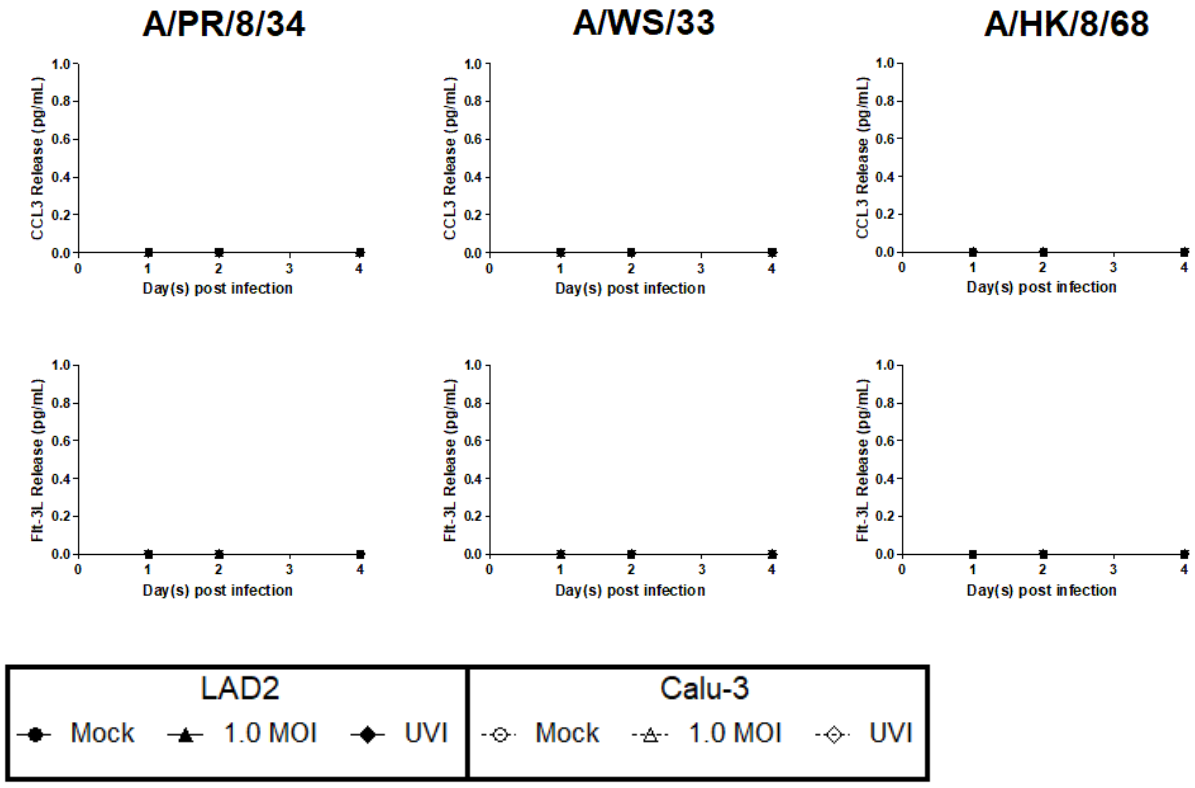

Figure B.1 Cytokine/Chemokine Release in LAD2 and Calu-3 Measured by ELISAs. CCL-3 and Flt-3L release was not detected in LAD2 and Calu-3 when exposed to three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68; 1.0 MOI). Supernatants were harvested on 1, 2 and 4 days post-FluA exposure (n=3).

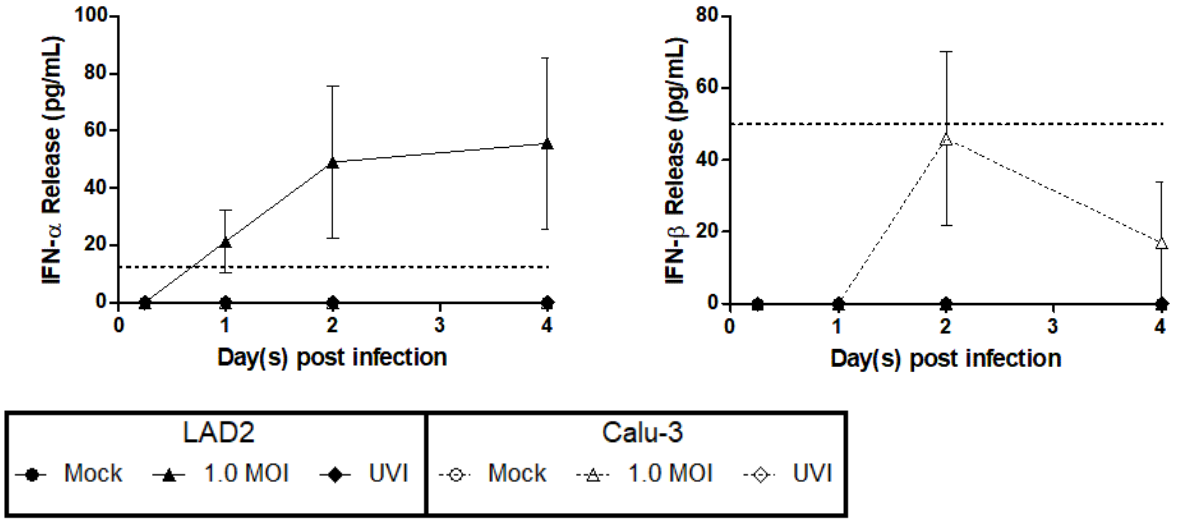

Figure B.2 Type I Interferon Release in LAD2 and Calu-3 Measured by ELISAs. CCL-3 and Flt-3L release was not detected in LAD2 and Calu-3 when exposed to FluA/PR/8/34 (1.0 MOI). Supernatants were harvested on 1, 2 and 4 days post-FluA exposure. Dotted line denotes detection limit of the ELISAs for the respective IFNs (n=3, performed by Chris St. Laurent).

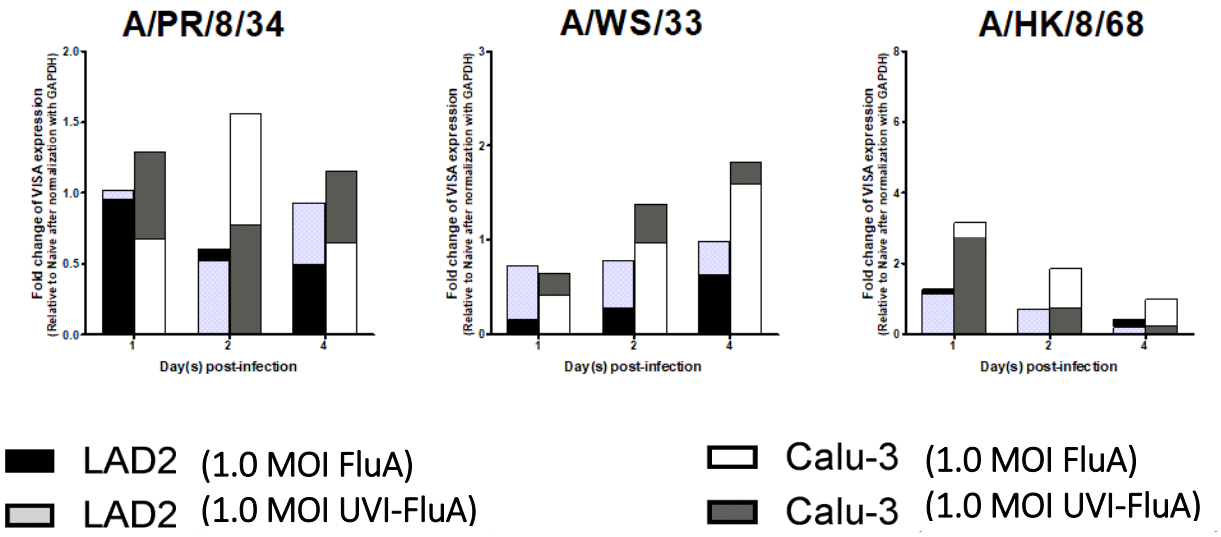

Figure B.3 MAVS mRNA Expression in LAD2 and Calu-3 After FluA Exposure. Changes in MAVS mRNA expression was not significant in LAD2 and Calu-3 when exposed to three FluA strains (A/PR/8/34, A/WS/33 and A/HK/8/68; 1.0 MOI). UV-inactivated virus was used as control. GAPDH was used as house-keeping gene to normalize data. All data are shown as relative fold change to naïve treatment (no viral exposure) (n=3; performed by Javeria Raheem).

Appendix C

Results of 41 Cytokine/Chemokine Multiplex Assay from EVE Technologies (Calgary, AB) were obtained from experiments performed by Tae Chul Moon. The following cytokine/chemokine release was measured in EC only cultures and EC-MC co-cultures:

EGF, FGF2, Eotaxin, TGF-α, G-CSF, Flt-3L, GM-CSF, Fractalkine, IFNα2, IFNγ, GRO pan, IL-10, MCP-3, IL-12, MDC, IL-12 P70, PDGF-AA, IL-13, PDGF-BB, IL-15, sCD40L, IL-17A, IL-1RA, IL-1a, IL-9, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, CXCL-10, CCL-2, CCL-3, CCL-4, CCL-5, TNF, TNFβ, VEGF-A.

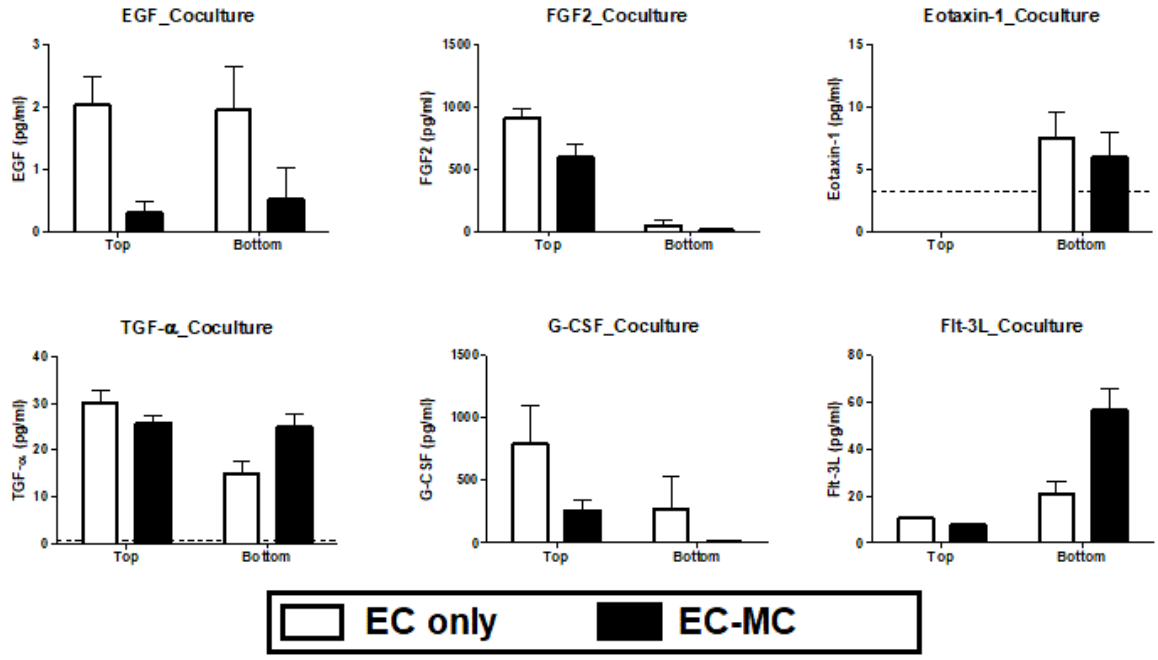

Figure C.1 Cytokine/Chemokine Release in EC-MC Co-cultures. EGF, FGF2, Eotaxin, TGF-α, G-CSF and Flt-3L release in EC only cultures and EC-MC co-cultures. FluA/PR/8/34 (0.04 MOI) were used to infect EC. Supernatants were harvested on day 5 post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

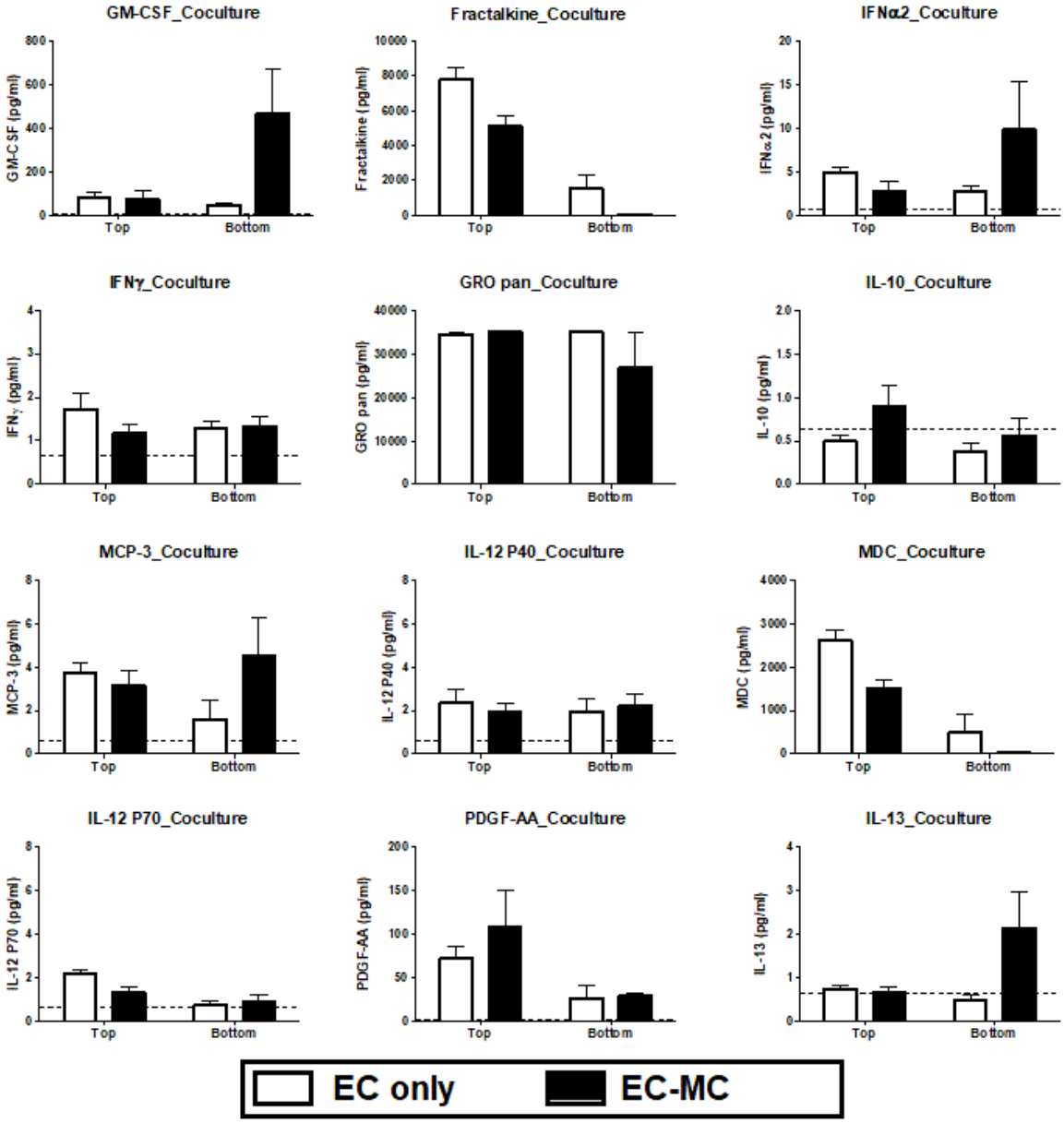

Figure C.2 Cytokine/Chemokine Release in EC-MC Co-cultures. GM-CSF, Fractalkine, IFNα2, IFNγ, GRO pan, IL-10, MCP-3, IL-12, MDC, IL-12 P70, PDGF-AA and IL-13 release in EC only cultures and EC-MC co-cultures. FluA/PR/8/34 (0.04 MOI) were used to infect EC. Supernatants were harvested on day 5 post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

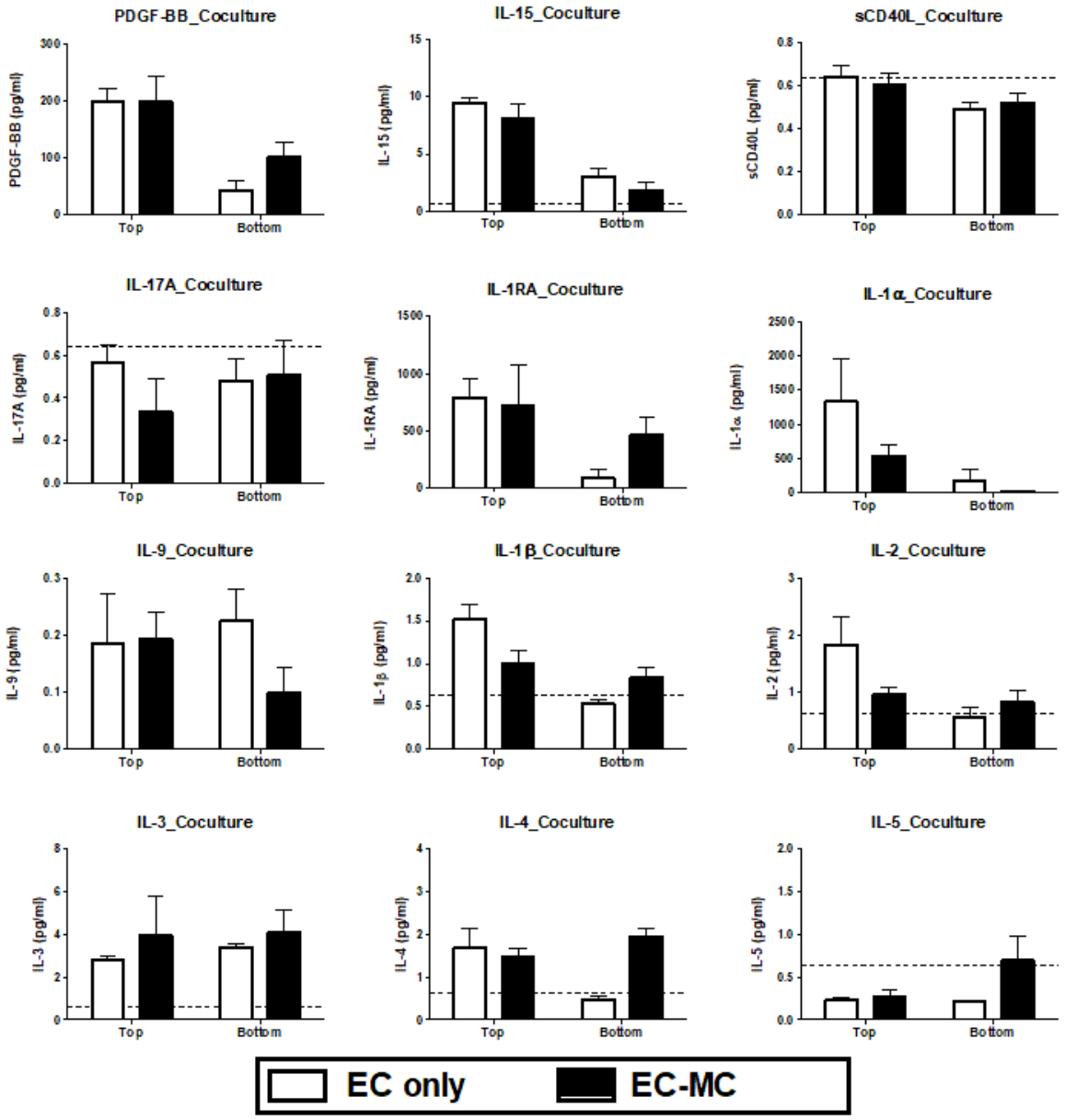

Figure C.3 Cytokine/Chemokine Release in EC-MC Co-cultures. PDGF-BB, IL-15, sCD40L, IL-17A, IL-1RA, IL-1a, IL-9, IL-1b, IL-2, IL-3, IL-4 and IL-5 release in EC only cultures and EC-MC co-cultures. FluA/PR/8/34 (0.04 MOI) were used to infect EC. Supernatants were harvested on day 5 post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).

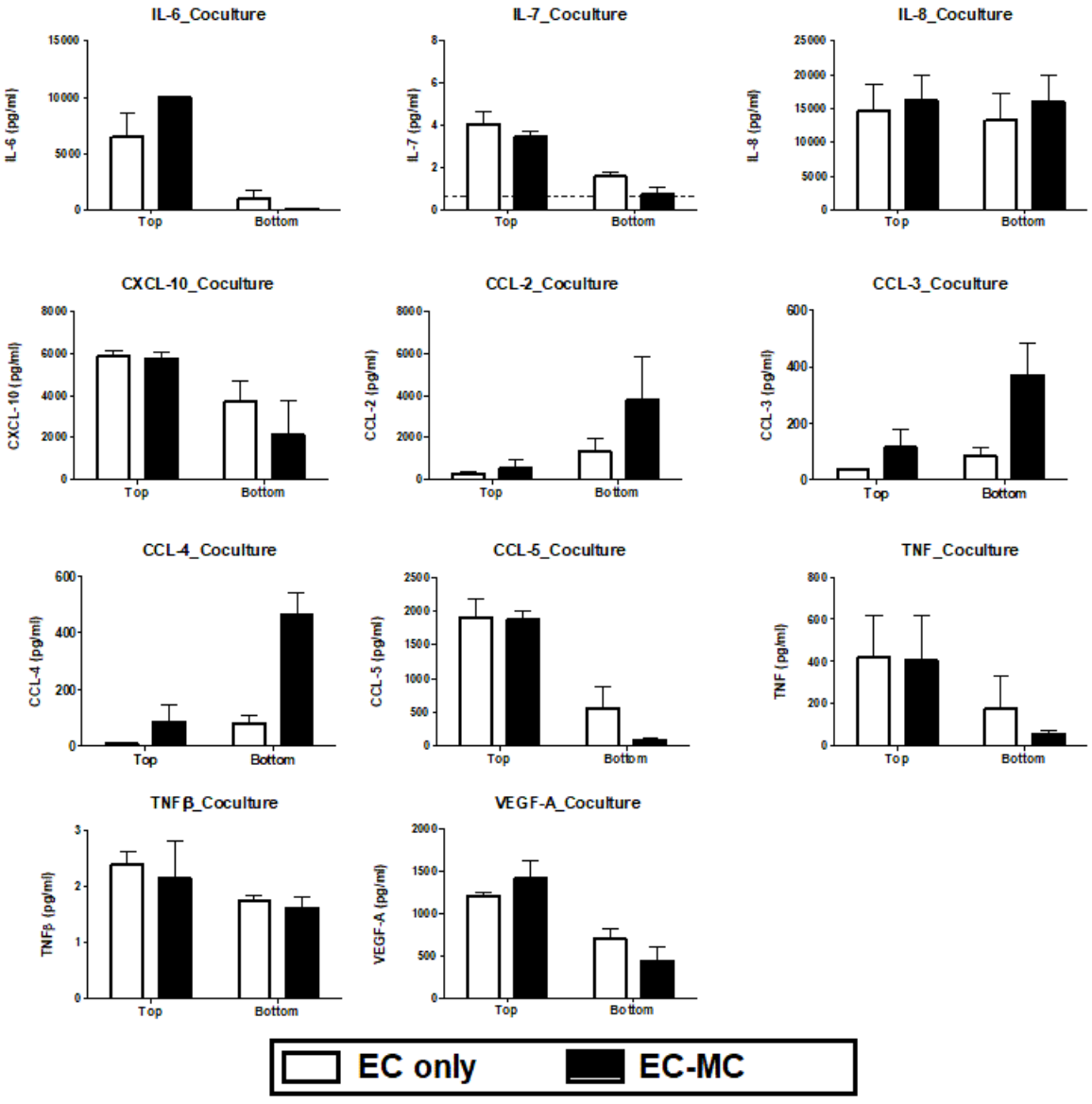

Figure C.4 Cytokine/Chemokine Release in EC-MC Co-cultures. IL-6, IL-7, IL-8, CXCL-10, CCL-2, CCL-3, CCL-4, CCL-5, TNF, TNFβ and VEGF-A release in EC only cultures and EC-MC co-cultures. FluA/PR/8/34 (0.04 MOI) were used to infect EC. Supernatants were harvested on day 5 post-FluA exposure. Dotted lines denote detection limit of the multiplex assay for the respective cytokine/chemokine (n=4).