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INTERFERON-γ MEDAITES LUNG DAMAGE THROUGH THE HYPER-ACTIVATION OF CCR2+ INFLAMMATORY MONOCYTES DURING INFLUENZA VIRUS INFECTION

by

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Bachelor of Science, University of Colorado at Colorado Springs 2017

A Thesis
Submitted to the Graduate Faculty

of the

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in partial fulfillments of the requirements

for the degree of

Master of Science

Grand Forks, North Dakota

August 2020

This thesis, submitted by Mitchell Anthony Klomp in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

_____
Chris Nelson
Dean of the School of Graduate Studies

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Date

PERMISSION

Interferon-γ Mediates Lung Damage Through the Hyper-Activation of $CCR2_+$ Inflammatory monocytes During Influenza Virus Infection

Department of Biomedical Sciences

Master of Science

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Mitchell Anthony Klomp
July 17, 2020

TABLE OF CONTENTS

LIST OF FIGURES

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DEDICATION

This thesis is dedicated to my family and loved ones who have supported me along the way.

I would like to thank my fiancé Ashley for her continued support and guidance throughout the years. The current pandemic postponed our wedding but my love for you grows more and more each day and it will continue to do so throughout our unexpected, prolonged engagement. Not only I but the world is a better place with you in it.

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ABSTRACT

Influenza viruses (IV) have caused untold morbidity and mortality throughout the previous centuries. These viruses have caused several pandemics that have witnessed the loss of millions of lives. Today, IVs continue to circulate annually, and the efficacy of influenza virus vaccines are limited. As IV pandemics in 1918, 1957, and 2009 have shown the world, the push for a universal influenza vaccine has never been more relevant. While much research has been devoted to understanding the virology around IVs, the immunopathology of the host-pathogen axis has been less studied. Recently, there has been much attention on the role of the immune response during IV infections as they pertain to damage to the host's lungs. Therefore, pathological immune mechanisms implicated in lung damage represent targets of therapeutic interventions aimed at mitigating the lung inflammation and at increasing resistance to influenza-associated bacterial pneumonia.

In the current study, we identified an interferon (IFN)-γ-regulated subset of monocytes, $CCR2_+$ monocytes, as a critical component facilitating the crosstalk between leukocytes and barrier cells, and a driver of lung damage in a mouse influenza model. IFN-γ regulated the infiltration and inflammatory phenotype of $CCR2_+$ monocytes in influenza-infected lungs. At the transcriptional level, IFN-γ deficiency attenuated the inflammatory phenotype of monocytes. Effector CD8 T cells were identified as the dominant cell type associated with pathogenic IFN-γ response in our influenza model. These previously unappreciated immune mechanisms highlight the immunoregulatory roles of IFN-γ response in regulating the monocyte inflammation and a molecule of therapeutic target in the future.

Chapter 1: Introduction

Influenza viruses (IVs) have caused death and destruction of pandemic proportions in both humans and animals. Seasonally in the United States, IVs infected over 35 million people in 2018-2019 and resulted in over 30 thousand deaths. IVs are spread through aerosol droplets via coughing or sneezing from an infected person. Contact with contaminated surfaces, like hands, and then coming into contact with the eyes or mouth is also a mode of transmission. The dry air of winter makes the spread of IVs much more common. Early in infection, IVs can be shed prior to the onset of symptoms making the spread of the virus much more likely.

The purpose of this study was to research the immune-related mechanisms that result in damage to the lungs during the acute phase of viral infection. We developed the hypothesis that IV results in the migration and accumulation of specific pro-inflammatory immune cells that are vital to viral clearance but damage the lung epithelium architecture as a consequence of viral infection. Further, the lung damage mediated but immune cells promote commensal bacterial adhesion, proliferation, and subsequent infection in the aftermath of IV infection.

This first chapter details the background of the study, specifies the problem, describes the significance of the problem, and briefly features the methodology used to show the importance of elucidating a mechanism for immune cell-mediated lung damage.

Background

Influenza viruses are negative sense, single stranded RNA viruses in the family *Orthomyxoviridae* (1). Their genome consists of seven or eight segments of RNA that encode for 11 genes and 11 proteins (1). Influenza A viruses (IAV), the family of IVs that have caused the

largest pandemics from the *Orthomyxoviridae* family, contain eight segments of RNA (1). IAV virions are approximately 80 to 120 nanometers in diameter depending on their genetic content and contain a membrane with embedded glycoproteins (1). Hemagglutinin (HA), a lectin, and neuraminidase (NM), an enzyme, further extend from the outside of the viral membrane and are intertwined within the genomic material inside of the membrane (1, 2). Along with the nucleoprotein intertwined with the genomic material, HA and NM serve to protect the viral RNA prior to viral entry into a cell (1, 2). These glycoproteins are also responsible for both the entry and release of the virus into and from the target cell respectively. Hemagglutinin on the surface of the virion binds to sialic acid residues on the surface of lung epithelial cells facilitating viral entry via endocytosis (2). Once endocytosed, the cellular compartment fuses with a lysosome and the pH decreases rapidly thereby releasing the viral genome, the nucleoprotein, and auxiliary proteins into the cytoplasm of the cell for replication (2).

IAV genomes are negative sense and single-stranded meaning they require an RNA-dependent RNA polymerase (RdRp) for replication (1). The viral genome is trafficked into the nucleus of the cell and subsequently transcribed via the viral RdRp (1-3). The new positive sense IAV RNA is released into the cytoplasm of the cell where the host's cellular machinery will translate the message into viral proteins (1-3).

Once the virus has replicated its genome from the newly synthesized positive sense RNA into negative sense RNA, the new virion begins the process of assembling at the rough endoplasmic reticulum and Golgi for export (3). NM on the surface of the new virions cleaves the cytoplasmic side of the sialic acid residues and triggers the release of the virion (2, 3). From there, the virus begins the process of infecting more lung epithelial cells.

The immune response to the viral infection often times indicates the outcome of the infected individual. Robust production of inflammatory cytokines increases the vascular permeability of capillaries that lead to the lungs. The subsequent influx of pro-inflammatory immune cells results in epithelial cell death and further production of cytokines. As a whole, acute respiratory distress syndrome (ARDS) can set in when the respiratory epithelial barrier that lines and protects the lungs becomes leaky. An increase in fluid into the lungs makes breathing difficult for the infected individual as more and more epithelial cells are directly killed by immune cells or undergo cellular death (apoptosis or necroptosis) from internal signals of infected cells. The end is result is significant morbidity and mortality caused by influenza virus infection.

Statement of the problem

In 1918, an H1N1 influenza A virus caused a global pandemic that lasted for 15 months from the spring of 1918 into the summer of 1919 (4). Over 500 million people, just over one-third of the world's total population became infected during this time (4). With a death toll ranging from 17 to 50 million people, this pandemic remains one of the deadliest viral outbreaks of all time (4). Today, there are seasonal influenza virus vaccines and a few notable anti-viral therapies that lessen the morbidity of the virus, but the efficacy of each treatment is limited. In 2019, the efficacy of the available seasonal vaccines was estimated by the CDC to be 45% (5). In 2009, another H1N1 influenza A virus caused a pandemic that brought significant morbidity but limited mortality (5). The CDC estimates that 60 million people became infected in 2009 and up to 500,000 people died across the globe (5). While limited in disease burden, the 2009 H1N1 pandemic underscores the capability IAVs have to cause pandemics thus, understanding how

they spread from person to person, replicate inside of their host, and subsequently cause disease is vital in hindering the next influenza virus pandemic.

Acute respiratory destress syndrome (ARDS) is a result of an over-zealous, damaging immune response initiated by the host (6). The mortality associated with influenza viruses during the 1918 pandemic was mostly the result of 20-59-year-old adults becoming sick and their immune response causing damage to their lungs (6). Since 1918, the field of immunology has sought to better understand the mechanisms employed by the host's immune response that both limit viral replication and limit damage to the host. Specifically, detailing both the innate and the adaptive immune response as they pertain to cellular damage during IAV infections has been limited in the field of influenza biology. Further, uprooting the mechanisms by which specific cells of both branches of the immune response, those being direct cell-to-cell interactions as well as intracellular signaling, is required to produce better therapies for people infected with IAV.

Purpose of the study

The purpose of this study was to elucidate the cause of pathology as a result of the host's immune response during IAV infection. The phenotype of key innate immune cells that have been associated with lung pathology were assessed during the height of the viral infection in mice. Thorough immune profiling of mice infected with a pandemic strain of H1N1 influenza virus resulted in a robust model by which innate immune cell recruitment, activation, and subsequent immune cell-mediated lung damage could be studied. From these studies, a subset of innate immune cells, termed inflammatory monocytes (IMs) were thought to cause damage in virus-infected lungs.

The specific protein chemoattractants (chemokines) that were required for innate immune cell recruitment were detailed via specific genetic knock out mice. Using the same model that was described early in the study, the lack of immune chemokines allowed for the study of influenza virus in the lungs of mice where damaging cells were absent. The lack of IMs provided a unique model by which pathology that results from immune cells could be analyzed. These studies provided insight into the roles of each subset of innate immune cells during viral infection.

While chemokines are responsible for the recruitment and migration of immune cells, how these damaging cells become activated is an important mechanism of study. IMs were found to be recruited into the lungs of IAV-infected mice. An influx of these cells coincided with an increase in the pro-inflammatory cytokine, IFN-γ. Without IFN-γ, the frequency of IMs in the lungs decreases significantly which correlated with a decrease in lung pathology.

As a whole, the purpose of this study was to analyze immune cell subsets that directly cause damage to the respiratory epithelium during IAV infection. The mechanism by which these cells clear influenza virions subsequently damages the host. Going forward, the study presented here provides a novel mechanism by which the host's immune response inadvertently causes significant morbidity during IAV infection and further suggests possible areas of intervention to decrease the observed seasonal morbidity caused by IAVs.

Research questions

The following research questions were used to guide the research as it progressed: (a) how do you model influenza virus infections in mice? (b) what is the role of ablating inflammatory monocyte recruitment to the lungs by global CCR2 knockout? (c) does IFN-γ

activate inflammatory monocytes and predispose them to be damaging? (d) what are the transcriptomic changes that occur during influenza virus infection and does knocking out IFN-γ alter the inflammatory state of monocytes? (e) what immune cells are the largest producer of IFN-γ during IAV infection? (f) what role does the adaptive immune response have in damaging the respiratory epithelium during IAV infection?

Conceptual framework

Influenza virus has been a staple in the modern vernacular for centuries. With reservoirs in both birds and swine, the opportunity for these viruses to continue to infect humans will not decrease. As H1N1 Influenza A viruses remained a constant threat from their emergence in 1918 until H2N2 viruses caused the next pandemic in 1957. In 2009, the swine flu pandemic brought the reemergence of H1N1 viruses. While not as deadly as the 1918 pandemic, the total number of infected people ranged from 500 to 700 million people. A century later, scientists have studied and mapped virtually every aspect of influenza virus in terms of its replication, transmission, virulence factors, assembly, and epidemiology. However, the mechanisms by which our immune systems react to influenza virus in regard to initial viral recognition, clearance of the virus, and subsequent immune memory has left more questions than answers.

The studies that have provided the framework for studying influenza virus, as well as studying host-pathogen interactions, will be reviewed in chapter 2. The focus of this study is aimed around analyzing several of the early immune cell-virus recognition events to determine what drives lung pathology during acute viral infection. To assess lung pathology during acute viral infection several working hypotheses were tested under the framework discussed below.

1. Mice infected with influenza virus A/PR8/H1N1 will experience significant morbidity via weight loss, posture, and behavior.
2. Cellular influx into the lungs during PR8 infection will be assessed via hematoxylin and eosin (H&E) staining. Determination of large airway inflammation will also be determined via H&E staining.
3. The identify, or phenotype, of the cells in the lungs of mice infected with PR8 will be analyzed via flow cytometry.
4. Changes in the transcriptome of monocytes will be analyzed via RNA sequencing and the data will be analyzed in an unbiased, blinded manner.
5. Cellular source of pro-inflammatory cytokines that contribute to immunopathology will be determined via intracellular cytokine staining.

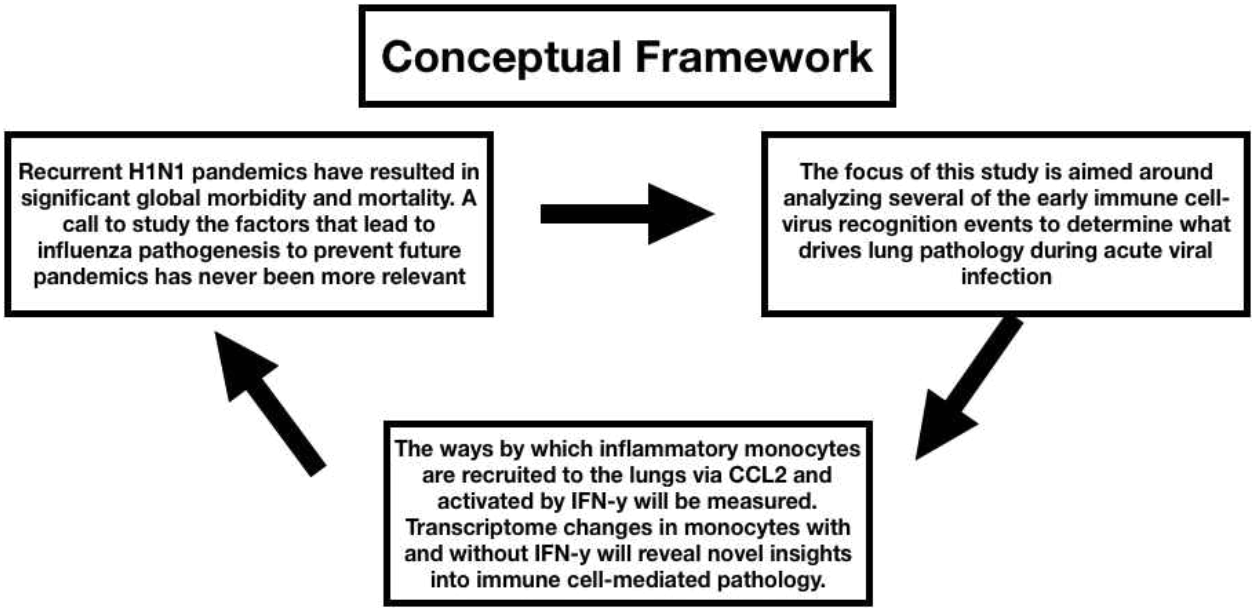

Figure 1. Conceptual Framework

Design and procedures

This study used C57BL/6 mice as an animal model to study influenza virus infections as it pertains to viral-host interactions in the lungs. Mice were lightly anesthetized using isoflurane and 250 plaque-forming units (PFU) of IAV were intranasally administered in 100 µL cold PBS into each mouse. For several experiments, CCR2$^{-/-}$ and IFN-γ$^{-/-}$ mice were used. Knock-out mice were bred on a C57BL/6 background and bred in-house or purchased from Jackson Labs. Knock-out strains were verified using PCR for presence or absence of the gene of interest. In all experiments, an equal number of control mice that were lightly anesthetized with isoflurane (in the same manner as experimental mice) and administered 100 µL cold PBS without IAV. Control mice were age and sex matched with experimental mice for every experiment. Mice were monitored daily to assess morbidity via changes in body weight. The percentage of body weight lost in IAV-infected mice was measured where a 20% decrease from the starting weight was deemed the cut-off point for euthanasia. After the mentioned days of infection, mice were euthanized, and their lungs were processed for a variety of experiments.

The large lobe of the lungs was perfused with 1 mL 4% formalin and subsequently paraffin embedded for histological staining. Hematoxylin and eosin (H&E) staining was performed to analyze the (a) structure of the airways post-IAV infection (b) cellular influx into the lungs (c) inflammation around the large airways.

The three smaller lobes were excised using sterile surgical tools. One lobe was fixed in RNA-Later solution for RNA extraction and analysis. Another lobe of the lungs was homogenized in a solution containing protease and phosphatase inhibitors for protein quantification and protein analysis. The remaining lobe of the lungs was used to analyze cellular populations within the lungs of mice via flow cytometry. A single cell suspension was

performed, and single cells were immediately surface stained with pre-chosen fluorophore-conjugated antibodies and then analyzed on a flow cytometer.

Flow cytometric analysis was performed using a BD Symphony flow cytometer. All fluorophore-conjugated antibodies were purchased from BioLegend. Briefly, single cells were washed and then stained with the optimal, pre-determined concentration of antibodies in 50 µl of wash buffer (2% FBS in cold PBS). Cells were stained for 40 minutes in the dark at room temperature before being washed twice with wash buffer. For intracellular staining, cells were permeabilized after surface staining using the BD fixation/permeabilization kit (BD) and then an optimal concentration of anti- IFN-γ antibody was added in 50 µL permeabilization buffer. The cells were then washed with permeabilization buffer and then normal wash buffer. Cells were immediately analyzed on a flow cytometer using pre-set conditions. The flow cytometer was compensated for each color in order to minimize spectral overlap between colors. The data acquired from the flow cytometer was analyzed using FlowJo (TreeStar) software.

Bronchoalveolar lavage (BAL) fluid was also collected from mice after euthanasia. Cells and cellular debris were removed from the BAL fluid and cytokine and chemokines were measured via LEGENDPlex bead array (BioLegend). Bead arrays are a novel technique that allows for the measurement of proteins concentration in 12 analytes in a single experiment.

This study also utilized RNA sequencing from sorted monocytes to determine total transcriptomic changes in inflammatory profiles. Seven days post-IAV infection, uninfected C57BL/6 mice (mock) C57BL/6 mice infected with IAV (WT-PR8), and IFN-$\gamma^{-/-}$ mice infected with IAV (IFN-$\gamma^{-/-}$-PR8) were euthanized and whole lungs were aseptically collected. After preforming a single cell suspension, single cells were sorted via magnetic separation and column filtration (Miltenyi). RNA from the sorted monocytes was extracted using a Qiagen RNA

extraction kit (Qiagen) and subsequently PolyA RNA-Seq libraries were prepared using a NEBNext Ultra II RNA-Seq library kit. Once the adapters from each read were trimmed, the reads were aligned to a mouse genome (mm10) with HISAT2. Differentially expressed genes (DEGs) between groups were identified using the R/Bioconductor package DESeq2 v.1.24.0. The cutoff for DEGs was determine using an adjusted p value of less than 0.05. Heatmaps were created using Log2-fold change of individual genes from each group of monocytes. Pathway enrichment analysis was performed using Ingenuity Pathway Analysis (IPA). Canonical pathways were identified with a $\log_{10}$ significance (p-value) cutoff of 1.3.

Data are presented as the mean ± SEM. Multigroup analyses were performed using one-way ANOVA, followed by Tukey's *post hoc* analysis. Comparisons between two groups were performed using the Mann-Whitney nonparametric test. Statistical analyses were performed with Prism 7.04 (GraphPad Software, La Jolla, CA, USA).

Significance of the study

This study is significant because it shows how IFN-γ drives lung damage mediated by $CCR2_+$ monocytes during influenza virus infections. The field of influenza virology has long tried to decipher the mechanisms by which the host's immune response damages the lungs in order to clear the viral infection. The development of an influenza virus mouse model, coupled with knock-out mouse models to study the role of CCR2 and IFN-γ during IAV infection, have provided insight into IAV pathogenesis and lung pathology mediated by the host's immune response. Information can be drawn from this study to impact the outcome of people with severe immunological reactions to IAV. Further, these findings lay the groundwork for understanding the pro-inflammatory versus anti-inflammatory paradigm of inflammatory monocytes, and

potentially lung-resident macrophages, as they pertain to IAV infections. Going forward, this study will held elucidate mechanisms employed by the immune response that clear viral infections in the lungs but also cause severe cellular damage as well.

Limitations of the study

This study tested the hypothesis that IFN-γ drives lung damage through the activation of $CCR2_+$ monocytes during influenza virus infection. As with any study, there are inherent limitations that must be considered.

The researcher has noted the following limitations: (a) the long-term consequences of removing IFN-γ from IAV-infected mice, and thus, a large component of viral clearance, must be considered and the ramifications of potentially chronic rather than acute viral infections noted (b) transcriptome changes via RNA-sequencing analysis from sorted monocytes must also be tested on the protein level rather than the just the RNA level in order to test for the observed differences (c) *in vitro* studies of direct cell-cell interactions where IMs induce apoptosis via TRAIL or FasL should be performed to analyze the extent to which these leukocytes have in a controlled setting (d) subsets of helper T cells should be analyzed to address their role in the activation of the humoral immune response, the anti-inflammatory cellular response, and the production of various cytokines that further interact with all branches of the immune response (e) without IFN-γ, the class-switching capabilities of B cells to produce IgG against IAV should be analyzed as means of preventing future IAV infections.

Delimitations of the study

The researcher imposed the following delimitations to this study: working with model organisms can be difficult to control for as each living organism is inherently different thus producing implicit bias into each study. The researcher treated each mouse as if they were the exact same but limitations on differences in immune function between mice cannot be altogether dismissed. Further, interpretation of the data may also produce limitations.

The researcher made the following assumptions throughout the study: (a) collecting 100,000 events for flow cytometry is unbiased and provides an accurate representation of the total number and frequency of cells in the lungs. (b) the collection of 10,000 events for cytokine bead array analyses provides an accurate representation of the analytes measured in the experiment via flow cytometry. (c) both control and experimental mice were treated in a similar manner such that isoflurane and carbon dioxide were held constant when anesthetizing and euthanizing mice respectively. Isoflurane and carbon dioxide did not skew the data or alter the findings in any meaningful way. (d) only mice bred on a C57BL/6 background were used and knock-out mice bred on this background were the same as wild-type mice with the exception being the gene/protein that was knocked out. (e) measuring weight loss in mice throughout infection is a measure of morbidity and severity of disease. (f) the processing of tissues for protein analysis, RNA extraction and measurement, fixation and histological analysis, and single cell suspensions were the same through each experiment and any observed differences are due to the independent variable rather than experimental factors. (g) 100 μL of PBS containing 250 PFUs of PR8 for experimental mice or simply PBS were administered to each mouse and as such, the virus reached beyond the nasopharynx into the deeper alveoli of the lungs.

Definition of terms

Throughout the course of experimentation, the researcher has chosen terms and phrases that are common and are clarified below. The origin of each term is cited following the definition of the term:

Acute Respiratory Distress Syndrome. Type of respiratory failure characterized by the onset of inflammation into the lungs causing an increase in fluid and organ failure due to the lack of oxygen (Fan, 2018).

Alveolar Epithelium. The alveolar epithelium is comprised of alveolar type (AT) I and ATII cells where ATII cells primarily produce pulmonary surfactants essential for efficient gas exchange and for maintaining alveoli's structural integrity and contribute to lung defense by secreting antimicrobial factors, cytokines, and chemokines (Herzog 2008, Crouch 2001).

Apoptosis. Programmed, controlled cellular death (White 2011).

C-type lectin receptor. CLR, family of transmembrane and soluble receptors that contain carbohydrate recognition domains to recognize a wide variety of glycans (Lavelle 2010).

Chemokine. A class of cytokine that serves as an attractant for white blood cells (Stegelmeier 2019).

Co-infection. Simultaneous infection of a host with multiple pathogen species (Keller 2016).

Cytokine. Proteins, such as interleukins, interferons, and growth factors, that are secreted by cells of the immune system to affect the function of either themselves or other cells (Stegelmeier 2019).

Flow Cytometry. Experimental technique used to measure and detect the physical and chemical properties of a population of cells or particles (Herzenberg 2006).

Hemagglutinin. Hemagglutinin is the major glycoprotein responsible for viral entry into cells of the upper respiratory tract by attaching to sialic acid residues (Wilson 1981, Boonsta 2018).

Leukocytes. White blood cell derived from hematopoietic stem cells in the bone marrow; primarily innate immune cells: monocytes, macrophages, eosinophils, neutrophils, mast cells, and basophils (Lamichhane 2019).

Necroptosis. Cell-programmed necrosis is the phenomenon by which cells uncontrollably die and release their intracellular content into the periphery (Majno 1960, Dhuria 2018).

Neuraminidase. Also called *Sialidase*, an enzyme on the surface of Influenza Viruses that cleave glycosidic linkages of neuraminic acids (McCullers 2003).

Nucleotide binding and oligomerization-domain like receptor. Intracellular pattern recognition receptor that recognizes conserved molecules via phagocytosis or endocytosis; expressed on immune cells and epithelial cells in the lungs (Crystal 2008, Lavelle 2010).

Pandemic. An outbreak of a disease that is not limited to one area but rather, has spread globally (Pleschka 2013).

Pattern Recognition Receptor. PRR, germline encoded sensors of the innate immune response that recognize conserved molecules typical of pathogens or damaged tissue (Choi 2019).

Respiratory epithelium. Ciliated columnar epithelium that lines the respiratory tract and mucosal membranes in the lungs to moisten and protect the airways (Hansen 1975).

Retinoic acid inducible gene-1-like receptor. RLR, recognition of conserved viral molecules in the cytoplasm of immune cells (Lavelle 2010).

RNA-dependent RNA-polymerase. Enzyme responsible for the replication of RNA from an RNA template, typically coded for in the genome of a virus or encapsulated within the viral capsid/envelope (Hay 2001, Pleschka 2013).

Toll-like receptor. TLR, expressed both intracellularly and extracellularly by cells of the innate immune response and recognize structurally conserved molecules typical of pathogens or host proteins (Lavelle 2010).

Summary

Chapter 1 presented background for the study, specified the unique problem with host-pathogen interactions, the significance of that problem, and concluded with a brief overview of the context and methodology used for the study. The first chapter described the limitations of the study and why these limitations are important. In chapter 2, a review of the relevant and current literature will be presented. The definitions of key words and phrases were included in chapter 1 for brevity and clarity prior to the review of the literature. The virology of influenza virus, the physiology of the respiratory epithelium, and the immunological aspect of the host-pathogen interactions is summarized in chapter 2. The related impact of different immune cells on targeted epithelial cell death as well as effector cytokines that prolong the pro-inflammatory immune response will also be analyzed in the next chapter. Chapter 3 presents the method design of the study including the animal model that was used and the relevant. The methodology behind each experiment as well as how the data were collected is also discussed. The results derived from the experimental design described in chapter 3 will be presented in chapter 4. Each experiment as well as statistical analyses used to determine the significance of the data is explained as well. Chapter 5 will conclude with the limitations and further research that can be done after detailed

analysis of the data. The research described throughout aims to provide a mechanism by which interferon-γ activates inflammatory monocytes in the lungs during acute influenza A virus infection thereby creating a hyper-inflammatory response in the lungs that damages the respiratory epithelium of the host in addition to clearing the virus.

Chapter 2: Literature Review

Purpose of the review

In reviewing the extant literature regarding the mechanisms by which influenza virus promotes lung damage during acute infection, numerous labs have published a variety of studies that detail different aspects of this medically-relevant problem. However, correlating the specific role monocytes and early innate immune cells with influenza virus infection is poorly understood and thus, this review serves as the framework by which the hypotheses tested were conceived.

The purpose of this review is to identify background information regarding the virology and immunology of influenza viruses so as they are better understood from a functional aspect as well as fully understand how influenza viruses a) are transmitted b) how they enter a cell c) how they replicate inside of cells and, d) how influenza virions escape from their host cells to infect other cells. Next, this review serves as a basis for non-human influenza virus infection models. Influenza viruses infect a broad range of creatures but determining the most cost-effective, ethical, and useful animal model is of great importance when conducting virus research. This review details how the field of influenza virus research as well as research into the immune system has used specific mouse models to study influenza viruses. The last goal of this literature review is to underline the significance of the studies performed here and specify the gaps in our current understanding of influenza virus-mediated lung damage.

The intent of this review is to provide members of the public, educational leaders, and research scientists alike on the medical relevance of lung damage during influenza virus infection that is a result of the host's immune response as well as highlight where the field of virus-based immunology is heading.

This review will cover a variety of topics and will be guided by the questions: (a) how does influenza virus infect its host and spread from person to person?(b) What are the mechanisms of lung damage that are mediated by the virus, monocytes and macrophages, other innate cells that are recruited early in infection, and the role of the adaptive immune response in damaging the lungs? (c) How do interactions between epithelial cells in the lungs and early immune cells mediate inflammation? (d) What are currently used and potential therapeutic interventions that may disrupt the crosstalk between epithelial and immune cells during acute influenza virus infection?

The body of literature regarding influenza viruses and the innate immune response separately is quite large and thus, they are separately beyond the scope of this review. Further, there are many other respiratory viruses besides influenza virus that cause acute respiratory infection and damage. The focus of this review is solely based on influenza viruses. Further, the damage mediated by influenza virus infection can often times be the result of other non-viral factors such as immunodeficiencies or recurrent lung damage, like allergies or asthma. This review focuses only on acute influenza virus infections and the damage caused by the innate immune response. Through a thorough review of the literature, the framework by which influenza virus mediates damage in the host's lungs and the role of the host's immune response in further damaging the lungs will be displayed and result in a greater understanding of such a medically and economically relevant problem.

Literature search procedures

The literature reviewed for this chapter were accessed through several online databases including PubMed and Google Scholar. Print editions of several articles were also used when the

specific article could not be accessed online. Every article used for this review is experimental and data driven. Qualitative research was not included. Non-peer-reviewed or pre-published articles were not used in this review as every article that was used for this review was accessed from a published, peer-reviewed scientific journal.

Methodological issues in the research

During the review of the literature, a recurrent problem emerged where correlative evidence of epithelial cell damage in the lungs mediated by early, innate immune cells was scarce. Further, detailed analyses of monocytes and the mechanisms by which they are recruited to the lungs during influenza virus infection was limited in both characterization of these cells in the lungs and recruitment of monocytes into the lungs. Therefore, multiple sources for each topic were used to piece together the gaps in the literature. The synthesis of virology, immunology, and in some instances, pulmonology represented in this review were needed to better understand how the host's response to acute viral infection mediates the majority of the damage observed in the lungs.

Another problem that presented itself was the lack of epithelial cell studies in response to ligands on functional monocytes, influenza virus envelope proteins, and a variety of pro-inflammatory cytokines. This review details a variety of articles that use these effector molecules separately as a means to synthesize the mechanisms of lung damage.

Inclusion and exclusion criteria for literature review

As noted earlier, quantitative, published, peer-reviewed articles from distinguished journals were used in this literature review. The recency of the articles was limited to the past 20

years. When addressing influenza viruses, studies that used animal models, typically mice, or human clinical trials were used. Other animal models, such as ferrets or apes, were excluded on the basis of cost, ethics, and availability of the animals. Typically, studies that used adult subjects were used and young or elderly subjects were excluded due to the biological relevance of pandemic influenzas viruses causing mortality in adults (ages 20-59 typically), the ethics of working with juvenile mice, and the length of time and ethics of working with aged mice.

Influenza Virus Overview

Influenza viruses belong to the viral family, *Orthomyxoviridae*, and contain a multi-segmented, negative-sense, single stranded RNA genome (1). Influenza is also an enveloped virus derived from the target cell that includes two major glycoproteins and the M2 ion channel (1). These two glycoproteins are hemagglutinin (HA) and neuraminidase (NA) (1-3). The envelope surrounds the viral core which houses the viral genome (1). The proteins inside of the envelope form intricate interactions with the viral genome to create the viral nucleoprotein (NP) (1). The nucleoprotein encapsulates the viral genome until the protein components become active (1). Influenza virus genomes have eight different segments that encode for between 11 and 14 genes depending on the strain of virus (1).

Hemagglutinin is the major glycoprotein responsible for viral entry into cells of the upper respiratory tract via the attachment of sialic acid residues. There are 18 different subtypes of hemagglutinin but HA1, HA2, and HA3 are found in human influenza viruses (1-3). Hemagglutinin is an integral membrane trimer consisting of three monomeric subunits (3). Each monomer consists of HA1 and HA2 regions that are connected by two disulfide bridges (3). Taking a cylindrical shape, the function of HA is pH-dependent where at neutral pH, the peptides

at the N-terminus responsible for membrane fusion between the virus and the host cell is concealed in a hydrophobic pocket (1-3). Upon recognition of monosaccharide sialic acid residues of target upper respiratory cells, the HA1 portions of HA will bind and attach the viral envelope to the outside of the target cell (3). Once attached, the cell will engulf the viral particle via receptor-mediated endocytosis and form an endosome inside of the cell (3). Once endocytosed, the endosome will begin to acidify after fusing with a lysosome (3). As the lysosome containing the virus acidifies (and the pH decreases), the fusion peptide component of the HA1 subunit will dissociate with the H2A domain which allows for a conformational change to the H2A subunit (3). Through this conformational change, the H2A subunit brings the inner membrane of the lysosome into close contact with the virus's envelope which allows for the fusion of the two membranes (3). Simultaneously, the acidification inside of the lysosome allows for the viral RNA and viral ribonuclear proteins to detach from the matrix protein 1 (M1) inside of the nucleoprotein (3). Once the viral envelope fuses with the cell membrane, the viral proteins inside of the envelope are released into the cytoplasm of the infected cell (1-3).

Influenza virus is unique in the fact that its eight viral RNA segments must traverse the contents of the cellular cytoplasm and find their way into the nucleus of the cell to replicate (1, 4). Viral RNA wraps around viral proteins where they form ribonucleoprotein (RNP) complexes (1, 4). Once the RNP complexes have been released into the cytoplasm they must be trafficked into the nucleus. Influenza proteins PB2 and NP have nuclear localization signals that interact with host karyopherins, a broad family of cargo molecules that import and export molecules into and out of the nucleus respectively (4, 5). Specifically, karyopherins α and β, and the small GTPase, Ran, collectively identify the nuclear localization signal on PB2, M1, and NP proteins

then in a GTP-hydrolysis-dependent manner, Ran translocates the viral RNP complexes into the nucleus for replication (4, 5).

Once inside of the nucleus, the viral RNA must be replicated and thus, influenza brings along its own RNA-dependent RNA polymerase (RdRp) in the form of the RNP complex (5, 6). Once inside of the nucleus, the RNP complex dissociates from the viral RNA and forms the RdRp (5, 6). The 5' and 3' ends of influenza RNA are conserved and are complementary thus, they transiently bind forming double stranded viral RNA for a brief period of time (5, 6). This allows for the RdRp to bind and begin synthesizing a complementary strand of influenza RNA that can be used as a template for negative-sense influenza RNA. For transcription and subsequent influenza protein synthesis, the host's RNA polymerase II is co-opted (5, 6).

Once the viral RNA has been replicated and transcribed, viral M1 assists with the translocation of viral RNA into the cytoplasm (1, 5, 6). The nucleoprotein once again forms and encapsulates the virus' genomic RNA as well as the viral proteins (1, 5, 6). At the late stages of viral assembly, HA, NA, and M2 integrate into the plasma membrane while M1 once again forms associations with the envelope and the RNP complexes inside of the nucleoprotein (1, 5, 6).

Neuraminidase has 11 different subtypes thus; the serotype of influenza virus is termed based on the combination of hemagglutinin and neuraminidase glycoproteins present on the envelope surface (1, 7). The infection of airway epithelial cells in the upper respiratory tract is dependent on NA activity (7). NA possesses receptor destroying activity and virion escape from a cell is dependent upon NA activity (7). In order for virion release to occur, NA cleaves sialic acid moieties from cellular receptors thus allowing for viral budding and release. As both HA

and NA are able to cleave sialic acid residues, HA receptor attachment and NA receptor destroying activity must be balanced (1, 7).

The replication cycle of a virus requires many concerted steps in order to ensure the virus replicates its genome effectively and efficiently while subsequently hijacking cellular machinery to produce new viral proteins. Through many of these steps, the cell is able to recognize the virus or viral components and respond in kind. Recognition of double stranded RNA (dsRNA) in the nucleus by RNA-activated protein kinase (PKR) begins a cascade where RNase L cleaves the dsRNA as well as induces apoptotic cell death (8). Production of interferons by recognition of viral RNA in the cytoplasm also leads to a pro-inflammatory, pro-apoptotic state (8). Influenza virus has many different strategies to overcome host defenses against viruses. The NS1 protein of influenza virus binds to its own dsRNA effectively hiding it from the host PKR and RNase L response. In turn, this also blocks one of the major cascades for interferon production (8). NS1 can also bind to RIG-1 thereby inhibiting its binding to single stranded RNA and preventing degradation of the viral genome (8).

Once all of the components of influenza virus, those being the eight negative-sense RNA segments, HA, NA, M2, M1, and the RNPs, the virion forms together and buds from the cell. The mature and fully processed forms of HA, NA, and M2 become integrated into the host cell's plasma membrane (1, 9). M1 then assembles at the membrane and assists in the recruitment of the RNPs (9). During this process, HA activates the ERK cascade which further allows for the production of viral proteins (10). At the plasma membrane, the viral components become enveloped resulting in the formation of a new virion at the apical surface of the cell (1, 9). Here, NA cleaves sialic acid residues on the cell surface allowing for both efficient budding and release of the virion as well as preventing HA from re-binding to surface sialic acid moieties on

the surface of the previously-infected cell (1, 9). The newly released virus is then free to begin the infection process again when it comes into contact with sialic acid residues on neighboring cells.

Currently, there are seasonal tetravalent influenza vaccines that allow for moderate protection against a range of influenza A and influenza B viruses (11). However, in the most recent influenza season, the CDC estimated the efficacy of the vaccine to be 47% (11). Several antiviral treatments exist with their efficacy ranging from 60-90% (12). This estimate is based on providing antiviral treatment within the first 48 hours of onset of the disease (12). As mentioned previously, a person infected with influenza virus is asymptomatic and can spread the virus in the form of aerosol droplets for days prior to the onset of symptoms making antiviral therapy difficult to administer early on (12). Antivirals are an effective treatment but are not equipped to prevent others from becoming infected as individuals infected with influenza virus often experience an incubation period prior to the onset of symptoms where they shed infectious virus particles.

Influenza virus infection causes morbidity in several different ways. In humans, influenza virus infections are typically limited to the respiratory tract where epithelial cells are infected and subsequently lysed resulting in mucous production and induction of the innate immune response (13). At the site of epithelial damage, innate immune cells, such as neutrophils, monocytes, and macrophages, will secrete potent inflammatory cytokines that contribute to inflammation (13). Together, these events result in fever, cough, and mucous production. If the viral infection spreads from the respiratory epithelial cells of the upper respiratory tract to the alveoli deeper in the lungs, viral pneumonia may occur (13). The morbidity and mortality associated with pandemic strains of influenza virus most commonly follow this route of infection where viral

pneumonia occurs (12, 13). The host's immune response also contributes to morbidity via the over-zealous production of inflammatory cytokines that damage the respiratory tract and the alveoli. Epithelial cell death, over-production of inflammatory cytokines, and an influx of fluids into the alveoli may result in acute respiratory distress syndrome and subsequent death.

Acute respiratory distress syndrome (ARDS) is characterized by rapid infiltration of immune cells into the lungs where they secrete large amount of pro-inflammatory cytokines (14). As a result of IAV infection and subsequent immune activation, the respiratory system begins to fail. Specifically, gas exchange in the lungs becomes dysregulated as fluid rapidly impairs the function of the microscopic air sacs responsible for exchanging carbon dioxide for oxygen (14). Atelectasis, or collapsing of the lungs, and hypoxemia are often the result of ARDS and may require ventilation of the patient (14). Patients infected with IAV that progress to ARDS as a result of an increase in fluid caused by cytokine release typically have a poor prognosis (14, 15). The mortality rate associated with ARDS that progresses to hypoxemia and lung collapse is between 9 and 20%. Bacterial sepsis or multiorgan failure resulting from ARDS increases the mortality rate up to 30% (14, 15). ARDS is also an incredibly debilitating disease where lung function can be severely impaired as both the epithelial and endothelial layers of the respiratory epithelium, along with the air sacs responsible for gas exchange, become damaged and the quality of life for people afflicted by ARDS decreases drastically. The sum of the events surrounding viral infection and the response initiated by the host result in morbidity. While both viral and host factors contribute to morbidity in the human population, commensal bacteria also play a large role.

Secondary Bacterial Infections

Streptococcus pneumoniae (Spn), Klebsiella pneumoniae, and *Staphylococcus aureus (SA)* are commensal bacteria generally found on the skin and in the nasopharynx of humans (16). However, during the 1918 and 1957 influenza virus pandemics, the greatest mortality was associated with bacterial pneumonia caused by these commensal bacteria (1, 16). The host's immune response is adept at coexisting with these bacteria and very rarely are they able to colonize in large quantities and damage the host without some prior trigger. Influenza virus is one of those triggers. While *Spn* and *SA* are the most common bacteria that cause infections after IAV infection, other bacteria may also colonize the upper respiratory tract and cause significant morbidity. (16) For example, *Legionella pneumophila*, *Streptococcus pyogenes*, *Nesseria meingitidis, Moraxella catarrhalis, Haemophilus influenzae, Pseudomonas aeruginosa,* and other *Staphylococcus* and *Streptococcus* species have been observed to cause secondary infections (17).

Focusing on *Streptococcus pneumoniae* (*Spn*) because it caused the greatest amount of mortality in 1918 and 1957 pandemics, influenza virus infection causes damage to the nasopharynx and respiratory tract of the human lungs thereby allowing for *Spn* to adhere, colonize, and become infections to the host (17). Damage on the surfaces of airways mediated by IAV exposes potential attachment sites for bacteria via the deposition of fibrin and the inherent repair mechanisms that regenerate epithelial cells (17). The IAV-induced necrosis of epithelial cells impairs the mucociliary clearance mechanisms by which debris from deep in the small alveoli of the lungs is removed (17). Bacteria present in the oronasopharynx then have access to the lungs to colonize. The neuraminidase activity that cleaves sialic acid residues when

influenza virions are released from the cell also results in the exposure of cellular receptors that make the lung environment favorable for *Spn* adherence (9, 17).

Spn releases hydrogen peroxide (H2O2) which directly interferes with inflammasome formation thereby blocking important early-innate immune cytokines like IL-1β and IL-18 (18). Immune cells that experience prolonged or transient interactions with IAV antigens may induce cellular anergy and allow for a more permissible environment for bacterial adherence and colonization as the immune response becomes less active (18). Sustained desensitization to TLR ligands, specifically TLR2 on the outer membrane of immune cells, leads to a decrease in neutrophil recruitment following the clearance of IAV (18). While the decrease in TLR2 activation results in less immunopathology, the lack of neutrophil recruitment results in a more permissive environment for *Spn* and *SA* colonization (17, 18).

The synergy between influenza virus infections and bacterial adhesion is also recapitulated in mice where the IAV virulence factor, PB1-F2, is administered. PB1-F2 administration led to an increase in potent pro-inflammatory cytokines, IFN-α/β, IFN-γ, TNF-α, and IL-6. IL-10 was also increased after PB1-F2 administration which further decreases the recruitment of pro-inflammatory immune cells thereby making the site of IAV infection more permissible to bacterial adherence (1, 19). PB1-F2 decreases the phagocytic ability of neutrophils and macrophages as well which hinder the ability of the immune response to inhibit bacterial colonization (19). Further, as immune cells from all over the host's body begin to migrate to the site of infection, vascular permeability is increased (19). Lung damage makes the vascular endothelium leaky and *Spn* is able to spread and become septic.

Spn is the most common cause of community acquired sepsis worldwide (17). *Spn* is a Gram positive, facultative anaerobic bacterium that has the potential to infect the air sacs of the

lungs (17, 20). Risk factors for community acquired pneumonia via *Spn* infection include underlying pulmonary diseases, immunodeficiencies or immunosuppressive conditions, smoking habits, IAV infections, and age. Individuals who are over the age of 65 are four-times more likely to experience community acquired pneumonia compared to younger people (20). While pneumococcal vaccines exist, their efficacy in people over 65 years of age is variable as the immune systems of elderly patients are often what make them more susceptible to community acquired pneumonia to begin with (20). This phenomenon, termed secondary bacterial infection, can lead to toxic shock syndrome as even more cytokines are produced to inhibit bacterial superinfection and the host may die (20). In the worst influenza virus pandemics humans have accounted for, these secondary bacterial infections have led to the greatest amount of mortality. With several hundred strains of *Spn*, current vaccines do not cover all strains of bacteria and antibiotics may not be effective either as the spread and propagation of the bacteria increases due to the damaged host (20).

Mechanisms of lung damage

Influenza-mediated cell death

Influenza virus (IV) is able to directly damage and kill epithelial cells through a variety of viral escape strategies. Further damage to epithelial cells is caused by intracellular recognition molecules inherent to the immune response when components of the virion are recognized. Apoptosis of infected or damaged epithelial cells represent the main form of damage to the epithelium (airway epithelial cells, goblet epithelial cells, and airway basal cells) during the early stages of IV infection (21). Once the infected cell recognizes a portion of IV intracellularly, the cell begins to condense, compacting everything inside of the cell's cytoplasm (21). This limits

the ability of the virus to replicate. This also allows for the induction of initiator apoptotic proteins (caspases) to come into contact with viral proteins thereby beginning the apoptotic cascade (21). These initiator caspases dimerize which then allows for autolytic cleavage of effector caspases (21). Effector caspases then activate DNA proteolytic enzymes which begin to fragment cellular DNA (21). This fragmentation of cellular DNA stresses the cell and inhibits the further production of regulatory proteins. This stress response results in cellular blebbing and apoptosis of the cell. Here, epithelial cells attempt to limit the production and spread of IV through cell death (21). However, apoptosis also limits the activation of the innate immune response as apoptosis does not induce a large inflammatory response. Through necroptosis, the immune response becomes activated.

Necroptosis, or cell-programmed necrosis, is the phenomenon by which cells uncontrollably die and release their intracellular content into the periphery (22). The DNA-dependent activator of IFN regulatory factors (DAI) is required for receptor-interacting protein kinase 3 (RIPK3) induction which leads to type I IFN production (23). During IAV infection, IAV RNA is recognized through DAI and leads to either apoptotic or necroptotic cell death (1, 21, 23). IAV RNA recognition by DAI activates RIPK3 and subsequently recruits mixed lineage kinase domain like pseudokinase (MLKL) via phosphorylation to create the necrosome (23). Conversely, receptor-interacting protein kinase 1 (RIPK1) in the presence of caspase 8 and Fas-associated protein with death domain (FADD) leads to apoptosis rather than necroptosis (23). Influenza virus is able to mediate necrosome formation and necroptosis as the primary means of cell death during infection by limiting the presence of caspase 8 (21, 23). The matrix 1 (M1) protein of IAV specifically binds to caspase 8 thereby skewing the cell to undergo inflammatory necroptosis rather than apoptosis (21, 23). The content released from cellular necrosis consists of

DNA, DNA-associated proteins, and cellular organelles all of which elicit a robust immune response (23). The innate immune response recognizes conserved moieties of damaged cells (termed damage-associated molecular patters, or DAMPs) which in turn activates them (22, 23). Through this activation, innate immune cells begin to express pro-apoptotic ligands on their cell surface to induce apoptosis in IV-infected cells (23). These ligands and their role in the induction of cell death will be discussed in the next section.

Unlike other respiratory viruses like rhinoviruses and adenoviruses, influenza virus is able to directly damage epithelial cells. Viral escape is often times mediated by the apoptotic death of cells thereby offering a mechanism of viral escape via the blebbing of the cell membrane (21). Necroptosis and the subsequent recognition of cellular debris by the innate immune response increases vascular permeability in the blood vessels lining the lungs which allows for the enhanced spread of IV virions (23). While damaging, the death of cells in the respiratory epithelium caused by IV is incredibly small when compared to the role of the immune response in tissue damage during viral infection.

Innate immune response

Once influenza virus has begun to infect the host, the host's innate immune response elicits a robust, nonspecific response to the infection. When recognizing the structure of the influenza virion, it is clear that there are many unique, conserved structures that the innate immune response can recognize (24). Through these conserved structures, innate immune cells use toll-like receptors (TLRs), C-type lectin receptors (CLRs), nod-like receptors (NLRs), and Rig-I-like receptors (RLRs). (24) Much like the recognition of DAMPs, this nonspecific

recognition of conserved pathogen -associated molecular patterns (PAMPs) is what makes the innate immune response non-specific, effective, and sometimes dangerous (24).

During the early stages of an IV infection, RLRs or NLRs inside of infected epithelial cells recognize viral RNA (24). As noted earlier, the recognition of IV RNA activates initiator apoptotic caspase (21). However, the threshold of initiator caspases required to cleave and activate effector caspases that commit an epithelial cell to undergo apoptosis can be large. Once the innate immune response has been activated by DAMPs or components of IV (proteins, glycoproteins, and/or RNA), immune cells begin to upregulate pro-death ligands that further commit a cell to undergo apoptosis when they come into contact with their respective pro-death receptors on a cell's surface. The recognition of IV by an epithelial cell results in greater production and upregulation of these pro-death receptors (25). Some innate immune cells do not upregulate pro-death ligands on their cell surface but rather, phagocytose infected and dying cells or aid in the slowing of viral spread (25). Even further, some innate immune cells bridge the gap between the innate and the adaptive immune response as a means to develop cellular memory against IV or provide the last push to finally clear the viral infection (25).

Cells of the Innate immune response

Monocytes and macrophages are pro-phagocytic leukocytes that also upregulate pro-inflammatory cytokines like tumor necrosis factor (TNF), IL-1 and IL-12 (26). Monocytes are produced in the bone marrow from their precursor, monoblasts, and then leave to circulate in the blood for several days (26). Under homeostatic conditions, half of circulating monocytes will stop circulating and differentiate into tissue-resident macrophages or myeloid-lineage dendritic cells (26). The other half of monocytes will migrate and be retained in the spleen. During an IV

infection, production of chemokines, CCL2 and CCL7 further recruit monocytes via interaction with the pro-inflammatory receptor, CCR2 (26, 27). CCL2 interactions with CCR2 facilitate calcium mobilization and inhibition of adenylyl cyclase thereby attracting monocytes from the bone-marrow (27). These inflammatory monocytes (IMs) migrate through the blood stream following the CCL2 chemokine gradient until they reach the site of infection (26, 27). During IV infection, the site of infection is the lungs. Monocytes and macrophage have three main anti-microbial properties: phagocytosis, cytokine production, and antigen presentation (26, 27).

Monocytes and macrophages recognize conserved PAMPs and DAMPs via their pattern recognition receptors (PRRs) and initiate uptake and subsequent phagocytosis of the target (26). Through the opsonization of pathogens or foreign molecules via complement or antibodies, these myeloid cells can also recognize and phagocytose the opsonized particles (26, 27). As it relates to IV, monocytes and macrophages are also able to directly kill virally-infected cells through direct killing (26, 27). The presentation of pro-apoptotic receptors (termed death receptors) on an IV-infected cell can interact with pro-death ligands on the surface of monocytes and macrophages (26, 27). The major ligands on the surface of monocytes and macrophages that induce death are Fas ligand (FasL) and TNF receptor apoptosis -inducing ligand (TRAIL) (27). It is here that much of the cellular damage to the host occurs.

Recruitment of IMs via CCL2 secretion results in a massive influx of pro-inflammatory cells that will secrete more pro-inflammatory cytokines thereby modulating the immune response to recruit more inflammatory cells and directly kill infected cells (26, 27). FasL and TRAIL induce apoptosis and necroptosis in epithelial cells (27). Mentioned earlier, the M2 protein of IV also directly damages and kills epithelial cells. The result of IMs killing epithelial cells coupled with viral-mediated cell death leads to massive cell damage and death, fluid build-up, and

potential complications (27). Further, the production of pro-inflammatory cytokines by IMs results in a negative feedback loop where more inflammatory monocytes and inflammatory cells are recruited (26, 27). The cellular damage perpetrated by IMs through direct cellular killing and further recruitment of inflammatory cells, being IMs or otherwise, leads to gross cell death and is a significant cause of morbidity seen during IV infection (26, 27).

Neutrophils are professional phagocytic cells and are the most abundant circulating leukocyte in the blood (28). They develop in the bone marrow from myeloblasts and are the first responding cells to most infections (28). Usually thought of as anti-bacterial cells, neutrophils play a large role in acute respiratory distress syndrome (ARDS) which can be a consequence of respiratory IV infection (28). Recognition of double stranded RNA via TLR7 on neutrophils results in the production of TNF and CCL3 which further recruit neutrophils to the IV-infected lungs (28, 29). Neutrophils serve several functions during viral infection via phagocytosis, the production of reactive oxygen species, and NETosis (28).

Neutrophils are able to phagocytose cellular debris during IV infection (28). Cells that have undergone apoptosis or necroptosis will be recognized by PRRs on the surface of neutrophils, internalized, and subsequently removed (28).

Neutrophils contain primary granules that directly damage or degrade components of bacteria (azurophilic granules) and secondary granules which form reactive oxygen species and lysozymes (28, 29). During IV infection, secondary granules and myeloperoxidase (MPO) are used to produce reactive oxygen species (ROS) via the induction of NADPH oxidase (30). Reactive oxygen species are produced when molecular oxygen is reduced which results in the production of superoxide (30). Dismutation of superoxide forms hydrogen peroxide which, when partially reduced by MPO, produces a hydroxyl radical (ROS). (30) Hydroxyl radicals have

many biological functions under healthy conditions but when a host is infected, the production of ROS increases (30). During IV infection, ROS play a variety of damaging roles. Viral-infected epithelial cells will undergo apoptosis via ROS signaling releasing virions into the periphery and destroying tissue (29, 30). ROS can also play roles in damaging rather than killing epithelial cells. Free hydroxyl radicals can remove electrons from lipids (lipid peroxidation) thereby weakening and damaging cell membranes, deactivate cellular co-enzymes or co-factors necessary for cellular maintenance, and damage both DNA and RNA inside of cells (29, 30). All of these processes drive epithelial cells closer to apoptosis and throughout the course of IV infection, lead to massive amounts of epithelial cell damage and cell death.

Neutrophil extracellular trap (NETs) formation also contributes to acute lung damage during IV infection, albeit indirectly. NETosis is the process by which dying neutrophils release their DNA containing antimicrobial peptides into the periphery in an effort to “catch” bacteria and cellular debris (28). In the context of IV infection, NETosis serves to produce mucous and cellular debris from dead neutrophils (28). NETs become entangled with alveoli which further leads to endothelial damage of the airway epithelium (28, 30). Also, when MPO is inhibited in neutrophils, NET formation decreased in influenza-infected lungs, suggesting that the generation of ROS via MPO is important in NETosis, again indicating the neutrophil’s function in influenza immunopathology (30).

Natural killer (NK) cells are aptly named after their ability to induce apoptosis in cells without activation steps (31). Through the recognition, or lack thereof, of MHC I molecules on the surface of stressed or infected cells, NK cells can directly induce apoptosis or redirect cell lysis (31). NK cells accomplish this goal by secreting granzymes in close proximity to their target cell (31). Perforins and other proteases puncture cell membranes allowing for osmotic lysis

to occur (31). During IV infection, this can result in an increase in the release of virions from a lysed rather than a cell that has undergone apoptosis. As it relates to the early immune response, NK cells are activated and trafficked into the infected lungs during an IV infection very early (31). Within three days of the beginning of the infection NK cells begin to mount an immune response (31). The early presence of NK cells makes them incredibly important in limiting an IV infection but there are several key evasion mechanisms IV possesses that allow them to continue the infection (31).

Influenza virus evades NK cells through direct infection or through the secretion of unbound HA (32). In the same manner as epithelial cell infection, HA of IV attaches the virion to sialic acid residues on the surface of NK cells (32). The virion then binds to the sialic acid residue, docks at the cell surface, and comes in close proximity such that the cell membrane of the NK cell fuses with the virion's envelope (32). The IV infection proceeds in the same manner in NK cells as epithelial cells. Free HA, that being not bound to influenza virions but is released from infected cells, can also inhibit NK cell cytotoxicity (32). Through the binding and subsequent internalization of HA into the cell, HA decreases NK cell cytotoxicity mediated by the downregulation of the zeta chain through the lysosomal pathway (32). The dysregulation of early NK cell signaling via the zeta chain diminishes the ERK pathway which is essential for granule production and exocytosis from the NK cell (32). While NK cells play a large role during the early stages of IV infection, they, along with other early innate cells often times cause more damage to the upper respiratory and lung epithelium.

Macrophages monocytes, and neutrophils comprise the largest group of innate cells recruited during the early stages of an IV infection. Macrophages and monocytes are capable of triggering apoptosis cascades in IV-infected epithelial cells via apoptosis-inducing ligands,

secrete potent pro-inflammatory cytokines that further the damaging inflammatory response, and phagocytose cellular debris (26-29). Neutrophils are not only able to phagocytose debris, but they also produce ROS and enzymes that can directly damage epithelial cells and induce apoptosis (30). Further, NETosis may result in damaging of endothelial cells and produce mucous that primes the lungs for ARDS. NK cells also induce apoptosis, and sometimes lysis, of epithelial cells thereby resulting in damage to the lungs. The early innate immune response not only shapes the adaptive immune response but also determines the extent of the damage the lungs and upper respiratory tract will face. Until the adaptive immune response begins to control the infection, the damage caused by the innate immune response may be lasting and have adverse effects after the clearance of the virus in the form of bacterial adherence, colonization, and super-infection.

Adaptive immune response

The innate immune response is comprised of the first cells to respond to a viral infection. During an influenza virus infection, pandemic strains or less virulent seasonal strains, viral clearance may be dependent on not the innate immune response, but the adaptive immune response. The adaptive immune response mediates both cellular and humoral immunity (33). Cellular immunity is derived from various subsets of T cells where activation via antigen presentation is required in the case of $CD4_+$ T cells or direct cell lysis via cytotoxic $CD8_+$ T cells (33). Humoral immunity stems from antibody-secreting B cells (34). The modulation of the adaptive immune response is dependent on the infectious agent, the innate immune response's ability to communicate with the adaptive immune response, and time.

T cell subsets play important roles in mediating the immune response as well as actively clearing virally-infected cells. $CD8_+$ T cells perform he latter function and induce apoptosis in infected cells (33, 35). In an effort to control the spread of viral particles and thus, continue the infection, $CD8_+$ T cells patrol the lungs and initiate contact with MHC class I (MHC I) molecules presented on the surface of the respiratory epithelial cells (33, 35). Under homeostatic conditions, non-immune cells and immune cells alike express self-antigens on MHC I molecules as a signal to $CD8_+$ T cells that they are alive and healthy (33, 35). Should the antigens expressed on MHC I molecules be abnormal or viral in origin, the $CD8_+$ T cell that comes into contact with it will release granzymes, perforins, and several serine proteases that induce apoptosis (33, 35). $CD8_+$ T cells also express the ligand, Fas (FasL) (35). FasL interacts with its receptor, Fas, on the surface of cells and recruits the death-associated signaling complex (DISC) which further interacts with the death domain, FADD (35). Activation of DISC results in the specific cleavage of procaspases into apoptotic effector caspases (35). Through these mechanisms, $CD8_+$ T cells control the spread and replication of IV.

$CD4_+$ T cells do not directly induce cell death in virally infected cells but rather, they coordinate the immune response against a specific threat. Professional antigen presenting cells (APCs), dendritic cells and macrophages, internalize viral antigens they encounter via endocytosis and travel to secondary lymph organs to encounter naïve $CD4_+$ T cells (33, 36). $CD8_+$ T cells are restricted to MHC I recognition while $CD4_+$ T cells are restricted to antigens presented on MHC class II (MHC II) molecules (33, 36). When IV antigens are processed and loaded onto MHC II molecules on the surface of APCs, and through cos-stimulatory surface ligand-receptor interactions coupled with cytokine signals, naïve $CD4_+$ T cells become activated against the presented antigen (33, 36). Helper T cells then coordinate with the innate immune

response and secrete cytokines that further the pro-inflammatory immune response or dampen the immune response depending on the lung micro-environment and the cytokines that are being produced by the multitude of immune and non-immune cells (33, 36). There are also a variety of T helper cell subtypes that play important effector roles during IV infection.

Th1, or type 1 helper T cells, elicit a cell-mediated response against intracellular pathogens, namely bacteria and viruses (37). Polarization of Th1 cells stems from recognition of IL-12 which activates the transcription factor, T-bet (37). T-bet is the hallmark transcription factor of Th1 cells. Once T-bet has been activated and the naïve T cell has polarized to a Th1 phenotype, Th1 cells begin to secrete IL-2 and IFN-γ which activate macrophages, $CD8_+$ T cells, and stimulate B cells to class switch and produce IgG (37). All of these cell types are indicative of a pro-inflammatory response against intracellular pathogens.

Th2, or type 2 helper T cells, are activated in response to parasitic or helminth infections. In a similar fashion to Th1 cells, specific cytokines activate naïve T cells and polarize them into the Th2 phenotype via the induction of transcription factors. For Th2 cells, IL-2 and IL-4 recognition upregulates GATA3, polarizes T cells into the Th2 lineage, and subsequently begins to secrete IL-4 and IL-10 (38). Th2 cells activate eosinophils, mast cells, basophils, and IgE production from B cells to combat helminth infections (38).

In the context of influenza virus infections, a Th2 response is observed only for IL-4 and IL-10 production as these cytokines are anti-inflammatory and serve to negatively regulate a pro-inflammatory response (39). A Th1 response is much more common during IV infections and as a result, monocytes, macrophages, and $CD8_+$ T cells become activated and induce damage to the epithelial cells in the lungs as noted earlier (26). However, there are other T cell subsets that play large roles during influenza virus infections.

Th17 cells secrete the effector cytokine, IL-17 (40). Upon recognition of damage-associated molecular patterns by Toll-like receptors or the cytokines, IL-6 and TNF-α, Th17 cells become activated (40). This activation of Toll-like receptors activates the transcription factor RORγt to increase expression of the gene, *RORC* (40). *RORC* encodes for the production of IL-17 which, once produced, is secreted from the cell. The secretion of IL-17 marks the beginning of a proinflammatory immune response against an infection. IL-17 is secreted from Th17 cells to assist with both autocrine and paracrine signaling in the environment (40). Inflammatory monocytes and macrophages that either reside in the location currently infected or recruited via secreted chemokines have receptors necessary for IL-17 activation as well as any other pro-inflammatory molecules that detail their course of action. Once IL-17 binds the IL-17 receptor, the inflammatory immune response against IAV begins.

There are six different cytokines in the IL-17 family (40). These are IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (40). Each cytokine binds to its respective receptor (IL-17RA-IL-17RF) but not exclusively. For example, IL-17RA binds to the heterodimer IL-17RA and IL-17RC. IL-17F can also bind this heterodimer (41). IL-17C binds the heterodimer IL-17RA and IL-17RE while IL-17E binds the heterodimer IL-17RA and IL-17RB (41). The receptors for IL-17B and IL-17D have not been fully elucidated. Furthermore, while homodimers of IL-17 receptors exist, it is through heterodimer receptors IL-17 activates a proinflammatory response (41). While subtle differences between each IL-17 isoform and IL-17 receptors are currently being studied, each cytokine and receptor share varying degrees of sequence homology. The similarities between each cytokine allow for variable binding on heterodimer receptors while heterodimer receptors are favored over homodimers in order to tailor the inflammatory immune response to the extracellular milieu.

Review of the NF-κB Signaling Cascade

IL-17A activates the NF-κB signaling cascade which is one of the largest and most rapid signaling cascades associated with the immune response (40-42). NF-κB responds rapidly to changes in the environment in the form of cytokines, free radicals, and/or pathogenic antigens recognized by the cell (42). Because of the rapid induction of the NF-κB pathway, it is the first cellular response to harmful pathogens and thus, the outcomes of NF-κB induction have consequences for the overall immune response. Due to the extent in which IL-17A activates NF-κB through the IL-17RA/IL-17RC heterodimer, it will be used to discuss the NF-κB pathway and the consequences of NF-κB activation (40-42).

IL-17A binding to IL-17RA has been found to be insufficient to begin the signal cascade for pro-inflammatory cytokine production (40-42). Therefore, once IL-17A binds to IL-17RA, the affinity for heterodimerization increases and IL-17RC binds to IL-17RA to enhance the signal from IL-17A (40-42). At the C terminus of the IL-17RA/IL-17RC heterodimer complex is a conserved region, termed similar expression of fibroblast growth factor genes and IL-17 receptors (SEFIR) (43). Included in the SEFIR domain is a coiled-coil loop that is essential for IL-17RA/IL-17RC and the first effector molecule in the cascade to bind (43). The C terminus end also consists of many variable polar amino acids further providing IL-17 receptor and the first effector binding. The first effector of the IL-17 cascade is NF-κB Activator 1 (Act1) which also contains a SEFIR domain on its C terminus (44). Through the coiled-coil loop on the IL17A receptor, Act1 can recognize and bind to the C terminal end of the IL-17A receptor (44). Through X-ray crystallography of the IL-17RB receptor, leucine 419 and leucine 422 residues on the SEFIR domain of the cytokine receptor are responsible for the IL-17 receptor and first effector interactions (44, 45). As for Act 1, leucine residues 474, 475, 477, and tyrosine 478 of the SEFIR domain on

Act1 are responsible and critical for their interactions which begin the signal cascade (44, 45). To stabilize the association between the C terminus ends of the IL-17 receptor heterodimer and Act1, heat shock protein 90 (Hsp90) binds to both at the plasma membrane (45). Act1 acts as a client protein for Hsp90 and there is evidence that Act1 and IL-17 receptor binding is drastically reduced in the absence of Hsp90 (45, 46). Once Act1 has bound to the IL-17RA/IL-17RC receptor on the cytoplasmic, C terminal end, there are two pathways in which IL-17A signals for a pro-inflammatory response; one pathway stabilizes newly transcribed mRNA and the other induces the activation of NF-kB to move into the nucleus and begin the transcription of pro-inflammatory genes (46, 47).

Once Act1, mediated by Hsp90, binds to the IL-17 receptor heterodimer, IkB Kinase (IKKi) can phosphorylate Act1 which activates several of its catalytic residues along the protein (48). Both IL-17A pathways are dependent on the phosphorylation state of Act1 where one pathway is activated when Act1 is phosphorylated while the other pathway is only activated when Act1 is dephosphorylated (48). IKKi is a major kinase in inflammation where it also serves two roles that will be discussed below. The major catalytic domain of Act1 is the U box consisting of roughly 55 amino acids (47, 48). Prior to the phosphorylation of Act1 by IKKi, the U box domain remains active due to the conformational status of Act1 when it is de-phosphorylated and Act1 can act as an E3 ubiquitin ligase while interacting with TRAF6 (49). Act1 ubiquitinates lysine residues at position 63 of TRAF6 and other proteins in order to facilitate further protein-protein interactions along the pathway. In order for the signal pathway to continue and activate NF-kB, TRAF6 must be polyubiquitinated at the lysine 63 position (50). Once TRAF6 has been poly-ubiquitinated, it becomes activated and can further interact with TRAF6-dependent TGF-β-activated kinase 1 (TAK1) (51). It is important to note that Act1 can ubiquitinate many TRAF6 molecules prior to

phosphorylation by IKKi thus, TRAF6 acts as the second messenger and amplifies the pro-inflammatory signal in the IL-17A pathway (49-51).

TAK1 is a member of the mitogen-activated protein kinase (MAPK) family that is activated by IL-1, tumor necrosis factor, and pathways through Toll-like receptors (including IL-17 receptors) (52). TAK1 binding proteins 2 and 3 (TAB2 and TAB3 respectively) associate with TAK1 as adapter proteins and are vitally important for TAK1 interactions with the polyubiquitinated TRAF6 complex (52). Once TAK1 and its adapter proteins interact and associate with TRAF6, TAK1 begins the canonical inflammation pathway which activates NF-kB for translocation into the nucleus (52). TAK1, being a kinase, phosphorylates IKKi, the same protein that was responsible for Act1 phosphorylation (52, 53). IKKi, once phosphorylated, phosphorylates Inhibitor of kappa B (IkBa) (53). During cell homeostasis, NF-kB is bound to IkBa which forms a protein complex that inhibits both NF-kB activation and translocation into the nucleus (53). Tight regulation of TAK1 phosphorylation of IKKi and subsequent phosphorylation of IkBa are necessary to prevent the activation of unnecessary inflammatory cytokines. Once IkBa is phosphorylated, it becomes an activated phosphoprotein and dissociates from NF-kB which is subsequently free to translocate into the nucleus and activate pro-inflammatory transcriptional machinery. Once inside the nucleus, genes that code for various pro-inflammatory cytokines (IL-6, TNFa, COX2, and GM-CSF) and chemokines (CCL2, CCL7, CXCL1, CXCL2, CXCL5) become upregulated and are transcribed at the behest of NF-kB (54-56). Several genes for tissue remodeling to allow for immune cell recruitment and aid immune cells' ability to move through tight junctions are also upregulated by NF-kB through the IL-17A signal cascade (40, 41, 57, 58). However, a major problem with IL-17 signaling of NF-kB becomes readily apparent. IL-17A is a very weak NF-kB activator and the transcripts produced are unstable and readily degraded in the

cytosol after they have been released from the nucleus. In order to solve this problem, Act1 activates a second branched signaling pathway (59).

The phosphorylation of Act1 by IKKi after Act1 associates with the IL-17 receptor heterodimer through their SEFIR C terminus domains occurs at serine 311 (59). Once this position has been phosphorylated, Act1 undergoes a conformational change that allows for TRAF2 and TRAF5 to bind but excludes TRAF6 (59). The docking and binding of TRAF2 and TRAF5 to Act1 forms an arginine and serine-rich splicing factor SRSF1 (59). SRSF1 prevents the human splicing factor, ASF, from binding to the 3' untranslated regions of cytokine and chemokine mRNAs in the cytoplasm (59, 60). By splicing ASF, pro-inflammatory mRNAs will not be degraded in the cytoplasm, rather, they will be translated at the endoplasmic reticulum, shuttled to the Golgi apparatus, and secreted out of the cell to serve their role in the immune response against IAV (61-63). Furthermore, the Act1/TRAF2/TRAF5 protein complex activates HuR, an RNA-binding protein that facilitates mRNA translocation from the nucleus to free ribosomes and then to the endoplasmic reticulum for translation (64). It is important to note that the Act1/TRAF6/TAK1 complex and the Act1/TRAF2/TRAF5 complex are independent and distinct from one another. During an immune response to an invading pathogen, other pro-inflammatory cytokines interact with immune cells and stimulate similar cascades that translocate NF-kB from the cytoplasm into the nucleus by cleaving IkBa (66, 62, 63, 65). The IL-17A cascade is important for an immune response because, while IL-17A is not the most potent activator of NF-kB, the Act1/TRAF2/TRAF5 complex stabilizes mRNA produced by NF-kB translocation.

The coordinated and highly regulated immune response to an invading extracellular or intracellular pathogen requires highly complex and intertwined signaling cascades in order to ensure the proper cytokines are produced and the concentration of cytokines produced does not do

more harm than good. In order to regulate the IL-17A inflammatory cascade, other cells and signals effectively dampen, then inhibit the cascade. The main mechanism by suppression of IL-17A signaling is through T regulatory cells (Tregs) (66, 67). Tregs prevent T cells (like Th17 cells) from staying constitutively active after an immune response has been resolved (66, 67). Tregs secrete specific immune cytokines, like TGF-β and IL-35, or directly interact with surface receptors on activated T cells which signal for the ongoing pro-inflammatory cascades to cease (66-69). The mechanisms by which these surface receptors signal is currently under intense study. However, it has been hypothesized that Tregs induce a negative feedback mechanism that constantly signals for the resolvent of pro-inflammatory signals as well as downregulates pro-inflammatory surface receptors, like IL-17 receptors (66-69). The lack of surface IL-17 receptors interferes with mRNA stabilization complexes and thus, pro-inflammatory mRNA would be cleaved in the cytoplasm faster than it would be produced thereby inhibiting pro-inflammatory signals. Beyond Tregs, there are other proteins in the IL-17A cascade that also inhibit NF-kB translocation into the nucleus as well as prevent the stabilization of pro-inflammatory mRNA transcripts.

The polyubiquitination of TRAF6 by Act1 acts as a major regulatory step in the IL-17A pathway. Deubiquitinating enzymes serve to control the rate in which TRAF6 proteins are ubiquitinated (70). Several deubiquitinating enzymes exist in the IL-17A pathway. The major deubiquitinating enzyme is ubiquitin-specific protease 25 (UPS25) (70, 71). UPS25 deubiquitinates both the TRAF2/5 complex that stabilizes pro-inflammatory mRNA transcripts as well as TRAF6, the second messenger in the NF-kB activation pathway (72). A second deubiquitinating enzyme, A20, has been suggested to be a critical negative regulator of the NF-kB pathway by deubiquitinating TRAF6. Outside of deubiquitinating enzymes, the IL-17-dependent

micro-RNA, miR-23b, also regulates the activation status of NF-kB (73). In particular, miR-23b targets the mRNA of TAB2 and TAB3 that associate with TAK1 in the MAPK family (73). As noted earlier, TAK1 association with the polyubiquitinated TRAF6 complex is unstable and will not result in the activation of downstream effector proteins unless TAB2 and TAB3 are associated with TAK1. Furthermore, miR-23b also degrades IKKi mRNA which results in the inhibition of stabilizing pro-inflammatory mRNA transcripts as well as cleavage of IkBa from the NF-kB complex (74). Through these mechanisms, the IL-17A pathway can be highly regulated by varying degrees of specificity in order to ensure the pro-inflammatory response can be controlled over time. However, the regulation of the pathway exists beyond deubiquitinating enzymes and micro RNA molecules.

In order to prevent Act1 from interacting with the SEFIR domain of IL-17RA prior to heterodimerization with IL-17RC and binding of IL-17A, TRAF3 inhibits the formation of the IL-17 receptor/Act1 complex (75). TRAF3 has been shown to inhibit the Act1/TRAF2/TRAF5 mRNA stabilization branch of the IL-17A signal cascade (75). As for the IkBa cleavage for NF-kB trans-localization pathway, TRAF4 acts as a competitive inhibitor of the TRAF6 binding site thereby preventing TRAF6 binding to TAK1 and TAK1 becoming activated (75, 76). An odd form of regulation is a direct, third signal from the IL-17RA/IL-17RC dimer itself (77). IL-17A signaling leads to the dual phosphorylation of C/EBPβ via the ERK pathway at residues threonine 179 and threonine 188 of C/EBPβ (77, 78). C/EBPβ regulates cellular metabolism and proliferation during an immune response (79). The inhibition of C/EBPβ stops signals to transcription factors in the nucleus that signal for continuous pro-inflammatory cytokine production.

The discussion regarding IL-17A and the induction of pro-inflammatory cytokines and chemokines through the IL-17 receptor cascade has been limited to only IL-17A. IL-17B, IL-17C,

and IL-17F all have similar pathways compared to IL-17A where NF-kB is released from IkBa via phosphorylation and subsequently translocates into the cytoplasm to induce the transcription of pro-inflammatory cytokines. IL-17C differs slightly in that interferon-stimulated genes are also upregulated in response to IL-17C binding to the heterodimer receptor complex, IL-17RA/IL-17RE (80). IL-17B induces the NF-kB pathway through the homodimer, IL-17RB (81). The fifth member of the IL-17 family, IL-17D, is thought to play a role in myeloid cell differentiation but evidence supporting this hypothesis is lacking and the true nature of IL-17D is unknown (82). The last IL-17 family member to be discussed has the least sequence homology to any other member of the IL-17 family and acts as more of an eosinophil recruitment cascade (83). IL-17E signals through the heterodimer complex: IL-17RA/IL-17RB (83). This receptor complex is found mainly in the kidneys, liver, and brain of mammals and upon cytokine-receptor recognition, a type 2 allergic response ensues (83). Stimulation of IL-17RA/IL-17RB with IL-17E initiates a similar cascade seen by IL-17A where Act1 binds to the heterodimer receptor complex and ubiquitinates TRAF6 (83, 84). TRAF6, most likely through a similar mechanism seen in IL-17A signaling, uses the MAPK and NF-kB pathways to upregulate the transcription of IL-4, IL-5, and IL-13 (85, 86). While hypothesized, this pathway for IL-17E has yet to be fully described. A second pathway that has been implicated in Th2 responses to large pathogens through IL-17E signaling, but has also not been fully elucidated, is a pro-apoptotic pathway through TRADD, FADD, and subsequent caspase cleavage (87). While a pro-apoptotic pathway for IL-17E in epithelial cells, macrophages, and immune cells after an infection has been hypothesized, the mechanism for this needs to be further understood.

Interleukin 17 is a vital and potent inflammatory activator in the immune system. It bridges the boundary between the innate and the adaptive immune system to help T cells coordinate a

response to pathogens while simultaneously activating neutrophils and macrophages to target infected cells (88). While inflammation is sometimes viewed to be a negative side effect during an infection, a lot of research has shown that IL-17-dependent inflammation is one of the most potent activators of inflammation and thus, one of the most important activating cytokines in the immune system. IL-17 signaling is responsible for inducing inflammation in such a way that its own signaling also acts as a negative regulator (89). By this method, IL-17 ensures the cell and/or system returns to homeostasis after infection by inhibiting the pathway it signals through. However, Th17 and innate lymphoid cells have been directly implicated in autoimmune diseases and inflammation dysfunction during infections (90). While the IL-17A pathway has been elucidated to an extensive degree, the role other IL-17 isoforms play during an infection and how they are regulated during homeostasis is an issue that is currently being studied. Discerning the pathway by which IL-17 activates other immune cells has proven to be of utmost importance in our understanding of how inflammation acts as a double edge sword to both assist in immune cell recruitment during an infection and how inflammation can result in inflammatory diseases.

For resolution and containment of an immune response, regulatory T cells (Tregs) become activated through the recognition of transforming growth factor-β (TGF-β). TGF-β activates the transcription factor, FOXP3, which increases the production of the anti-inflammatory cytokines IL-10 and IL-35 (66, 67, 68, 69, 91). During an influenza virus infection, these cytokines help to limit the production of pro-inflammatory cytokines like TNF-α, FIN-γ, IL-1β, and IL-6 (91, 92). Further, Tregs secrete Granzyme B which has the ability to induce apoptosis in effector T cells and pro-inflammatory cells further limiting the pro-inflammatory immune response (91, 93). The role in which Tregs play during an influenza virus infection, specifically the timing of their activation and when they limit the robust immune response requires further study.

At the end of the infection, activated $CD4_+$ T cells will become memory cells and become immediately activated against exposure to the antigen in the future (91-93). Further, $CD4_+$ T cells will coordinate with B cells in order to provide the robust and neutralizing humoral immune response (94).

B cells are capable of recognizing viral antigens in a T cell-independent manner and produce pro-inflammatory cytokines in response (94). They will also secrete IgM, the pentameric and low-affinity antibody against that antigen (94). However, T cell-B cell interactions are required for B cells to undergo class switching and somatic hypermutation (94, 95). In this respect, the $CD4_+$ T cell response directly influences the B cell response. Through somatic hypermutation, B cells become more specific against the antigen of interest (94, 95). Then, class switching from IgM to the high affinity IgG will occur (94, 95). Innate immune cells and complement recognize the Fc portion of IgG molecules that are bound to antigen and further their activation and viral clearance (96). Much like activated $CD4_+$ T cells, at the end of the viral infection, a small population of activated B cells will become memory B cells and reactivate during future infections against the same pathogen and stop the infection before it can cause damage (97).

The adaptive immune response is vitally important for clearance of virally-infected cells as well as tailoring the immune response to be anti-viral in nature. Memory adaptive cells ensure that subsequent IV infections do not occur and provide robust immunity against IV (98). However, the serotype of IV is constantly changing in part due to the high fidelity of its RNA-dependent RNA polymerase and its multipartite genome. The adaptive immune response provides specificity against an ongoing IV infection but, much like the innate immune response, often times damages the host in order to clear the pathogen.

Immune cell mediated lung damage: the dark side

Directly inducing apoptosis in virally infected cells contributes to the inhibition of viral propagation and spread during an infection (99). While the immune response may successfully clear the virus, the indirect and direct damage that is caused can often times be more devastating than the viral infection. Macrophages, monocytes, and $CD8_+$ T cells can induce cell death in epithelial cells which dysregulates the respiratory epithelium and promotes damage to the underlying basolateral membrane (100). Cell apoptosis also promotes the production of DAMPs in the lung (99, 100). This furthers the pro-inflammatory immune response and the damage to the epithelial cells in the lungs continues. As the respiratory epithelial barrier becomes damaged and dysregulated, the subsequent growth and proliferation promotes scarring and impacts the respiratory function of the lungs (99-101). Infection in the smaller alveoli of the lungs has a drastic impact on gas exchange and breathing in the host (99-101). Damage and scarring to the alveoli often times impact respiratory function in the host long after the viral infection is cleared. Even without long-term architecture changes in the lungs, acute respiratory distress syndrome via fluid build-up in the lungs is also mediated by immune cells (102). As epithelial cells secrete cytokines to alleviate the viral infection, fluid begins to build up in the lungs (102). Neutrophils exacerbate this phenomenon through NETosis and cytokine production (102, 103). While the immune response is vital for clearing an influenza infection, it can often times be overzealous and mediate much of the damage done to the host.

Inflammation of epithelial barrier crosstalk

The interface of host-pathogen interactions is usually characterized as a disease followed by the reciprocal immune response. While true, this paradigm neglects the interactions of non-

immune cells with immune cells in response to an infectious agent. The first line of defense against any infectious agent is the host's barrier; the skin, intestinal epithelium, and respiratory epithelium (102-104). During an IV infection, the respiratory epithelium plays a large role in modulating the immune response against the viral infection (102-104). This is accomplished through early and robust recognition of viral components via PRRs, secretion of migratory chemokines, and direct ligand-receptor interactions with immune cells (105). However, the consequence of crosstalk between immune cells and the respiratory epithelium can have detrimental effects. Previously, we have addressed the role the host's immune response plays in causing damage during IV infection via cytokine production, direct cell lysis, and cell death. Recent evidence has suggested that the modulation of the respiratory epithelium during acute lung infection via immune cell—epithelial barrier cross talk is also a site of host damage and might be a potential target for therapeutic intervention (105, 106).

The respiratory epithelium: first line of defense

The major epithelial cell types in the respiratory tract are ciliated, columnar, secretory, basal, and undifferentiated (107). The architecture of the respiratory epithelium is a function of the location in the airway. The upper respiratory epithelium is composed of ciliated pseudostratified columnar epithelial cells that are anchored to the basement membrane of the extracellular matrix (107, 108). Moving deeper into the lungs, the epithelial cells change from columnar to simple cuboidal epithelial cells and then to simple squamous epithelial cells in the alveolar ducts and alveoli respectively (107-109). The simple cuboidal epithelial cells of the alveolar ducts serve connect respiratory bronchioles to the alveolar sacs containing the alveoli (107, 110). The simple squamous epithelial cells that comprise the alveoli exist to diffuse oxygen

across the cell membrane and release carbon dioxide from the capillaries back into the alveoli to be breathed out and released from the body in a process termed gas exchange (107, 110). As a whole, the respiratory epithelium plays a role not only in gas exchange but is the lung's central defense against pathogens and damaging chemicals inhaled from the environment (1107-110).

Many foreign particles and pathogens, damaging and non-damaging alike, are inhaled and deposited into the lungs. In order to remove these objects before they can cause lung damage, the mucociliary transport mechanism acts as a self-cleaning escalator to remove the debris (111). Airway surface liquid (or epithelial lining fluid) is composed of a *sol* layer and a *gel* layer (111, 112). The *sol* layer surrounds the epithelium in the respiratory tract while the *gel* layer sits overtop, bathing the respiratory epithelium in mucous (111, 112). Mucous is 95% water with the remaining 5% being comprised of proteoglycans, lipids, other proteins, and DNA (mucous composition) (112). Submucosal glands and goblet cells in the respiratory tract secrete mucous and serve to trap foreign particles before they reach the deeper, more fragile regions of the lungs (111-113). Mucociliary transport is both mucous productions coupled with the beating of cilia on the columnar epithelial cells in order to remove debris and protect the lungs of the host (111-113).

As mentioned earlier, the tissue tropism of IV favors cells with sialic acid residues on their cell surface. Therefore, the ciliated columnar epithelial cells in the upper respiratory tract are the predominant cell type influenza virions infect (114). The cilia that are responsible for continuously moving debris and mucous out of the lungs decrease in number as the columnar epithelial cells undergo apoptosis or stop producing their normal surface proteins due to the viral infection. Mucous production also increases during viral infection as goblet cells upregulate the *MUCIN* genes responsible for mucous production (115). This poses a problem as the mucous

cannot be cleared via the cilia as efficiently prior to viral infection and leads to an increase in coughing (115). Coughing serves to forcefully eject mucous-ridden debris from the lungs which can be damaging and debilitating if it continues for a prolonged period of time. The changes in nominal functions of the respiratory epithelium are examples of ways in which the epithelial framework in the lungs become dysregulated (115). In order to limit the damage to the host through the loss of columnar epithelial cells and the increase in mucous production, the respiratory epithelium finds ways to communicate with the immune response against viral infection.

The respiratory epithelium is the first major class of cells to recognize the IV infection (116). Through the utilization of toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs), the columnar epithelial cells recognize various conserved portions of IV which elicits a specific anti-viral response (116-118). Viral RNA is a major target of intracellular TLRs and as such, TLR3, TLR7, and, in humans, TLR8 are used to recognize either single stranded RNA or double stranded RNA (116, 119). TLR3 is expressed both on the cell surface and in early endosomes (the compartment by which IV is internalized as mentioned previously) (116, 119). Through the recognition of double stranded RNA (the intermediate step of viral RNA replication mediated by the viral RNA-dependent RNA polymerase), columnar epithelial cells begin to produce the pro-inflammatory cytokines IL-6, TNF-α and type I interferons (119). TLR7 is also a potent producer of IL-6 upon recognition of single stranded RNA in the cytoplasm of the cell (119). The culmination of these pro-inflammatory cytokines is the polarization of resident immune cells in the lungs towards an inflammatory and damaging phenotype.

RLRs recognize double stranded RNA in the cytosol and activate interferon stimulated genes (116, 120). Through interactions with the mitochondrial anti-viral signaling (MAVS), interferon regulatory factors (IRFs) three and seven become activated which increase the transcription of interferon stimulated genes to induce a pro-inflammatory response (116, 120). In fact, when MAVS signaling is abolished, the levels of interferon drastically drop indicating the viral-sensing capabilities of RLRs in alveolar epithelial cells results in the early and robust interferon response (120). RLRs are also capable of inducing the inflammasome in epithelial cells via MAVs interactions with CARD9 and the NF-kB pathway (121). The RNA-sensing capacity of epithelial cells exist in low levels under homeostatic conditions but upon the recognition of double stranded viral RNA, this recognition mechanism increases to result in a robust interferon response that further polarizes the immune response to be pro-inflammatory (120, 121).

The internal recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) induce the NF-kB pathway to produce immature forms of IL-1β, IL-18, and caspase-1 (122). Recent evidence has suggested that NLR-recognition of the internalized PAMPs and/or DAMPs leads to the formation of the inflammasome and, often times, pyroptosis, in epithelial cells (123). Cleavage of pro- IL-1β, pro-IL-18, and gasdermin-d into the active forms of these cytokines which potently induce a pro-inflammatory response to IV infection (124). Interestingly, studies on the lack of inflammasome signaling in IV-infected mice have found a reduction in the survival rate and effectiveness of the innate immune response (124, 125). However, much of the information regarding the role of the inflammasome during IV infection has been done in myeloid cells thus, more work must be done to determine the effects of the inflammasome in epithelial cells early in IV infection.

The cytokines produced by epithelial cells are the result of early recognition of IV infection. These cytokines are meant to send a signal to the resident innate immune cells that there is a current viral infection occurring. However, the activation of resident innate immune cells is often not enough to control the infection and thus, the respiratory epithelial cells also produce chemokines that aid in recruiting more potent innate immune cells as well as cells of the adaptive immune system (126). For example, the major monocyte chemoattractant, CCL2, is produced early during IV infection by epithelial cells (126, 127). Monocytes are produced in the bone marrow and are a major driving force in viral containment and clearance. However, as noted earlier, monocytes are also major drivers of pathology in IV infection thus, epithelial cell production of CCL2 early in infection often times results in their own pathology.

The cytokines and chemokines produced by the respiratory epithelium drive a damaging pro-inflammatory response mediated by monocytes and macrophages during the early stages of infection and transitions to the adaptive immune response interactions during the waning stages of infection (126). The recruitment of monocytes directly induces cell death in infected epithelial cells an dysregulate the intracellular junctions that form the barriers between epithelial cells. Through this dysregulation, more lung damage occurs as iNOS, ROS, and damaging cytokines gain access to fragile tissue layers of the lungs (127). The respiratory epithelium is one of the largest physical barriers against infection and damage. As such, the respiratory epithelium aids in preventing unwanted or unwarranted communication between cytokines and epithelial cells of the basal membrane under the respiratory epithelium (107). During IV infection, the intracellular junctions that create a non-permissive barrier to cytokines breaks down as cells die via apoptosis or cell-cell interactions (107, 108). Cells of the basolateral membrane underneath the respiratory epithelium express the receptor for epidermal growth factor (EGF) (107, 128). EGF mediates the

proliferation, migration and repair after epithelial cell damage (128). Under homeostatic conditions, the EGF receptor and EGF are spatially separated to ensure the EGF response is limited (128). As respiratory epithelial cells die, and the intracellular junctions become dysregulated, EGF and other cytokines gain access to the basolateral membrane and begin the process of rebuilding the respiratory epithelium (128). However, the new columnar epithelial cells grown in on scar tissue or around decaying/damaged epithelial cells. The resulting airway remodeling changes the architecture of the lung and may result in ARDS if the IV infection continues or enhanced pneumococcal infections as the normally protective barriers were dysregulated (128, 129).

Therapeutic intervention to epithelial barrier dysregulation

The dysregulation of the respiratory epithelial barrier results in long-term consequences for the host. Current therapeutic strategies to alleviate this dysregulation as a means to protect the host have been ineffective (130). After IV infection, the damage that has accrued also allows for the colonization and subsequent proliferation of commensal bacteria like *Streptococcus pneumoniae* (*Spn*) or *Staphylococcus aureus* (*SA*) (131). Much work has been done to protect IV patients against these bacterial infections but given the large number of *Spn* serotypes and the multi-drug resistance in many *SA* strains, therapeutics have been lacking in effectiveness (131, 132). Kostadinova and colleagues found that knocking out the NLRP3 inflammasome in myeloid cells further dysregulated the alveolar barrier in the lungs and made the lungs more permissive to *Spn* infection (133). Through specific induction of the NLRP3 inflammasome in myeloid cells during IV infection, it may be possible to persevere the epithelial barrier integrity. Future studies

in co-infection models of IV and *Spn* will have to be done to test the efficacy of NLRP3 activation.

Cytokines produced by immune cells and epithelial cells during IV are also targets in therapeutic intervention. Potent pro-inflammatory cytokines like TNFα, Interferons α and β, and Interferon-γ perpetuate the pro-inflammatory response by activating immune cells. Chemokines that specifically target damaging cells, like CCL2, CCL5, and CCL7 also damage the integrity of the respiratory epithelial barrier (134). By potentially blocking and inhibiting the production or recognition of these molecules, the damage done to the epithelial barrier might be lessened and long-term damage to the host may be avoided.

Vascular permeability caused by trafficking cytokines, chemokines, and migrating cells is also consequential during IV infection. Fever during IV infections can lead to cellular and tissue damage until the virus is cleared. Further, the influx of fluid into the lungs due to the increase in vascular permeability is a major contributing factor to ARDS (135). Neutrophils and monocytes migrate to the lungs via increased vascular permeability as well. *Spn* also utilizes the increase in vascular permeability to traffic into other organs and induce sepsis. By finding a way to mitigate the spread of bacteria as well as the massive influx of immune cells into the lungs via the vascular system would aid in lessening the damage caused during infection.

We sought to merge the concepts of interactions between the respiratory epithelium and the immune response through influenza virus infection in a testable way. To better understand the integration of these branches of biology, a review article that details the finer points is currently under revision from our lab. The goal of the review article is to combine the pulmonary immunology with influenza virus virology to better articulate the problems both fields face in limiting acute lung damage during viral infection. In order to begin understanding the gaps in

knowledge listed above, several studies had to be done to test each hypothesis. The next section will detail the strategies used to explore many of the gaps in knowledge that have been described above.

Chapter 3: Methodology

Introduction

Given our hypothesis that inflammatory monocytes are the driving force behind much of the immune-mediated epithelial damage in the lungs, assessing the parameters surrounding monocyte recruitment, lung damage via direct cell-cell interactions or protein-mediated destruction, and the loss of barrier integrity underlying the respiratory were analyzed. In order to directly test our central hypothesis, techniques such as immunoblotting, histological analysis of paraffin embedded mouse lungs single cell suspensions, flow cytometry, and *ex vivo* cell assays were performed. Robust statistical analyses were performed at an alpha level of 0.05 to ensure differences seen between samples were not due to sampling error.

Research design

This study was conducted utilizing hypothesis-driven experimental research to establish a cause and effect relationship. This was accomplished by testing the central hypothesis using classical and modern immunological techniques. The basis of the experiments that were encompassed in the study begin with infection model that was used throughout the study. Mice on days 4, 7, 10, 14, and 21 were infected with 250 plaque forming units (PFU) of A/PR/8/34 (PR8, H1N1) influenza virus in 100 µL of cold PBS. This volume was chosen to ensure the virus was delivered not only into the lungs but deep into the small alveoli of the lungs as well. Mice were monitored every few hours for signs of disease (ruffled fur, hunched over, shallow breathing). Further, the body weight of each mouse was taken at the same time throughout the

course of the experiment. Age-matched mice of the same sex were given 100 µL of PBS without virus at the same time as the infected mice to be used as controls.

For early experiments, the lungs from mice were analyzed on days 4, 7, 10, 14, 21, and 28 in order to determine the time points that coincided with the greatest observed morbidity. On the day of the experiment, mice were euthanized via carbon dioxide asphyxiation, washed with 70% ethanol to remove contaminants, and the lungs were aseptically removed with sterile dissection scissors and forceps. Bronchoalveolar lavage (BAL) fluid was collected by inserting a small catheter into the trachea of the euthanized mouse and washing with 1 mL cold PBS a minimum of three times. This was performed for cytokine analysis and to assess lung damage. The large lobe of the lungs was perfused with 1 mL of 4% formalin and then placed into 4% formalin for 24 hours at room temperature for histological analysis. The three smaller lobes were placed into 1 mL RNA-Later for RNA analysis, tissue protein extraction reagent with protease and phosphatase inhibitors for immunoblot analysis, or RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Once these tissues were collected, the lobe-specific analysis occurred for flow cytometry.

For RNA sequencing studies, monocytes from mock, WT-PR8, and IFN-$\gamma^{-/-}$-PR8 were magnetically sorted and total RNA was extracted. PolyA RNA-Seq libraries were prepared using a NEBNext Ultra II RNA-Seq library kit. Adapters from the sequencing process were trimmed and and reads were aligned to a mouse genome (mm10) with HISAT2. Differentially expressed genes (DEGs) between groups were identified using the R/Bioconductor package DESeq2 v1.24.0 with a significance cutoff of adjusted p-value <0.05 as well as a minimum absolute log2-fold change value of 1. Pathway enrichment analysis was carried out using Ingenuity Pathway Analysis (PMID: 24336805). Canonical pathways were identified with a $\log_{10}$ significance (p-

value) cutoff of 1.3. Selected pathways and their Z-scores are shown as a bar graph using ggplot2.

Context of the study

These studies were conducted using C57BL/6 mice or specific knockout mice bred on a C57BL/6 background. In every experiment, there were even amounts of age-matched male and female mice for each experimental and control group. For every experiment, control mice received the same treatment as the experimental mice (light anesthesia via isoflurane) were given 100 µL of PBS instead of 250 PFU of PR8 in 100 µL of PBS. When knockout mice were used, the same proportion of age and sex matched mice were given 100 µL of PBS instead of 250 PFU of PR8 in 100 µL of PBS. The knockout mice were compared to C57BL/6 (WT) mice that were given either PBS alone or PR8. The sample sizes of each group were calculated prior to the experiment using a power analysis in order to ensure statistically reliability but remain within the ethical limitations of animal usage.

For flow cytometric analyses, the same flow cytometer (BD Symphony) was used for every experiment. The antibodies used for flow cytometry were also the same throughout the studies. The clones and colors of each cellular marker were used and titrated prior to use.

Instrumentation

To assess relative frequencies of inflammatory monocyte populations in the lungs, many instruments must be used. The primary instrument utilized in these studies was a BD Symphony flow cytometer. Flow cytometers are powerful machines in that, they can decipher the size, granularity, and relative fluorescence of single cells as they pass through a capillary tube termed

a flow cell. In order to determine size and granularity of cells, single cells pass through the flow tube and disrupt lasers that are adjacent to one another. The time in which one laser is disrupted can calculate the size of the cell. The adjacent laser is also disrupted at the same time however, the amount of light that passes through the cell is calculated and this provides the granularity of the cell. Using both measurements of size and granularity, specific populations of immune cells can be determined. For example, lymphocytes are relatively large in size, but their nucleus comprises the vast majority of the cell's cytoplasm thus, there is little granularity. Conversely, neutrophils are large cells with a bi-lobed nucleus and thus, are able to be differentiated from lymphocytes. While these measures are the first important step, flow cytometers are also able to determine the relative fluorescence intensity of surface receptors on cells or even the relative fluorescence intensity of transcription factors and intracellular cytokines. To achieve this, fluorophores, molecules that are excited by specific wavelengths of light, are conjugated to antibodies that are specific to a surface receptor, transcription factor, or cytokine. As the single cells pass through the flow cell, their size, granularity, and antibody-specific markers are measured via lasers that emit the visible spectrum of light. While some flow cytometers have lasers that can measure fluorophore-conjugated antibodies outside of the visible spectrum of light, namely UV and IR, the FACS Symphony does not use these lasers.

To ensure the data acquired from the flow cytometer is accurate, a compensation panel must be performed to minimize spectral overlap as many markers are analyzed simultaneously. Compensation is the process by which spectral overlap between two or more fluorophores is teased apart and any overlap between colors is negated. Photons emitted by one fluorophore are converted into electrons by photomultiplier tubes in order to obtain the emission wavelengths. However, the photomultiplier tubes can interpret signals from more than one fluorophore. The

spectral overlap created is what causes false positives or severely skewed data. To overcome this spectral overlap, compensation of spectral overlap calculates the relative emission of a single fluorophore without the emission of any other fluorophores. The numerical value of a single fluorophore is then added to a matrix. This process is then carried for every fluorophore that is going to be used during an experiment. Once all of the individual emissions have been calculated and added to the matrix, automatic compensation, as derived by Bagwell and Adams or Herzenberg *et al*. is then utilized to calculate spectral overlap and subsequently remove it from the analysis (1993, 2006). By following these steps, spectral overlap and false positives in the data acquired by the flow cytometer can be negated and the results trusted.

When compensating samples for flow cytometry, there are several rules that need to be followed. Initially, the material that is used to compensate samples is extremely important. Either cells or beads that bind to any and all antibodies can be used to compensate fluorophores for flow cytometry. With this come a few caveats. For cells, the cells must express the markers of interest to a relative intensity that will be seen for the experimental samples. If the markers on the compensation cells are too few or too plentiful, the relative intensity could result in more or less spectral overlap when examining the experimental samples which would result in false positives or false negatives in the experimental data. To assess the correct fluorophore emission, the proper concentration of antibodies to cell markers should be analyzed. When using beads, there must be strong negative and strong positive populations in order to correctly assess the relative intensity of each fluorophore. Also, the proper forward scatter area and side scatter areas must be calculated prior to using beads due to the varying sizes of cells in experimental samples. Last, the colors of the fluorophores used for any given experiment must match the fluorophores used in the control samples. For example, a ubiquitously expressed cell surface marker will have

a very bright emission wavelength and the control must have a similar emission wavelength for proper compensation.

Another way to compensate for flow cytometry are fluorescence minus one (FMO) controls. When using multicolor panels, FMOs can be important when setting gates and calculating spectral overlap using flow cytometry software. FMOs are samples of cells that contain every antibody in a panel except one. For example, if a panel contains nine colors, there would be ten FMOs; each lacking a specific color and one sample without any antibodies. These samples are recorded and after automatic compensation with the compensation matrix, FMOs allow for precise gating and assurance that there is no spectral overlap in the data.

When performing a flow cytometric experiment with multiple fluorophores, especially when using fluorophore-conjugated antibodies, compensation must be performed in order to prevent false positive data. When two fluorophores emit photons at similar wavelengths, some of this light can spill over into the detector of other fluorophores. This spillover, or spectral overlap, may result in a false positive and skew the flow cytometry data. To account for this, mathematical corrections using individual emission spectra collected in a matrix can separate fluorophores that are very close in emission. Certain fluorophores, however, cannot be fully compensated (like several brilliant violet colors or APC and PE-Cy7) and should be considered prior to the experiment, during the design of the panel. Through compensation, the reproducibility and validity of any flow cytometer can be assured.

Data collection

Data were collected in the same fashion for every experiment. For the primary collection of mouse tissues, buffers were prepared using the same aseptic procedures the day prior or the

morning of the experiment. Mock-infected mice were always euthanized first to ensure there was no contamination between groups and experimental groups (infected) were subsequently euthanized. One set of dissection tools were used to open the chest cavity of the animal and another set was used to remove tissues. Prior to the removal of any lung, the dissection scissors were doused in 70% ethanol. As mentioned previously, bronchoalveolar lavage (BAL) fluid was collected by inserting a small catheter into the trachea of the euthanized mouse and washing with 1 mL cold PBS several times. This was performed for cytokine analysis and to assess lung damage. The large lobe of the lungs was perfused with 1 mL of 4% formalin and then placed into 4% formalin for histological analysis. The three smaller lobes were placed into 1 mL RNA-Later for RNA analysis, tissue protein extraction reagent with protease and phosphatase inhibitors for immunoblot analysis, or RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Once these tissues were collected, the lobe-specific analysis occurred. Single cell suspensions were then performed as noted earlier and only samples that had a viability of at least 70% were included in flow cytometry studies or cell stimulation-based studies.

Data analysis

The results from the studies were analyzed to determine the changes, if any, were witnessed between each experimental and control group of mice. Disease morbidity and disease mortality were analyzed using linear regression analysis where the slopes of each line were tested for significance. Non-biased scoring via histological analyses of influenza virus-infected mice were analyzed by a pathologist not involved in the studies. Statistical analysis between mock-infected and WT mice was determined using a t-test with an alpha level of 0.05 while a

one-way ANOVA was used for histological analysis involving more than two groups. A Tukey's post-hoc test was then calculated to determine where the statically significant differences, if any, were found.

For frequencies of monocytes between groups, 100,000 individual cells, or events from the flow cytometer, were analyzed. The frequency of cell populations acquired from the flow cytometer were analyzed using the same gating strategy to ensure the analyzed area of cells were the same. Between mock-infected and WT mice, a t-test with an alpha level of 0.05 was used to determine statistical significance. When analyzing more than two cell populations, a one-way ANOVA with an alpha level of 0.05 followed by a Tukey's post-hoc test was used to determine if there was any statistical significance among the groups and where the differences between groups was.

The researcher recognizes that there are assumptions that must be made when using a one-way ANOVA. A one-way ANOVA may only be used when investigating a single factor and a single dependent variable. This statistical test is designed to determine if there are any statistical differences, but it does not determine where those differences are from. To correct for this assumption, every statistical test involving more than two groups was accompanied with a post-hoc test to determine where the statistical differences are. Further, the alpha level of 0.05 is suggestive of differences between groups 95% of the time. In other words, 95% of studies would find the observed difference. It is possible the null hypothesis is true in each scenario. To account for this assumption, each experiment was run in duplicate, if not in triplicate, to ensure any and all observed differences were robust.

Reproducibility

The experiments that were required months of work and many individual experiments that were subsequently analyzed to test the central hypothesis. Given the range of time in which these experiments were performed, the reproducibility of data from one experiment to the next was of vital importance. Mice were used in compliance of NIH recommendation for use of the mice published in the guide for the care and use of lab animals. The animal protocol detailing the procedures and techniques utilized was reviewed and approved by the University of North Dakota Institutional Animal Care and Use Committee (IACUC) under the protocol 1808-8. Mice were routinely infected in the morning and subsequently weighed at the same time each following day. Mice were routinely euthanized in the mornings and tissues were collected in the same manner for every experiment. Single cell suspensions were performed in similar fashions from one experiment to the next and the flow cytometer was compensated prior to running any samples for each experiment. Standard curves and mock-infected controls were included for every cytokine bead array and the experiments were performed as per the manufacturer's directions. By taking these vital steps, the reproducibility of the data and the experiments was ensured.

Summary

This chapter describes the context, instrumentation, methods, procedures, and the reliability of each study. Further, the data collection process and the data analysis were also presented. The data collected was subsequently analyzed following the parameters presented in this chapter. In the following chapter, the results of the collected and analyzed data will be discussed. A summary and discussion of the findings, first described in this chapter and

presented in chapter 4, as well as limitations of the studies, implications for future studies, and recommendations of future research will be described in chapter 5.

Chapter 4: Analysis and Presentation of the data

The severe morbidity and mortality associated with acute influenza A virus infection (IAV) results in $11.2 billion annually to the healthcare system of the United States. Further, the CDC estimates that as many as 61,000 people die each year in the United States due to IAV infection. From secondary comorbidities to the induction of cytokine storms within the lungs of IAV-infected individuals, the role of the host's immune response in containing, but also damaging the host cannot be understated.

As mentioned in chapter 2, the exact mechanism by which the innate immune response promotes a hyper-inflammatory state has been documented but not well defined. Here, we tested the central hypothesis that the host's immune response is responsible for the respiratory epithelium damage observed in acute influenza A virus infection. Further the direct damage to the lungs during IAV infection is caused by inflammatory monocytes ($CD11b_+$ $Ly6C_+$ $CCR2_+$, IMs). The purpose of this study was to elucidate the mechanisms by which IMs are recruited during IAV infection, detail their role in lung pathology, and identify specific pro-inflammatory cytokines that aid in IM-lung damage.

This chapter begins with an overview of the procedures used to analyze the data acquired over the course of one and a half years. Many techniques including flow cytometry, histological staining, cell sorting, RNA sequencing, and mouse work was necessary to test the central hypothesis. The next section details the reliability of the instrumentation that was used. Namely, flow cytometry is a very useful and robust instrument but the controls, machine maintenance, and reliability are all important and vary between users. It is of the upmost importance to ensure the flow cytometer was accurate and the machine settings were similar across each experiment.

The third section details the influenza mouse model that was used for each study. There were many different knock-out mouse strains that were available to test the central hypothesis.

The bulk of this chapter discusses in great detail the data that was acquired from testing the central hypothesis and the subsequent research questions that branched from one experiment to the next. There is a total of six research questions that were tested beginning with the overview of modeling influenza A virus infections in mice and identifying an important leukocyte that is recruited during the acute phase of the disease. The second research question tests the importance of these leukocytes on viral clearance and disease severity in knock-out mice. The third research question is in regard to an antiviral cytokine that plays an important and novel regulatory role of the aforementioned leukocytes and the fourth research question details data acquired from RNA sequencing of sorted and isolated monocytes. The fifth research question tests the source of IFN-γ during influenza virus infection. The sixth research questions detail the role of the adaptive immune response in mice and how the innate and the adaptive immune responses overlap. Together, these data support the hypothesis that an over-zealous immune response during influenza A virus infection is responsible for much of the pathology associated with the disease. The end of the chapter provides a summary of the data as they relate to the research questions.

Data analysis procedures

The researcher performed experiments over the course of one year and half and collected data from each experiment. Each experiment contained at least two groups, one experimental and the relevant controls for the experimental group. For some experiments, there were as many as four groups with an experimental group, a comparison for the experimental group, and the

relevant control groups. Each group contained at least five mice to ensure statistical power (detailed in chapter 3). Every experiment was done at least twice for rigor.

The six research questions were examined using descriptive statistics including means and standard error of the means. The mean provided the central tendency for each variable studied while the standard error of the mean measured variability between groups for each distribution. The data with two groups were analyzed using a t-test with an alpha level of 0.05 or less being statistically significant. Where more than two groups were compared, a One-Way ANOVA with a Tukey's post-hoc test for multiple comparisons was used with an adjusted alpha level of 0.05 or less being statistically significant.

Instrumentation reliability

As noted in chapter 3, the setting for the flow cytometer were optimized prior to each and every experiment. These optimal settings were saved and used for each experiment. Prior to each experiment, compensation controls using compensation beads, as well as single cells, were run to ensure optimal laser distribution. Compensation controls were also used to minimize spill-over between laser channels. A minimum of 106 cells from each sample of any given experiment were stained for flow cytometry. 10^5 cells were collected by the flow cytometer and analyzed

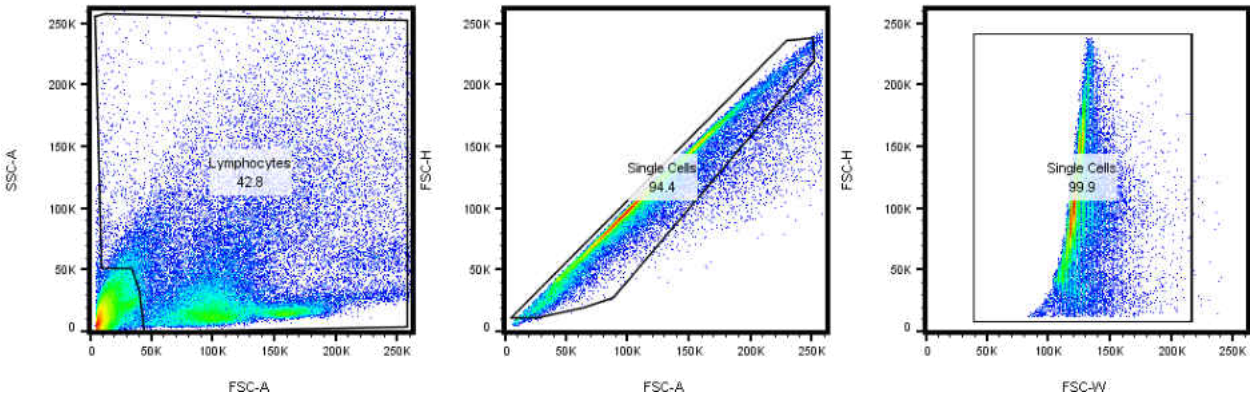

Figure 2. Gating strategy for flow cytometric analysis of single cells from the lungs of mice.

using FlowJo (Tree Star) software. Figure 1 provides an over view of the gating strategies employed for each experiment where total alive cells, singlets, and $CD11b_+$ cells were gated.

For cytokine bead array experiments, the setting of the flow cytometer was optimized prior to each experiment. When determining the concentration of each cytokine/chemokine, the mean fluorescence intensity (MFI) value from the flow cytometer was compared to the MFI standard curve of the known analyte.

Animal Model

Six to eight-week-old C57BL/6 mice (B6), or single protein knockout mice on a C57BL/6 background ($CCR2^{-/-}$, IFN-$\gamma^{-/-}$) were used in compliance of NIH recommendation for use of the mice published in the guide for the care and use of lab animals. The animal protocol detailing the procedures and techniques utilized was reviewed and approved by the University of North Dakota Institutional Animal Care and Use Committee (IACUC) under the protocol 1808-8. As mentioned previously, control mice were matched in both sex and age. The B6 mouse model was used for this study for several reasons. B6 mice are the most widely used inbred mouse strain to model human disease. Further, the B6 mouse is very easy to breed and inexpensive to house when compared to larger animal models like rats or ferrets (as influenza virus research has been performed in). These mice are also less sensitive to pain which minimizes animal suffering and cruelty when infected them with viruses.

Mice were anesthetized via light isoflurane inhalation and given a sub-lethal dose of 250 PFU H1N1 (PR8) intranasally in 100 µL sterile PBS. Control B6 mice were anesthetized and given only 100 µL sterile PBS. All mice were allowed to eat and drink *ad libitum* for seven days post-PR8 infection and monitored for loss of body weight. Seven days or a 20% loss in body

weight was considered to be the endpoint for each experiment. After seven days of infection, mice were euthanized, and their lungs were aseptically collected for protein analysis or flow cytometric analysis.

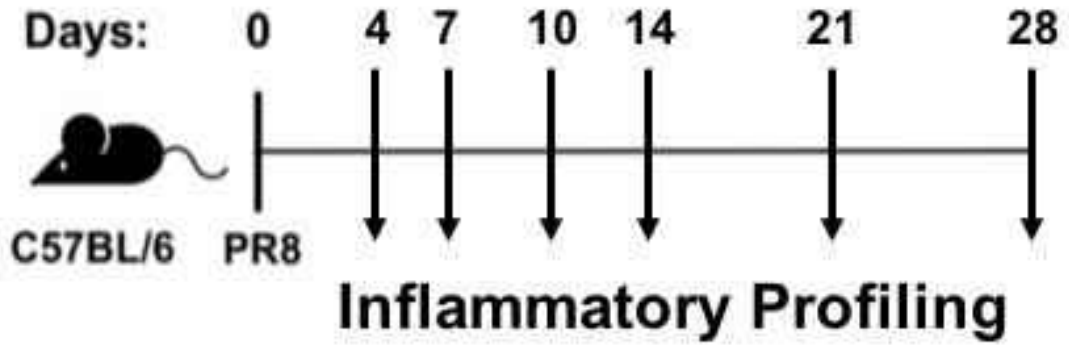

Figure 3. Timeline of all infections for mice.

Modeling Influenza A Virus Infection in Mice

Mice were infected with 250 PFU of influenza A virus (IAV) in 100 µL PBS as previously described. To evaluate the morbidity associated with IAV infection, mice were euthanized on day 4, 7, 10, 14, 21, or 28 post-PR8 infection. The largest decrease in body weight was observed between days seven and nine (figure 4). The body weight in PR8-infected mice was completely regained by day 14 post-infection. To assess the early immune response between days 4 and 10, inflammatory cytokines were measured. TNF-α, IFN-γ, and IL-6 were all elevated on days 4 and 10 compared to uninfected mice but these cytokines peaked at day 7 (figure 4) suggesting seven days post-PR8 infection is when the immune system mounts the largest response. Further, the concentration of CCL2 was highest at day 7 (figure 4). Hematoxylin and eosin (H&E) staining of the large lobe of the lungs revealed a large influx of cells infiltrating into the lungs via the large airways at day 7 post-infection compared to mock-infected mice (figure 4). By days 14 and 21 post-infection, the massive cellular infiltration into

the lungs had been ablated suggesting resolution of the disease by day 14. Due to the highest morbidity occurring seven days post-infection via percent of body weight lost, the influx of cells into the lungs, and the inflammatory burden as detailed by cytokine analysis, this time point was chosen for future experiments.

Due to the high influx of cells into the IAV-infected lungs of B6 mice as shown in Figure

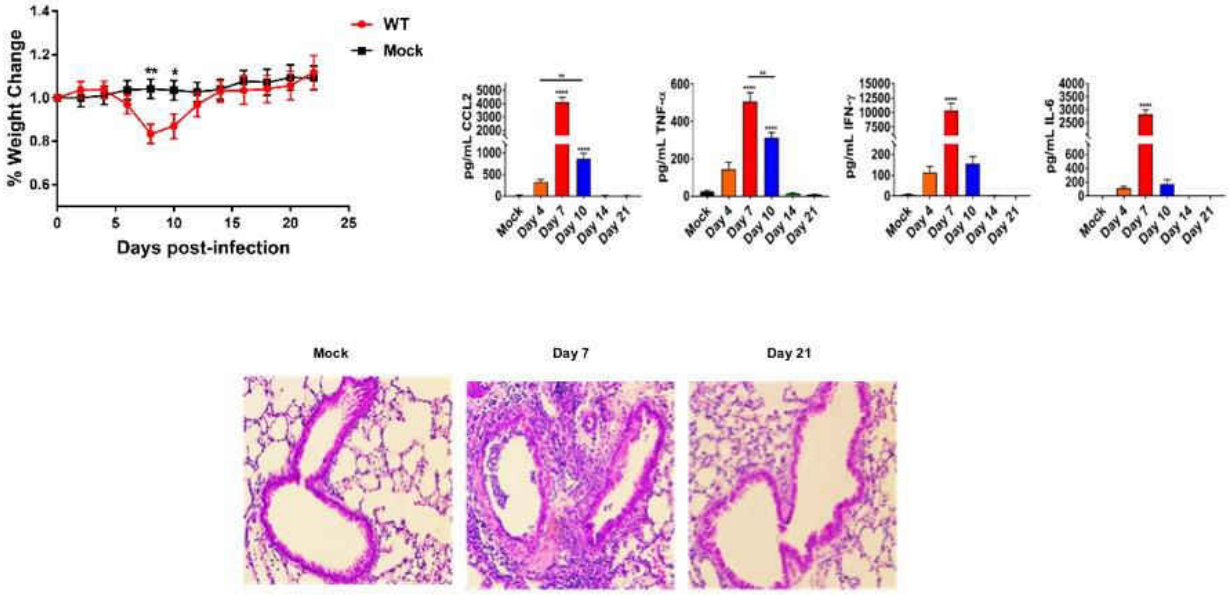

*Figure 4. Percent weight change, cytokine analysis for data 4, 7, 10, 14, and 21 post-PR8 infection in mice, and H&E staining of mouse lungs on days 7 and 21 post-PR8 infection. Data is representative of two independent experiments using 6-8-week-old sex-matched mice. Six mice per group were used in each experiment. Data are presented as ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 using a one-way ANOVA with Tukey's post-hoc test for variance.*

4, the phenotype of the immune cells was analyzed by flow cytometry. The frequency of monocytes ($CD11b_+$ $Ly6C_+$) drastically increased on day 7 compared to mock-infected controls as shown by comparing mock-infected mice and PR8-infected mice seven days post-infection (figure 5). Further, the increase in CCR2 expression also suggested the influx of monocytes was pro-inflammatory in nature. As evidenced by figure 4, the time point for inflammatory profiling in mice was determined to be day 7 due to the increase in pro-inflammatory cytokines. Similarly, the recruitment of inflammatory monocytes into the lungs was determined on days 4, 7, 10, 14, 21, and 28 post-PR8 infection. The frequency of IMs in the lungs were compared to mock-

infected mice at each timepoint. In an identical manner to the inflammatory cytokines, the peak of IM recruitment into the lungs was day 7 with a modest increase at day 4 (figure 5). By day 10, IMs had returned to levels comparable to mock-infected controls with a trend that continued to remain low through day 28 post-PR8 infection. (figure 5).

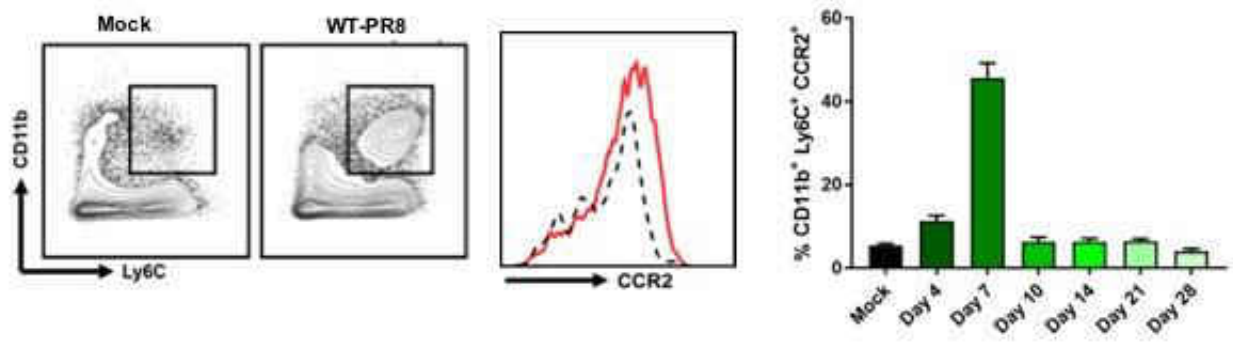

*Figure 5. Identification of inflammatory monocytes via flow cytometric analysis. Data is representative of three independent experiments using 6-8-week-old sex-matched mice. Six mice per group were used in each experiment. Data are presented as ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 using a one-way ANOVA with Tukey's post-hoc test for variance.*

To determine if the increase in monocytes were inflammatory or macrophage-like, the pro-chemotactic, pro-inflammatory receptor, CCR2, was measured on the monocytes via flow cytometry. $CCR2_+$ monocytes, termed inflammatory monocytes (IMs), were elevated drastically in PR8-infected mice compared to mock-infected controls (figure 5). Further, more than 50% of the monocytes in the lungs were $CCR2_+$ suggestive of a robust inflammatory response (figure 5). Further phenotypic analysis for inflammatory profiling of the IMs in the lungs showed an increase in CD80 (activation molecule), IL-17RA (pro-inflammatory receptor for IL-17), CX3CR1 (mediated adhesion of leukocytes in tissue), and CD11c (integrin on macrophages and monocytes) in PR8-infected mice compared to mock controls (figure 6). These data suggest that the influx of immune cells into the lungs during acute PR8 infection more closely resemble inflammatory monocytes and no other leukocytes. Further, the inflammatory profile of these IMs is suggestive of a hyper-inflammatory response.

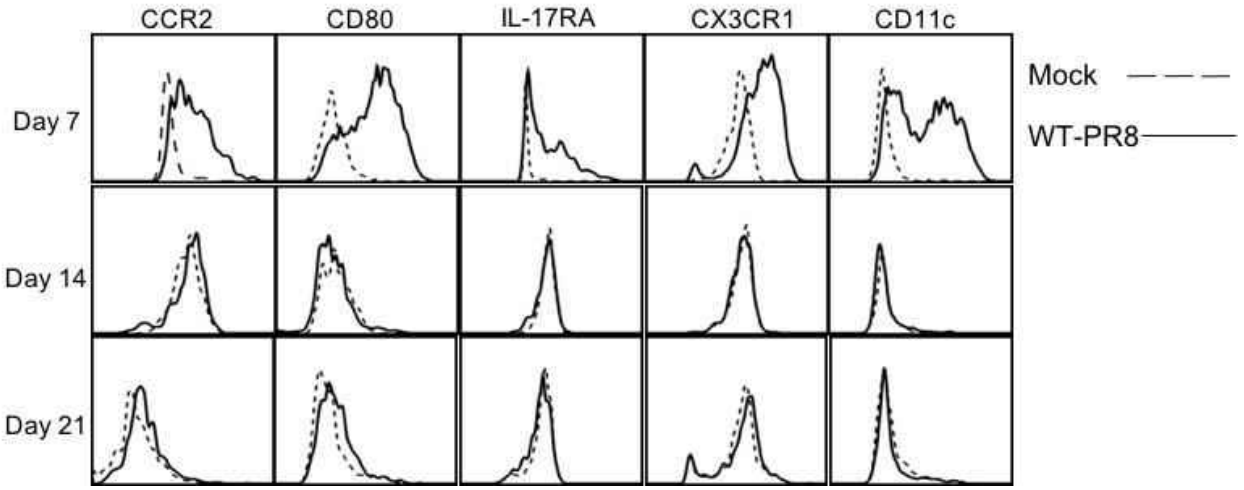

*Figure 6. In depth phenotyping of surface receptors on inflammatory monocytes via flow cytometry on days 7, 14, and 21 post-PR8 infection. Data is representative of three independent experiments using 6-8-week-old sex-matched mice. Six mice per group were used in each experiment. Data are presented as ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 using a one-way ANOVA with Tukey's post-hoc test for variance.*

To test the hypothesis that CCR2+ monocytes are responsible for the damage to the lung epithelium, we infected mice deficient in CCR2 with PR8 (CCR2-/--PR8) and analyzed the recruitment of IMs in the lungs and lung damage via the production of inflammatory cytokines and chemokines. Previously, the morbidity in mice infected with PR8 resulted in nearly 20% body loss within seven days of infection. In CCR2-/- mice, the observed morbidity was statistically less than WT-PR8 mice with total weight loss in CCR2-/- mice being 10% from their starting weight (figure 7). Further, the infiltration of total immune cells into the lungs was less in the CCR2-/--PR8 mice compared to the WT-PR8 mice (figure 6) suggesting the reduced weight loss was due to a less severe immune response. As a control, flow cytometric analysis revealed that CCR2-/--PR8 mice had a reduction of IMs in the lungs that resembled that of mock-infected mice (figure 7). Further, the concentration of CCL2 in the lungs was elevated in the CCR2-/--PR8 mice compared to the WT-PR8 and mock mice suggesting the production of CCL2 and thus, the ability to recruit CCR2+ cells was present in the CCR2-/--PR8 mice but the lack of the CCR2

receptor did not stop the production of the chemokine (figure 7). Instead of CCL2 being internalized and by recruited IMs as they migrate to the lungs, the concentration of CCL2 did not decrease and thus, there was an increase in CCL2 in CCR2$^{-/-}$-PR8 mice compared to WT-PR8 mice (figure 7). These data suggest the CCR2$^{-/-}$ mice were deficient in CCR2 signaling and the overall morbidity associated with PR8 infection was lessened without inflammatory monocytes.

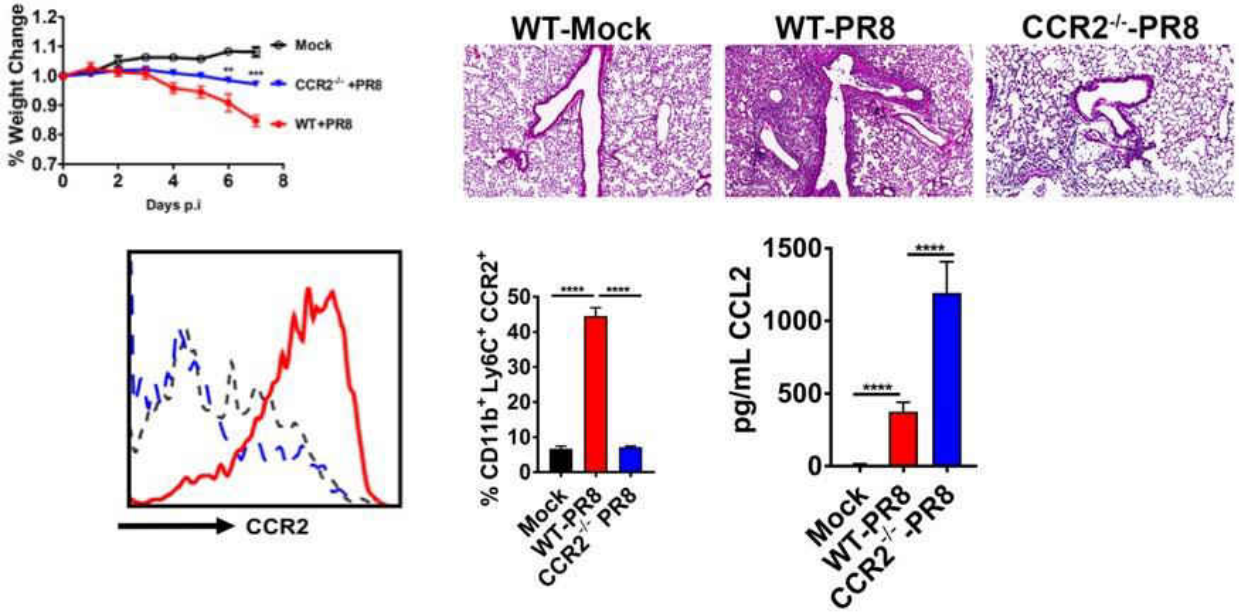

*Figure 7. Percent weight change, H&E histological staining, flow cytometric analysis, and measurement of CCL2 in WT-mock, WT-PR8, and CCR2 knock out mice seven days after PR8 infection. Pulmonary inflammation observed by H&E staining of mock, PR8-infected, and CCR2$^{-/-}$-PR8 mice 7 dpi. Data is representative of three independent experiments using 6-8-week-old sex-matched mice. Five to six mice per group were used in each experiment. Data are presented as ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 using a one-way ANOVA with Tukey's post-hoc test for variance.*

Role of IFN-γ in IAV Immunopathology: IFN-γ$^{-/-}$ Mouse Model

While monocytes may be the cells responsible for lung damage early in IAV infections, the observed morbidity without CCR2^{+} cells suggested another mechanism by which IAV damages the host. During the initial immune surveillance in figure 3, high concentrations of the anti-viral cytokine, IFN-γ, were observed (figure 8). To test the role of IFN-γ during PR8 infection, IFN-γ$^{-/-}$ mice were infected with 250 PFU of PR8 (IFN-γ$^{-/-}$-PR8) and seven days later, euthanized to assess the immune cells in their lungs as well as inflammatory cytokines and large

airway damage. IFN-γ$^{-/-}$-PR8 mice lost less than 10% of their body weight at day seven post-infection while WT-PR8 mice lost nearly 20% of their body weight suggesting a damaging role for IFN-γ during PR8 infection (figure 7). Further, the early pro-inflammatory cytokines, TNF-a and IL-1β were decreased in IFN-γ$^{-/-}$-PR8 mice compared to WT-PR8 mice (figure 8). The anti-inflammatory cytokine, IL-10, was increased in IFN-γ$^{-/-}$-PR8 mice suggesting less of a robust inflammatory response in the absence of IFN-γ (figure 8). The concentration of CCL2 in IFN-γ$^{-/-}$-PR8 mice was also decreased when compared to WT-PR8 mice (figure 8). These data suggest a shift from the hyper-inflammatory state observed in WT-PR8 mice to a milder immune response in IFN-γ$^{-/-}$-PR8 mice. To test whether the decrease in CCL2 in IFN-γ$^{-/-}$-PR8 resulted in a decrease in IMs in the lungs, mice were euthanized seven days post-PR8 infection and single cells from their lungs were prepared for flow cytometry. The frequency of IMs in the lungs of IFN-γ$^{-/-}$-PR8 mice was less than that of WT-PR8 mice suggesting IFN-γ modulates the recruitment of CCR2^{+} monocytes from the bone marrow during PR8 infection (figure 8). Taken together, the data suggest a relationship where IFN-γ production in the lungs during PR8 infection drives the recruitment of CCR2^{+} inflammatory monocytes by increasing the production of CCL2 and subsequently priming IMs to become hyper-inflammatory and damaging to PR8-infected epithelial cells. To test whether the lack of IFN-γ also resulted in a decrease in lung pathology, large lobes of lungs from mock-infected mice, WT-PR8 mice, and IFN-γ$^{-/-}$-PR8 mice were fixed with 4% formalin for at least two days, paraffin embedded, sectioned into 5 µm sections, and subsequently stained with H&E to analyze the infiltration of cells into the lungs in addition to the pathology associated with large airways. H&E staining revealed a decrease in cell infiltration in the IFN-γ$^{-/-}$-PR8 mice compared to the WT-PR8 mice. Further, the damage to the brachial epithelial and endothelial cells in the IFN-γ lungs was less severe when compared to the

WT-PR8 mice (figure 8). Less damage to the pulmonary epithelium when mice are systemically deficient in IFN-γ supports the hypothesis that IFN-γ is a key recruiter of pro-inflammatory cells and mediates the damage done to the lungs during PR8 infection.

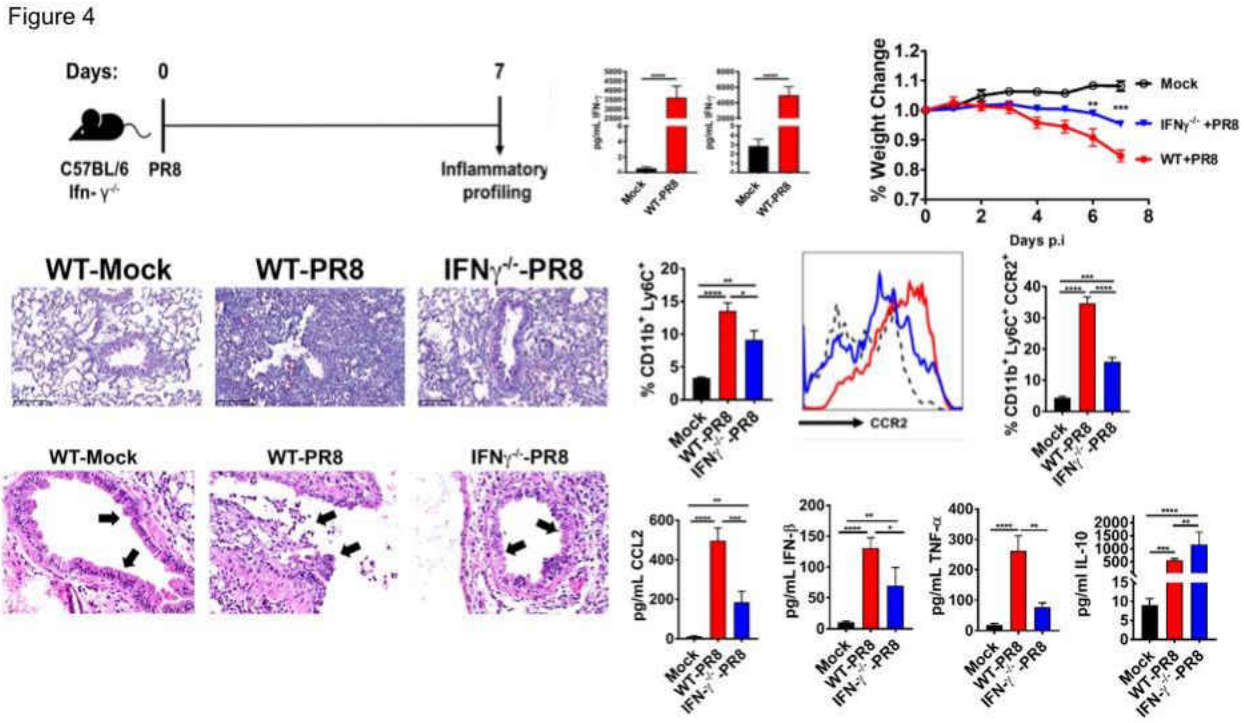

*Figure 8. Overview of experiments with IFN-γ-/--PR8 mice. Concentration of IFN-γ seven days post-PR8 infection in WT mice suggested a pro-inflammatory role for this anti-viral cytokine. Percent weight change, histological analysis via H&E staining, flow cytometry analysis of inflammatory monocytes, and concentrations of pro-inflammatory cytokines or anti-inflammatory cytokines. Data is representative of three independent experiments using 6-8-week-old sex-matched mice. Five to six mice per group were used in each experiment. Data are presented as ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 using a one-way ANOVA with Tukey's post-hoc test for variance.*

RNA-sequencing of IFN-γ-Proficient and IFN-γ-Deficient Mice During IAV Infection

The recruitment of inflammatory monocytes to the lungs by IFN-γ during an influenza virus infection causes direct damage to the respiratory epithelium. To better understand the factors that inherently allow IMs to interact with infected epithelial cells, as well as canonical inflammatory pathways, inflammatory monocytes from uninfected, WT-PR8, and IFN-γ-/--PR8 mice were magnetically sorted. RNA from the magnetically sorted monocytes was extracted and

RNA sequencing analysis was performed. Principle Component Analysis showed that the sorted cells clustered within their respective groups thereby suggesting the sorted cells were similar to their respective group (figure 9).

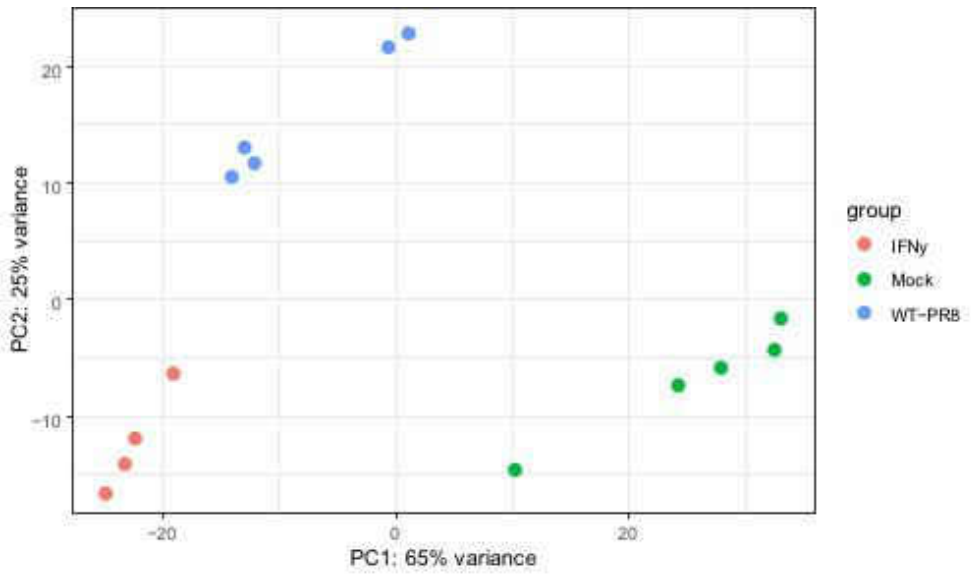

Figure 9. Principle Component Analysis of magnetically sorted monocytes from WT-Mock, WT-PR8, and IFN-γ$^{-/-}$-PR8 mice seven day spost-PR8 infection. Five mice, sex-matched and aged 6-8 weeks, were used for RNA-sequencing analysis except the IFN-y sorted monocytes where four mice were used.

Differentially expressed gene (DEG) analysis revealed each group had a distinct subset of genes that were expressed during PR8 infection. The largest amount of DEGs was between the mock and the IFN-γ$^{-/-}$-PR8 monocytes where 1241 genes were differentially expressed (figure 10). Between mock and WT-PR8 monocytes, 469 genes were differentially expressed (figure 10). These data suggest that mice infected with PR8 had genes that were either significantly upregulated or significantly downregulated when compared to mock mice. The final comparison between WT-PR8 monocytes and IFN-γ$^{-/-}$-PR8 monocytes revealed 335 DEGs (figure 10) which suggests that without IFN-γ, IMs express a different subset of genes. To determine what the roles of the observed DEGs are, canonical immune pathways were calculated using pathway enrichment analysis Canonical pathways were identified with a $\log_{10}$ significance (p-value)

cutoff of 1.3 and determine dot be upregulated or downregulated between groups (mock vs WT-PR8, mock vs IFN-γ$^{-/-}$-PR8, and WT-PR8 vs IFN-γ$^{-/-}$-PR8). A wide variety of canonical inflammatory pathways were significantly decreased between the WT-PR8 and IFN-γ$^{-/-}$-PR8 monocytes (figure 10). Specifically, CCR5 signaling in macrophages, chemokine signaling, complement fixing, cytotoxic T lymphocyte-mediating apoptosis, inflammasome pathways, leukocyte extravasation, the NF-kB pathway, and iNOS and ROS production in macrophages were also significantly decreased in the IFN-γ$^{-/-}$-PR8 monocytes when compared to WT-PR8 mice (figure 10). The drastic reduction in the pathways listed are indicative of an attenuated M1 inflammatory response in the IFN-γ-deficient monocytes as evident by a reduction in these monocytes' ability to undergo chemotaxis and recognize inflammatory chemokines, recognize complement proteins and target opsonized protein for phagocytosis, produce IL-1β and IL-18 via inflammasome induction as well as type I interferons, and directly damage influenza virus-infected cells via nitric oxide and/or reactive oxygen species production respectively (figure 10). Together, the DEGs and the pathway analysis revealed a dampened inflammatory response in monocytes that were not activated in the presence of IFN-γ compared to monocytes that were (WT-PR8 monocytes). These data correlate with the *in vivo* data from research questions two and three where IMs are activated by IFN-γ and recruited to the lungs by CCL2 where they directly decrease the integrity of the respiratory epithelium by committing epithelial cells infected with influenza virus to undergo apoptosis and by secreting pro-inflammatory cytokines that further exacerbate the over-zealous anti-viral, pro-inflammatory state.

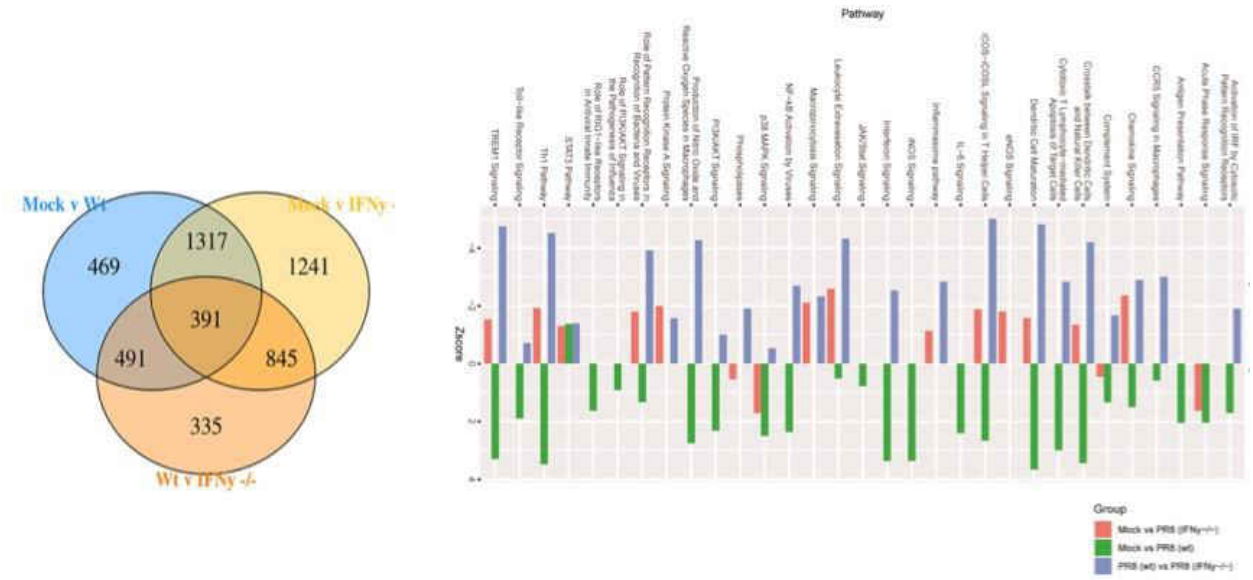

Figure 10. Differentially expressed genes between WT-mock, Wt-PR8, and IFN-$\gamma^{-/-}$-PR8 sorted monocytes on day seven post-PR8 infection and pathway analysis of specific immune-related pathways as shown by changes in Z scores. Five mice, sex-matched and aged 6-8 weeks, were used for RNA-sequencing analysis except the IFN-y sorted monocytes where four mice were used. Venn diagram of Differentially Expressed Genes (DEGs) identified by pairwise comparisons of groups in an RNA-Seq analysis of purified lung monocytes from uninfected (mock) and PR8-infected WT and IFN-$\gamma^{-/-}$ mice. ***C****. Pathway enrichment networks between DEG sets generated using the in-house package RichR. Commonly enriched pathways in two or more DEG sets are considered nodes, while edges represent shared genes between pathways. The Cytoscape app MCODE was used to systematically analyze the network for highly interconnected regions or clusters as described in the FAQ. The clusters are presented as colored regions around groups of nodes in the network.*

To better understand the specific genes that are differentially expressed between the three groups of monocytes (mock vs WT-PR8, mock vs IFN-γ-/--PR8, and WT-PR8 vs IFN-γ-/--PR8) a heatmap that quantified the log-fold change of individual inflammation-associated genes, or genes involved in inflammatory pathways, were analyzed (figure 11). In WT-PR8 monocytes, apoptosis-related genes (Tnf and Fasl) and pro-inflammatory signaling cascade proteins that mediate cytokine production (Stats, Socs, Irf) were increased compared to mock mice (figure 11). The same expression patterns of pro-inflammatory genes in IFN-γ-/--PR8 monocytes were decreased relative to WT-PR8 mice further suggesting a shift from a pro-inflammatory, M1-like state in WT-PR8 monocytes to an anti-inflammatory, M2-like state in IFN-γ-/--PR8 monocytes. This notion of a shift from M1 to M2 (pro-inflammatory to anti-inflammatory) monocytes is further shown via network analysis and pathway mapping. An in-house R package, RichR, was

used to generate pathway enrichment networks between comparisons. Commonly enriched pathways in two or more comparisons were included as nodes, while edges represent shared genes between pathways. Pathways related to immune signaling via cytokine production, diapedesis, and T cell activation/differentiation pathways were enriched all suggesting a pro-inflammatory role in WT-PR8 monocytes.

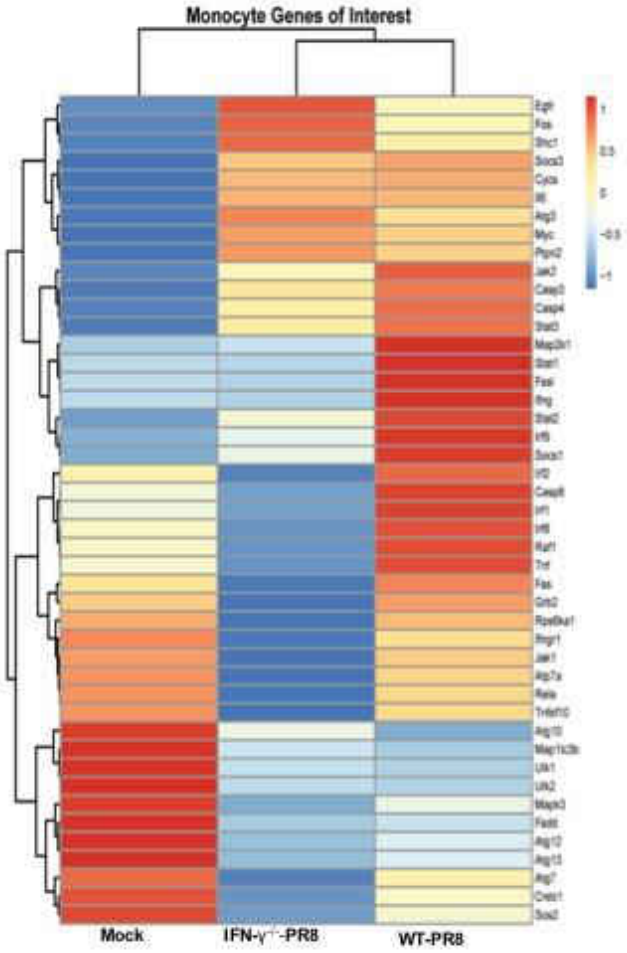

Figure 11. Heatmap analysis of specific pro-inflammatory, anti-inflammatory, or signaling genes from WT-mock, WT-PR8, and IFN-$\gamma^{-/-}$-PR8 sorted monocytes seven days post-PR8 infection. DEGs in the heatmap have their relative directionality normalized across rows to represent the change in expression levels across models.

Cellular Source of IFN-γ During IAV Infection

Thus far, the role of IFN-γ on recruiting and activating inflammatory immune cells has shown the mechanism by which the lungs are damaged during PR8 infection. However, the cells that produce IFN-γ during PR8 infection are poorly understood. To test the hypothesis that resident immune cells in the lungs produce IFN-γ early in infection to recruit damaging inflammatory immune cells, intracellular cytokine staining of IFN-γ was performed in WT mice seven days post infection. Single cell suspensions of aseptically removed lungs from WT-PR8 and mock-infected mice were performed and intracellular staining for flow cytometry was immediately done. Once the cells had been stained for surface markers for NK cells (CD3- NK1.1+), innate-like NK cells (CD11b+ NK1.1+), leukocytes (CD11b+), CD4+ T cells (CD3+ CD4+), and CD8+ T cells (CD3+ CD8+), the cells were permeabilized and stained for IFN-γ in the cytoplasm of the cells. The cells were then fixed with formaldehyde to prevent the production and subsequent release of IFN-γ, and the cells were analyzed via flow cytometry. While each cell type expressed a small amount of IFN-γ, it was only marginally so when compared to their respective mock controls. However, CD8+ T cells expressed over 14% of IFN-γ- when compared to the mock control (figure 12). CD8+ T cells have been shown to play a role in early PR8 recognition in the lungs (figure 12) as well as direct viral-infected cells to undergo apoptosis. These data suggest that CD8+ T cells are the major source of IFN-γ at day seven post-PR8 infection.

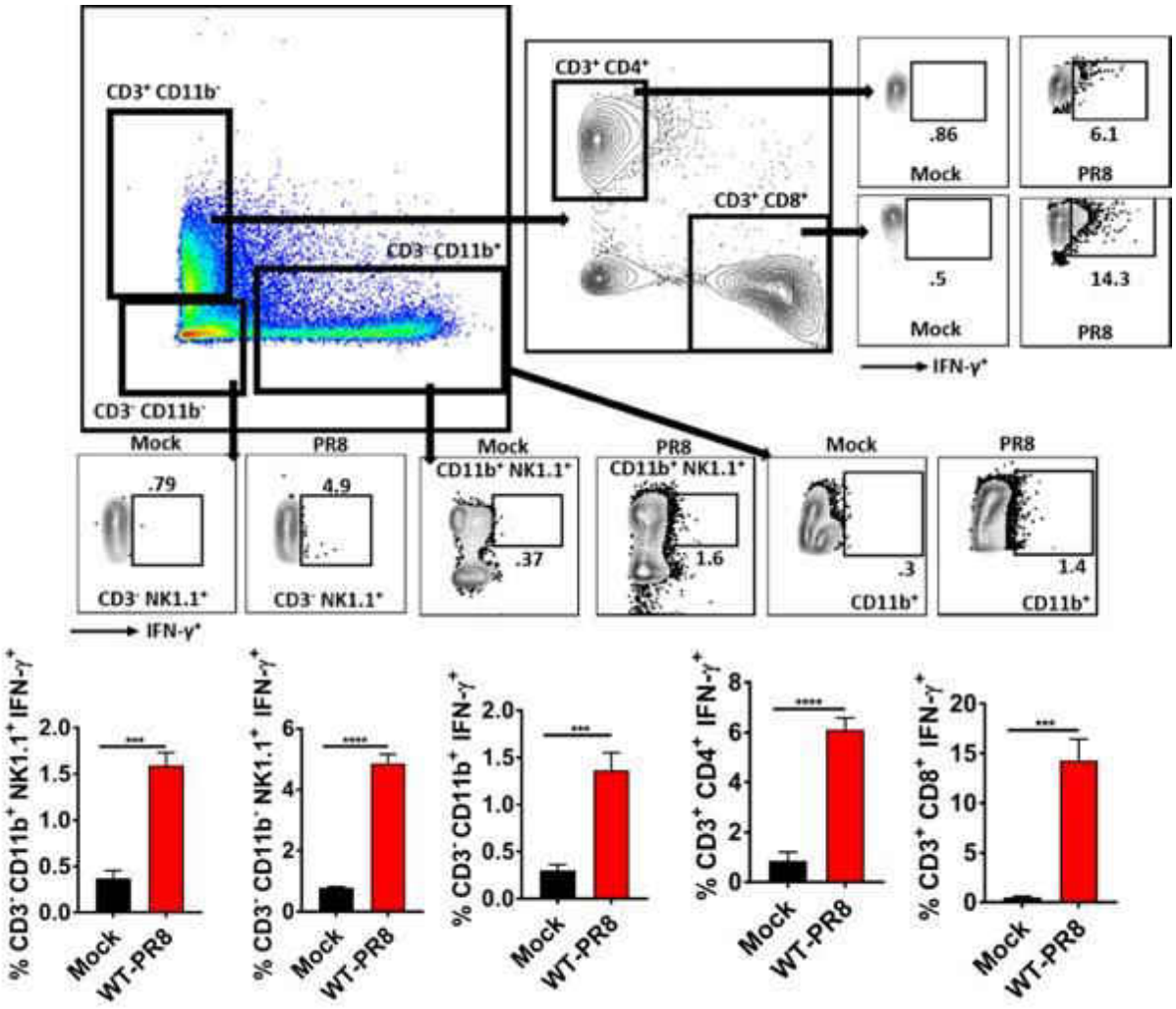

*Figure 12. Intracellular cytokine staining for IFN-γ in WT-PR8 mice seven days post PR8 infection as compared to mock-infected mice (baseline). Cells analyzed were CD4+ T cells, CD8+ T cells, leukocytes, and NK cells. Data is representative of two independent experiments using 6-8-week-old sex-matched mice. Six mice per group were used in each experiment. Data are presented as ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 using a one-way ANOVA with Tukey's post-hoc test for variance.*

Role of the Adaptive Immune Response During IAV Infection

While CD8+ T cells produce IFN-γ, the role of the adaptive immune response in our influenza mouse model, namely CD4 and CD8 T cells, has yet to be elucidated. Using the same infection timeline as done previously, T cells in the lungs of PR8-infected mice were analyzed on days 7, 14, 21, and 28 post-PR8 infection via flow cytometric analysis. The frequency of CD4+

helper T cells ($CD3_+$ $CD4_+$) was marginally increased on day seven post-PR8 infection when compared to the mock-infected mice (figure 12). The frequency of $CD4_+$ T cells at days 14-28 post-PR8 infection were unchanged from mock-infected mice (figure 13). The lack of $CD4_+$ T cell expansion coupled with the limited production of IFN-γ by $CD4_+$ T cells suggests a limited inflammatory role during influenza virus infection. Conversely, cytotoxic $CD8_+$ T cells were drastically increased at day 7 post-PR8 infection when compared to mock-infected mice (40% more $CD8_+$ T cells, figure 13). Similar to helper T cells, the robust expansion of $CD8_+$ T cells in the lungs at day 7 post-PR8 infection was diminished and unchanged to mock-infected mice at days 14-28 (figure 13). A larger frequency of T cells does not outright mean they are activated. To test whether the observed increase in the frequency of T cells also resulted in an increase in the activation of the same T cells, CD62L was measured on the surface via flow cytometry. CD62L is an integrin and a marker for naïve T cells that have yet to be activated. The lack of CD62L on T cells suggests they have been activated via their migration from secondary lymphoid organs. It was observed that at day 7 post-PR8 infection, both $CD4_+$ and $CD8_+$ T cells had a drastic reduction in CD62L expression when compared to the mock-infected controls (figure 13). These data suggest that $CD8_+$ T cells are the main type of effector T cell at day 7 post-PR8 infection in terms of total frequency and activation. $CD4_+$ T cells, while activated, do not increase in frequency relative to mock-infected controls. Coupled with the lack of IFN-γ production, these data also suggest a minimal role for $CD4_+$ T cells in the pathogenic state of the lungs during influenza virus infection and rather, cytotoxic $CD8_+$ T cells being the culprit.

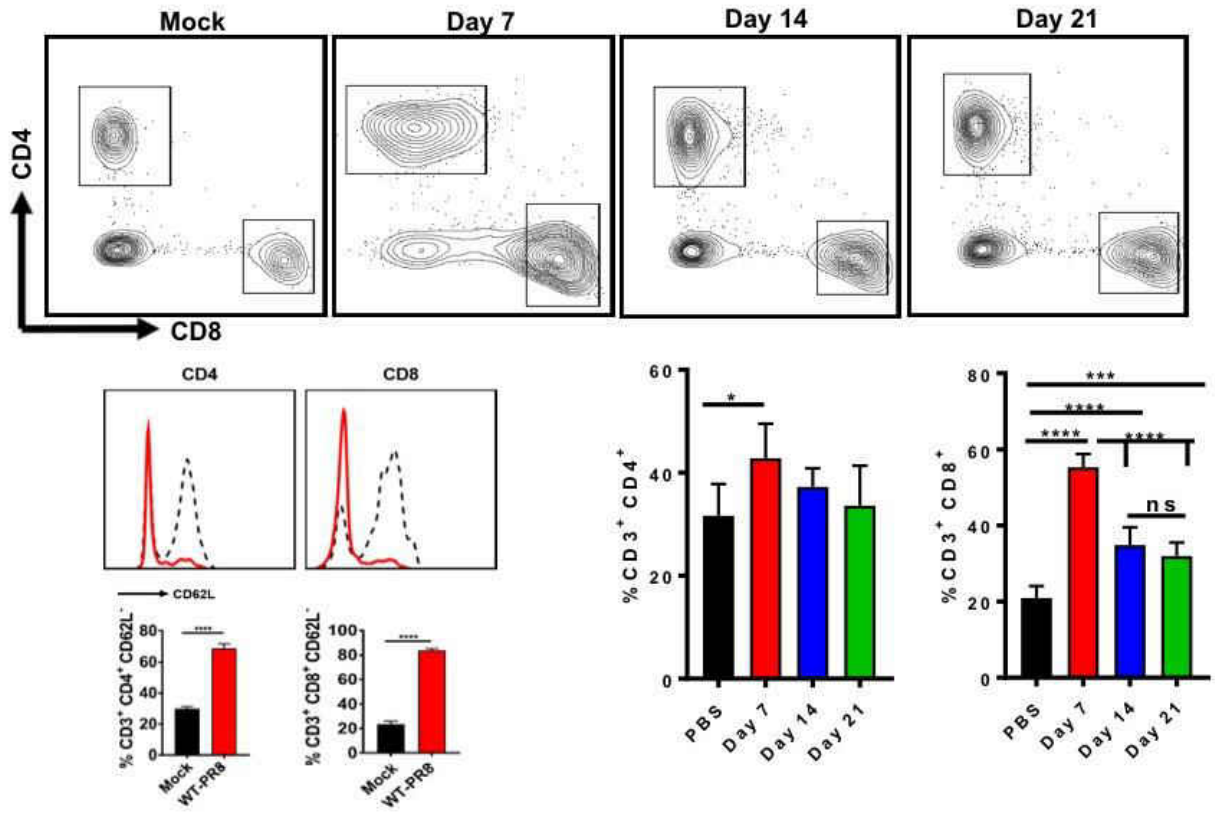

*Figure 13. Adaptive immune response analysis of CD4⁺ and CD8⁺ T cells on days 7, 14, 21, and 28 post-PR8 infection. CD62L expression of the surface of T cells was measured on day 7 to assess the activaiton states of the T cells. Data is representative of two independent experiments using 6-8-week-old sex-matched mice. Five to six mice per group were used in each experiment. Data are presented as ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 using a one-way ANOVA with Tukey's post-hoc test for variance.*

Summary

This chapter began with a brief overview detailing the procedures for analyzing the data, the reliability of the instrumentation used for each experiment, and the infection model used to test the central hypothesis. The focus of this study sought to test how the host's immune response is responsible for the respiratory epithelium damage observed in acute influenza A virus infection. Descriptive statistics were used to ensure rigor and account for variance between the groups.

The data suggested that IMs are recruited to the lungs early in infection and their presence results in cell damage, cell death, and increased morbidity amongst mice infected with PR8. Their absence resulted in less morbidity in mice. Further, the antiviral, pro-inflammatory cytokine, IFN-γ, is a key regulator of CCL2 production, and thus, $CCR2_+$ IM recruitment into the lungs. IFN-γ-deficient mice resembled the lower morbidity observed in CCR2 knock-out mice in terms of weight loss, pro-inflammatory cytokine burden, and inflammatory cell recruitment. Finally, the data suggested that $CD8_+$ cytotoxic T cells are the main immune cell that produces IFN-γ and in the absence of an adaptive immune response, the inflammatory burden decreases.

The results from this experiment will provide key insights into the cross-talk between the immune response and epithelial cells during acute influenza A virus infection. The resulting increase in cell death correlate with an increase in morbidity. The increase in cell death is mediated by a key leukocyte that is activated by $CD8_+$ T cells via the production of IFN-γ. Further, cutting edge RNA sequencing of inflammatory monocytes provides a window into the transcriptome of these cells as they mediate much of the lung damage witnessed during acute respiratory infection. Therapeutic intervention to alleviate the damage to the respiratory epithelium by eliminating or decreasing the frequency of IMs in the lungs of IAV-infected individuals may reduce the observed morbidity and mortality associated with acute viral infections.

Currently, there is a paper in revision that details the data above. Many of the future considerations stem from the results of that paper and as such, there are several intriguing hypotheses that will be followed up.

Chapter 5 will provide an interpretation of the data as well as conclusions that can be drawn from this experiment. Further, the discussion in chapter 5 will expand the current

knowledge that was detailed in the literature review section. Considerations for future studies as well as limitations to this study will also be discussed.

Chapter 5: Summary, recommendations, implications, and conclusions

This research was conducted in order to further understand the mechanisms by which influenza A virus elicits an over-zealous, hyper-immune response in the host. The immune response limits viral propagation through the secretion of pro-inflammatory cytokines as well as direct cell-to-cell interactions which further limit the spread of the virus at the expense of the host's health. Understanding one of the key mechanisms by which inflammatory monocytes are recruited to the lungs during acute viral infection as well as the protein mediators in eliciting their damaging response are crucial to limiting the morbidity of influenza viruses.

Insights into the mechanisms of lung damage caused by the host's immune response may provide critical points for therapeutic intervention and reduce the observed morbidity and mortality the world experiences every year due to influenza virus infections. The link between the adaptive immune response and the innate immune response, as they relate to damage to the host's respiratory epithelium, is a novel mechanism in which influenza viruses inadvertently cause death.

This chapter will present a brief summary of the purpose of these experiments, summarize the procedures that were used in order to test the central hypothesis, and summarize the relevant findings. This chapter will conclude with limitations of the study as well as recommendations for future studies. The implications this study may have on the field of influenza virus research going forward will be addressed as it pertains to the broader literature.

Summary of purpose

The CDC estimates there are approximately 65,000 deaths every year due to influenza virus infections or complications related to influenza virus. (11, 12) Many of the global pandemics that have occurred over the previous century have been caused by influenza virus. (11, 12) Further, seasonal influenza vaccines exist but their efficacy can be limited and compliance with vaccinations are often low, ranging from 50 to 60 percent. (11) Antiviral therapies for influenza virus are often used for individuals infected with the virus but proactive treatment against viruses is often the best course of action. (12)

The deaths caused by influenza virus are the result of acute respiratory distress syndrome (ARDS) or secondary bacterial infections due to the damage elicited by the virus. (14-16) The mechanisms by which the virus makes the lungs permissive to bacterial infections in unknown and the evidence is scarce. Therefore, the purpose of this study was to elucidate the mechanisms by which influenza virus infection damaged the respiratory epithelium due to the host's immune response. ARDS is the result of a cytokine storm which then increases the vascular permeability of the lungs and fluid begins to leak in. (14) Once this occur, the individual's blood pressure drops, and this can result in death. (14, 15) Understanding how the cytokine storm is produced, and the cells responsible for mediating the lung damage, are two questions that the field has yet to answer.

This study showed that there is a large influx of $CD11b^+$ $Ly6C^+$ $CCR2^+$ leukocytes, namely inflammatory monocytes, that are recruited by the infected epithelial cells to the lungs in order to limit the spread of the virus. However, the inflammatory monocytes directly induce apoptosis or necroptosis in infected epithelial cells through the TNF receptor apoptosis inducing

ligand (TRAIL) of their cell surface. The direct cell damage increases the morbidity in the infected individual.

While inflammatory monocytes are the key mediators of cell damage in the lungs, the antiviral protein, IFN-γ, induces a pro-inflammatory state and is responsible for much of the recruitment and activation of inflammatory monocytes. The majority of IFN-γ is produced by lung-resident $CD8_+$ T cells early in infection and without IFN-γ or $CD8_+$ T cells, the observed morbidity caused by influenza virus infection can be limited.

Summary of procedures

For this study, the researcher used a mouse model in order to recapitulate an influenza virus infection in an ethical and expedited way. Using the C57BL/6 mouse (WT), mice were infected with a sub-lethal dose of H1N1 influenza A virus A/PR8/1934 (PR8) and monitored for seven days. Body weight loss was used as a measure of morbidity and, when left alone, 100% of mice recovered even after losing up to 20% of their starting body weight. The B6 mouse background was used in this study because these mice are less sensitive to pain compared to other animal models and they are easy to breed. The BALB/c mouse strain tends to skew towards an anti-inflammatory Th2 response while the B6 background does not further making it a better model for viral studies.

Protein-specific knockout mice were used to assess their role when compared to protein-competent controls. These knockout mice included $CCR2_{-/-}$ and IFN-$\gamma_{-/-}$ which allowed for the assessment of inflammatory monocyte recruitment, the role of the type II interferon response, and the role of the adaptive immune response respectively.

Flow cytometric analysis was employed to study cell populations in the lungs of PR8-infected and mock-infected mice as well as determine the concentration of inflammatory cytokines and inflammatory chemokines.

In order to determine statistical significance and account for variability between groups, a t-test with an alpha level of 0.05 was used for comparisons between WT-PR8 and mock-infected groups. For comparisons between more than two groups, a One-Way ANOVA was used with an adjusted alpha level of 0.05. A Tukey's post-hoc test was used after the One-Way ANOVA analysis to determine where the differences between groups was, if there was any difference at all.

Research questions

The first research question sought to determine the inflammatory phenotype of WT-PR8 mice before, during, and after acute influenza virus infection. To confirm the model was applicable to influenzas virus research, the weight loss, cytokines, chemokines, and lung damage were evaluated in WT mice infected with PR8. There was a statistically significant difference in weight loss compared to mock-infected mice and pro-inflammatory cytokine and pro-inflammatory chemokine production were elevated. Histological analysis showed severe loss of large airway structures and a large volume of immune cell recruitment into the airways all indicative of acute respiratory infection.

Flow cytometric analysis of single cells from the lungs of infected mice showed a large frequency of inflammatory monocytes relative to the mock-infected controls. The phenotype of the inflammatory monocytes was further indicative of their cell-damaging, pro-inflammatory

nature as macrophage activation markers and integrins were all upregulated in CCR2+ inflammatory monocytes.

The second research question tested whether CCR2+ inflammatory monocytes cause lung pathology during influenza virus infection. By using a CCR2-/- mouse model infected with the same plaque forming units (PFU) of PR8 as the WT mice, the morbidity associated with CCR2-proficient immune cells could be analyzed. Weight loss, inflammatory cytokines, inflammatory chemokines, and lung pathology were less severe in the CCR2-/--PR8 mice compared to the WT-PR8 mice suggesting inflammatory monocytes play a damaging role during influenza virus infection.

As controls, flow cytometry was used to analyze CCR2+ cells in mock-infected, WT-PR8, and CCR2-/--PR8 mice. The frequency of CCR2+ inflammatory monocytes in WT-PR8 mice was similar to what was observed in the experiments covering the first research question while there was only background from the CCR2-/--PR8 mice. The concentration of CCL2 in CCR2-/--PR8 mice was elevated when compared to WT-PR8 mice also suggesting the knock-out mouse model was truly deficient of CCR2+ expression. This is due to free CCL2 not being bound by its receptor and thus, not being internalized and removed from the periphery.

Research question three examined the role of the type II interferon response in the PR8 mouse model. In research question one, it was apparent that the antiviral protein, IFN-γ, was the most abundant pro-inflammatory cytokine measured. To assess the role of IFN-γ, an IFN-γ-/- mouse was used. For these experiments, 250 PFU of PR8 was used to infect IFN-γ-/--PR8 mice. When IFN-γ was absent, the concentration of CCL2 was decreased in addition to canonical pro-inflammatory cytokines, TNF-α and IFN-β while the anti-inflammatory cytokine, IL-10, was increased. IFN-γ-/--PR8 mice loss less body weight when compared to WT-PR8 mice but there

was still significant morbidity as they lost 10% of their starting weight compared to mock-infected controls.

The decrease in CCL2 suggested that IFN-γ$^{-/-}$ may play a role in recruiting CCR2^{+} inflammatory monocytes to the lungs. Flow cytometry experiments revealed a decrease in the frequency of inflammatory monocytes compared to WT-PR8 mice. This reduction in inflammatory monocytes in IFN-γ$^{-/-}$-PR8 mice corresponded with less severe immunopathology in the lungs as indicated by less large airway damage via histological staining.

The fourth research question involved sorting monocytes from mock WT mice, WT-PR8 mice, and IFN-γ$^{-/-}$-PR8 mice and subsequently extracting RNA for RNA sequencing analysis. Full transcriptome comparisons between groups revealed several hundred differentially expressed genes (DEGs) that comprised the severity of the pro-inflammatory response in monocytes. Interestingly, the largest variance in DEGs was between the mock control monocytes and the IFN-γ$^{-/-}$-PR8 monocytes (figure 9, 1241 genes). To better understand the role of the DEGs, pathway analysis was performed. It was determined that CCR5 signaling in macrophages, chemokine signaling, complement fixing, cytotoxic T lymphocyte-mediating apoptosis, inflammasome pathways, leukocyte extravasion, the NF-kB pathway, and iNOS and ROS production in macrophages were also significantly decreased in the IFN-γ$^{-/-}$-PR8 monocytes when compared to WT-PR8 mice. These data suggest that the discrepancy in DEGs of IFN-γ$^{-/-}$-PR8 monocytes is most likely due to the downregulation of pro-inflammatory genes that are regulated by IFN-γ. Further, the lack of pro-inflammatory gene induction in IFN-γ$^{-/-}$-PR8 monocytes suggests a more anti-inflammatory, or M2 macrophage-like state. To test the hypothesis of pro-inflammatory genes being negatively regulated in IFN-γ$^{-/-}$-PR8 monocytes, the log-fold change in gene expression between all three groups of monocytes was analyzed via

heatmap analysis. Pro-inflammatory, pro-apoptotic-inducing genes were increased in WT-PR8 monocytes compared to IFN-γ$^{-/-}$-PR8 monocytes. Further, pro-inflammatory signaling genes, like Stats, MAP kinase, and interferon regulatory factors (Irf), were all decreased in IFN-γ$^{-/-}$-PR8 monocytes compared to WT-PR8 mice further suggesting an M2 state without the presence of IFN-γ.

The fifth research question assessed which immune cells are the major source of IFN-γ production in the lungs during influenza A virus infection. Research question one detailed the robust increase in IFN-γ concentration in the lungs during influenza A virus infection compared to mock-infected controls. Research question three elaborated tested the hypothesis that IFN-γ, while an anti-virial mediator, also contributed to the recruitment of CCR2^{+} IMs in the lungs. To better understand which cells are coordinating this non-CCL2-mediated recruitment of IMs, the immune cell that produces IFN-γ was tested via intracellular protein staining.

A wide array of immune cells were analyzed for IFN-γ production. NK cells and NKT cells have been thought to produce IFN-γ. However, the robust expansion of CD8+ T cells in our model suggested these cytotoxic T cells may be the main producer of IFN-γ. NK and NKT cells were found to produce a very small amount of IFN-γ in the influenza-infected mice compared to mock-infected mice. Similarly, CD11b^{+} leukocytes barely produced IFN-γ above baseline levels compared to mock controls. When assessing IFN-γ production in T cells, CD4^{+} T cells produced 6% more IFN-γ compared to mock controls but overwhelmingly, CD8^{+} T cells produced the bulk of IFN-γ in the lungs of influenza-infected mice. More than 14% of CD8^{+} T cells produced IFN-γ at day 7 post-influenza virus infection suggesting these cytotoxic T cells are responsible for the interactions between epithelial cell damage and immune cell-mediated death.

The sixth research question described the expansion of the adaptive immune response in the lungs of influenza virus-infected mice. Because $CD8_+$ T cells were found to produce the majority of IFN-γ, the expansion of T cells in the lungs was analyzed.

The frequency of $CD4_+$ T cells was slightly increased seven days post-influenza virus infection when compared to mock-infected mice. The frequency of $CD4_+$ T cells was unchanged from mock-infected mice on days 14 and 21 post-influenza virus infection suggesting a minimal role during acute viral infection. Conversely, there was a pronounced expansion of $CD8_+$ T cells at day 7 post-influenza virus infection. This expansion remained elevated when compared to mock-infected controls at 21 days post-infection suggesting the $CD8_+$ T cell response is the predominant T cells response during influenza virus infection.

Further, both cytotoxic and helper T cell populations were activated seven days post infection relative to mock-infected controls as assessed by CD62L expression on the surface of the cells. These data suggest that not only is there an increase in the frequency of both types of T cells at day seven post-infection, but the infiltrating cells have been activated.

Limitations of the study

IFN-γ is an incredibly potent anti-viral cytokine that mediates the functions of monocytes, macrophages, B cells, and NK cells. Further, IFN-γ plays a role in priming the immune response, namely macrophages in the lungs, against bacterial infections caused by acute lung damage. The outcome of viral clearance and the subsequent lack of macrophage priming during viral infection without IFN-γ is unknown. The present study does not address viral clearance over time in IFN-$γ_{-/-}$ mice nor does it address subsequent influenza virus infections as is often witnessed seasonally. While this study does provide evidence to suggest the lack of IFN-

γ decreases the recruitment of inflammatory monocytes while concurrently reducing damage to the respiratory epithelium, the consequences of reduced viral clearance and reduced priming of important immune cells in bacterial infections is unknown.

RNA sequencing of sorted monocytes from WT-mock controls, WT-PR8, and IFN-$\gamma^{-/-}$-PR8 was performed in research question four. The data from RNA sequencing suggested differential inflammatory states of inflammatory monocytes from PR8-ifnected mice via transcriptome changes. However, transcriptome changes do not mean changes on the protein level. To determine if the observed changes in inflammatory genes translate to changes in protein synthesis and subsequent cellular interactions, *in vitro* studies that measure apoptosis of epithelial cells via sorted monocytes need to be performed. Increases in surface proteins like TRAIL and FasL on IMs and increases in sphingomyelin on the surface of epithelial cells as measured by Annexin V using WT-PR8 monocytes would be a good example of confirming the RNA sequencing data. Sorted IFN-$\gamma^{-/-}$-PR8 should have a decrease in surface expression of pro-apoptotic ligands and less death in epithelial cells would be observed to confirm the RNA sequencing data. However, the observed trends in the RNA sequencing data are highly indicative of changes in the phenotypes of monocytes from WT-PR8 and IFN-$\gamma^{-/-}$-PR8 mice. The RNA sequencing data confirms the observed flow cytometry, histology, and weight change data in research questions one, two, and three.

This study did not delineate which subsets of helper T cells were present in the lungs of PR8 infected B6 mice. Specifically, there was a minimal, but significant increase in the frequency of $CD4_+$ T cells in the lungs of mice infected with PR8 seven days post-infection. These T cells could be Th1, Th2, Th17, Th9, regulatory T cells (Tregs), or follicular helper T cells. The phenotype of T cells is suggestive of the type of inflammatory response where Th1,

Th17, and Th9 are indicative of a pro-inflammatory response while Th2 and Tregs are anti-inflammatory. While the frequency and expansion of $CD8_+$ T cells was much higher, the contribution to the overall immune response, even in a small way, is necessitated by the helper T cell response.

Recommendations for further study

The following recommendation for further research were derived from the finding of this research study: (a) this study did not test the specific interactions between inflammatory monocytes and IFN-γ. To test this relationship, deletion of the IFN-γ receptor (IFNγRa) on inflammatory monocytes would deduce the mechanism and role IFN-γ has on these cells in the bone marrow and lungs. In addition to the role of IFNγRa on monocytes, deletion or truncating the IFN-γ gene in only CD8+ T cells, thereby creating an IFN-γ knock out in only $CD8_+$ T cells would further address the relationship between two subsets of death-mediating immune cells. (b) The role of the adaptive immune response during influenza virus infection should be further studied through the analysis of adoptive transfer experiments between mice. Specifically, CD8+ T cells from PR8-infected mice should be intraocularly transferred into WT mice, IFN-$\gamma_{-/-}$ mice, and $CCR2_{-/-}$ mice that are either uninfected or infected with PR8 to assess the lung damage caused by PR8-activated $CD8_+$ T cells. These studies will address the role of activated CD8+ T cells and the cell death mediated by IFN-γ produced by CD8+ T cells as well as their recruitment of IMs. (c) IFN-γ is a major activator of B cells during infection and as such, assessing the role of the humoral immune response during influenza virus infection is extremely important. Antibody-mediated protection from subsequent influenza virus infections is thought to be a main target of influenza-based vaccines. As such, understanding the role of IFN-γ in B cell activation

during influenza virus infection would provide evidence for the necessity of antibody-based therapies. (d) One of the leading causes of death during the 1918 Influenza Pandemic was not specifically the virus but rather, secondary bacterial infections that led to pneumonia and bacterial sepsis. Today, bacterial infections result in similar morbidities in influenza-infected patients ever year. Future studies using the influenza model that was used in these studies to determine the role of IMs and IFN-γ in secondary bacterial infections may provide insight on the immune response's role in allowing commensal bacteria to invade, adhere to the respiratory epithelium, and colonize thereby increasing the disease state of those infected. The ways in which IMs damage the respiratory epithelium, and ablating much of that damage, may decrease the incidence and/or severity of secondary bacterial infections after the primary influenza virus infection. (e) Immunodeficiencies and influenza virus are also major health concerns. Individuals with selective immunoglobulin A deficiency have B cells that produce every antibody normally with the sole exception of IgA. IgA transiently protects mucosal surfaces and individuals that are deficient in IgA have a higher incidence of secondary bacterial pneumonia caused by acute respiratory viral infections. Thus, the role of IFN-γ, B cell activation, and the role of IgA during influenza infection as a means of protecting the mucosal respiratory epithelium warrants future study. (f) the current SARS-CoV-2 pandemic has raised many questions regarding the role of acute respiratory viral infections. SARS has been shown to evade the immune response by limiting the induction of the type I interferon response. Decreases in the type I interferon response have correlated with increases in IL-6 which was observed in the IFN-γ-/- mouse model (data not shown). Therefore, studies into each branch of the interferon response may provide insight into mechanisms by which influenza A virus evades the immune response, rapidly

advances the viral infection, and subsequently requires inflammatory monocytes via IFN-γ activation for viral clearance.

Conclusions

Influenza virus caused the worst pandemic in human history during the 1918-1919 pandemic. The loss of human lives, economic cost, and emotional toll the virus caused the world is still felt in the DNA of people today. The importance of understanding the virological, immunological, and molecular mechanisms involved in influenza pathogenesis cannot be understated. Further, as other acute respiratory viruses continue to cause pandemics and the loss of human life increases, a greater understanding of respiratory viruses in general will assist with navigating the coming pandemics.

In the data presented here, the interactions between the host's immune response and influenza virus were assessed through a variety of cutting-edge techniques that have helped grow our understanding of host-pathogen interactions. Monocytes, a precursor to resident macrophages, are recruited by the chemokine, CCL2, to the site of infection via the CCR2 receptor. Once there, these monocytes begin to aid in the defense against the virus by directly interacting with virally-infected epithelial cells. Now termed inflammatory monocytes for their ability to kill epithelial cells and respond to pro-inflammatory cytokines, inflammatory monocytes become further activated by the anti-viral protein, IFN-γ. Inflammatory monocytes then cause damage to the respiratory epithelium that increases the pulmonary vasculature. Here, much of the pathology associated with influenza virus occurs as acute respiratory distress syndrome (ARDS) may manifest and cause significant morbidity to the host.

This study provides a novel conceptual framework by which the host's immune response, when left over-activated, may cause more damage to the host than the initial viral infection. By understanding the molecular mechanisms that drive tissue damage via inflammatory monocytes through their recruitment to the lungs, their activation and functions at the site of infection, and the immune system's ability to limit an inflammatory state, the morbidity and mortality associated with influenza virus can be limited. Novel therapeutics against the host's own response might be the best choice we have to limit deaths cause by influenza virus. Further, vaccine development can be furthered by understanding the ways the host responds to influenza virus and potentially avoid the next influenza pandemic altogether.

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