EXPLOITING THE RESPIRATORY SYNCYTIAL VIRUS (RSV) FUSION PROTEIN TO POTENTIATE THE IMMUNE RESPONSE TO INFLUENZA VIRUS

by

TIFFANY MICHELLE TURNER

(Under the Direction of Ralph Tripp)

ABSTRACT

Influenza A virus and respiratory syncytial virus (RSV) are two of the leading causes of respiratory tract infections worldwide. Annually, influenza kills more than 250,000 people worldwide despite the availability of a safe and effective vaccine. RSV is primarily a pathogen of the very young and elderly populations. More than 90% of children are infected with RSV in their first year of life. We developed a dual influenza and RSV vaccine composed of the hemagglutinin (HA) protein of influenza and the fusion (F) protein of RSV to combat both viruses. Our vaccine is designed to be given in an annual vaccine regimen, like the current influenza vaccine, but unlike the current influenza vaccine, the addition of the RSV F protein will help to additionally protect vaccine recipients against RSV. A major aim of this research was to determine if the F protein could enhance the immune response to the poorly immunogenic HA protein, thus serving as an immunological adjuvant as well as a vaccine antigen. We found that the F protein was effective at enhancing the antibody response to the HA protein and dual vaccination reduced the influenza viral burden. During these vaccination experiments we discovered that, surprisingly, anti-F antibodies not only bound to influenza H3N2 viruses but also neutralized them. We were able to demonstrate that a peptide (120-140) generated from the

stalk region of the F protein was able to interfere with binding of anti-F antibodies to influenza X31 virus, an H3N2 influenza virus strain. Another aim of this research was to determine if prior exposure to the RSV F protein modulated the immune response to a subsequent influenza infection. We determined that immunization with the F protein modified the recruitment of innate and adaptive immune cells to the site of infection, as well as modified the chemokine and cytokine response to a subsequent influenza virus infection. Together, these findings demonstrate the dynamic properties of the RSV F protein as it pertains to influenza virus infection.

INDEX WORDS: Influenza, RSV, dual vaccination, immune modulation

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by

TIFFANY MICHELLE TURNER

B.S., University of Georgia, 2006

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013

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by

TIFFANY MICHELLE TURNER

Major Professors:	Ralph A. Tripp
	Donald Harn
Committee:	S. Mark Tompkins
	Kim Klonowski
	Zhen Fu

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2013

DEDICATION

This work is dedicated to my Lord and savior for giving me the strength to make it through this endeavor. All things are possible through Him. I would also like to dedicate this work to my family who provided moral support and encouragement.

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Ralph Tripp, for the opportunity to work in his laboratory and learn under his guidance. I have truly learned a great deal from this experience and I have grown as a scientist and an individual. I thank my family for their continued support and encouragement of the years. There were so many time when those words pulled me through difficult situations. I truly love my family and I am thankful that they love me so much. To my father who passed away before I could complete this journey, I know you would be proud of me for achieving this goal. I love you daddy. I would also like to thank current and past AHRC lab members including: Valerie Cadet, Victoria Meliopoulos, Julie Fox, Jenifer Pickens, Jon Gabbard, Jackelyn Crabtree, Patricia Jorquera, Scott Johnson, Les Jones, and Youngjoo Choi for their friendship and support. I have learned a great deal from each and every one of you, and I am truly appreciative. Thank you

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CHAPTER 1

INTRODUCTION

Influenza A viruses and respiratory syncytial virus (RSV) are leading causes of respiratory tract infections in young and elderly individuals worldwide [1-3]. Annually, influenza A virus infections are responsible for greater than 250,000 deaths worldwide [4], and in the United States, more than 180,000 individuals are infected resulting in greater than 32,000 deaths annually [5]. Influenza virus is a member of the *Orthomyxoviridae* family. It is a single-strand, negative-sense RNA virus having a segmented genome encoding 10 to 12 proteins. RSV, a leading cause of severe lower respiratory tract infections in infants [1, 6, 7] affects a majority of infants within the first year of life with greater than 90% of infants being infected by the second year of life [6-10]. RSV, a member of the *Paramyxoviridae* family, was first discovered in a colony of chimpanzees that developed a sudden respiratory illness that quickly spread throughout the colony [11]. RSV is a single-strand negative-sense RNA virus with a non-segmented genome encoding 11 proteins [12]. Both influenza virus and RSV have similar circulating seasons ranging from October to May [13-16].

Influenza vaccines have been available since 1945 [17]. There are three types of influenza vaccines approved by the food and drug administration (FDA) in the United States: trivalent inactivated vaccine (TIV), quadrivalent inactivated vaccine (QIV), and live attenuated influenza vaccine (LAIV). The TIV consist of two influenza A stains (H1N1 and H3N2) and one

influenza B strain. Since the emergence of a distinct lineage of influenza B viruses in 1985, the new lineage strains have, at times, dominated over the more established influenza B strains [18]. From the 2001-2002 influenza season to the 2010-2011 season, the predominant influenza B strain was mismatched [19]. For this reason, the new FDA approved quadrivalent influenza vaccine was introduced. The QIV consist of two influenza A strains (H1N1 and H3N2) and two influenza B strains (one from each lineage) [20]. The LAIV consist of two influenza A strains (H1N1 and H3N2) and one influenza B strain like TIV. Quadrivalent live attenuated influenza vaccines (Q/LAIV) are also being explored [20]. These vaccines have been shown to be safe and efficacious for their targeted age populations [19, 21, 22] and induce a hemagglutinin (HI) titer greater than 1:40 which has been shown to be the standard of protection for influenza vaccines [23].

There is currently no FDA approved vaccine for RSV. This is in part due to the inability of live virus infection to produce an immune response that is long lasting and protective against secondary infection [8]. For this reason, producing an attenuated or killed RSV vaccine has been a challenge. The first attempt at producing a RSV vaccine was in the 1960s with the introduction of the formalin inactivated RSV vaccine (FI-RSV) [24, 25]. Unfortunately there was vaccine associated disease associated with this vaccine, where 80% of children receiving the vaccine and then naturally infected by the virus were hospitalized compared to only 5% in the control group [26, 27]. Since the 1960s, there have been several attempts at creating a safe and effective RSV vaccine including purified F protein (PFP) vaccines 1, 2, and 3, cold passage temperature sensitive (cpst) mutants, and a chimeric RSV FG vaccine [28-33]. PFP vaccines, while safe and immunogenic during clinical trials, did not significantly reduce the incidence of lower

respiratory tract infections [29, 34-36]. The cpts mutants were either over attenuated or under attenuated and reverted back to wt phenotype [37].

Another concern surrounding RSV vaccine development is the virus target population. Although RSV can infect people of all ages, groups most affected are the very young and very old. Infants have underdeveloped immune systems, which leave them vulnerable to infection. In the case of RSV specifically, infants under 9 months have been shown to produce less antibodies to the F and attachment (G) proteins than older children [38]. Infants that are breast feed have the presence of maternal antibodies that have also been shown to interfere or suppress the infant's ability to mount a proper immune response to RSV [39, 40]. The elderly population is also readily infected by RSV [41, 42]. Overtime, elderly individuals lose the ability to respond properly to infections seen previously or respond improperly to those infectious agents [43]. Therefore, developing a RSV vaccine directed at these targeted populations poses a significant challenge.

The primary objective of this research project was to explore a dual vaccine approach for influenza virus and RSV that uses the RSV fusion (F) protein as a molecular adjuvant to enhance the immune response to the hemagglutinin (HA) of influenza virus. Current influenza vaccines are made to induce immunity to the strain-specific HA component, and are not effective in controlling outbreaks of new pandemic viruses [44]. Unfortunately, the HA for many influenza strains induces suboptimal immunity, e.g. H5N1 [45, 46]. Therefore, HA boosting by adjuvanting is often required [21, 22], as is higher concentrations of HA which is needed to achieve protection [47]. Currently, preparation of influenza vaccine takes approximately 6-to-8 months using embryonated hen eggs [48-50]. This prolonged production process is not amenable to problems that may arise during production such as issues with virus propagation, or changes in

the circulating virus strain for the upcoming influenza season. As the current vaccine approach relies on the poultry industry for vaccine production, new methods for generating safe and effective vaccines are being sought [51-53]. In this study, a mammalian cell-based system is used to produce influenza virus HA protein using a Freestyle ^{TM} 293-F human embryonic kidney cell system [54-57]. These cells do not require serum for expansion which reduces the introduction of foreign contaminates into the vaccine. These cells can be readily transfected with a plasmid encoding the HA from influenza virus stains, where the HA protein will be secreted into the supernatant for purification. The need for rapid and alternate means of generating influenza vaccines was highlighted during the 2009 influenza pandemic. In 2009, pandemic H1N1 influenza virus emerged in March in Mexico [58], and subsequently spread throughout Mexico and the rest of the world in just a few months. On June 11, 2009 this swine-origin strain was declared a pandemic by the World Health Organization (WHO) [59]. Despite the virus emerging in March and being declared a pandemic in June of 2009, the vaccine was not available to the public until months later [60]. In instances where viruses quickly emerge and a large portion of the population is at risk of being infected, it would be advantageous to be able to rapidly produce a safe and efficacious vaccine.

A goal of this project was to determine if the RSV fusion (F) protein can serve as a molecular adjuvant for the HA protein. Recently, the F protein was shown to be a Toll-like receptor-4 (TLR4) agonist [61]. Thus, the premise was to use the F protein to trigger TLR4 and enhance the immune response the HA protein during vaccination. Triggering of pattern recognition receptors (PRRs), such as TLR4 by pathogen associated molecular patterns (PAMPs), initiates the host immune response to react to invasion by foreign pathogens [62]. Activation of PRRs results in secretion of various chemokines and cytokines designed to recruit

immune effector cells to the site of infection as well as up-regulation of co-stimulatory molecules required for activation of the adaptive immune response [63]. The ability of the F protein to activate TLR4 was also considered as a way to enhance recognition and uptake of HA by antigen presenting cells (APCs) which bear TLR receptors essentially leading to enhanced activation of the adaptive immune response to influenza. The ability of the F protein to enhance the immune response to the HA protein was tested through several immunological endpoints, ie enhanced anti-HA antibody titers, increased neutralizing antibody titers, decreased viral shedding, and decreased lung immunopathology. Importantly, the RSV F protein would also act as a vaccine antigen in this dual vaccination strategy. The F protein contains neutralizing epitopes [34, 64, 65], and by example, palivizumab (Synagis) is a FDA approved drug for the treatment of severe RSV infection in infants that is based on human monoclonal antibodies reactive to the F protein which neutralizes RSV [66-68]. There has been several vaccine approaches aimed at targeting the F protein for neutralization of the virus [28, 33, 69]. For example, purified F protein (PFP) vaccines have gone through clinical trials and have been found to safe and effective at reducing RSV pathogenesis in humans [28, 34, 70, 71]. Vaccine efficacy was determined by the ability to the F protein to induce neutralizing antibodies against RSV and reduce RSV replication, with special emphasis on lung immunopathology. Thus, in the novel vaccine approach addressed in this study, the RSV F protein has two distinct and important roles: one as a novel protein adjuvant for HA protein, and secondly, as a vaccine antigen for RSV.

Another objective was to evaluate the immune response to influenza virus in the context of influenza-specific CD4+ and CD8+ T cell responses following RSV F protein immunization. Since the adaptive immune response to influenza is required to clear infection [72, 73], and provide heterosubtypic immunity [74, 75], aspects of the T cell response were evaluated. For

example, the CD4+ T cell compartment is important for driving both the cell mediated and humoral arms of the adaptive immune response to virus infection [76]. CD4+ T cells are responsible for aiding B cells in the production of high affinity antibodies capable of neutralizing influenza virus and preventing subsequent infection [77]. As adaptive immune responses are facilitated through innate immune mechanisms, cytokine and chemokine responses were also examined.

Consistent with addressing T cell responses, homotypic and heterotypic immune responses were investigated for the HA and F protein vaccine combinations, specifically examining the ability of the RSV F protein to adjuvant the immune response to the influenza while providing protection against homologous and heterologous challenge by RSV. The vaccines tested here were HA in equal concentration with F protein (HA+F), HA directly conjugated to F (HA-F), HA only, and F only. One objective was to determine if the F protein was more effective at adjuvanting the immune response to HA protein when directly conjugated to HA or was the individual presence of each protein sufficient. Finally, the ability of the F protein to enhance the T cell response to influenza through TLR4 activation was also evaluated. The specific aims of the study are:

Specific Aim 1. Generate mammalian cell-expressed HA protein using a 293 Freestyle system and evaluate the antigenicity of the protein. The *working hypothesis* is based on the ability of eukaryotic cells to produce recombinant HA protein that is antigenic, safe and protective. The native form of the F protein extracted from the viral envelope was used for all experiments to ensure proper folding and triggering of TLR4 [61].

Specific Aim 2. Evaluate the capacity of HA+F, HA-F, HA only and F only vaccines to protect against homologous (A/HK/X31) and heterologous (A/PR/8/34) influenza virus

challenge. The ability of the F protein to adjuvant the immune response to HA protein was evaluated to determine if antibody production was increased, neutralizing antibody titer was increased, and viral titer reduced to a greater degree when the F protein was directly conjugated to the HA protein (HA-F) or admixed with the HA protein (HA+F). The overall antibody response, neutralizing antibody response, viral burden, and histology were determined as measurements of protection.

Specific Aim 3. Evaluate novel HA and HA- F vaccines ability to protect against homologous (RSV A2) and heterologous RSV (RSV B1) viral challenge. The F protein contains neutralizing epitopes. Antibodies directed against the RSV F protein have been shown to reduce the lung viral burden and decrease morbidity associated with RSV infection in animal models [28, 69]. Thus, this aim encumbers examining the neutralizing antibody response, associated lung viral burden, and lung histopathology following RSV challenge as measurement of protection and disease.

Specific Aim 4. Evaluate the influenza-specific T cell response in RSV F protein primed mice challenged with A/HK/x31 (X31) influenza virus. As the F protein activates TLR4, the pattern and level of pro-inflammatory cytokines and chemokine expression will be determined with regard to recruitment and activation of pulmonary T cell responses.

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CHAPTER 2

LITERATURE REVIEW

Overview

Respiratory tract infections are one of the most common recurrent infections in the human population [1]. Influenza virus and respiratory syncytial virus (RSV) are two viruses that cause serious respiratory tract infections in humans. Influenza virus causes substantial morbidity and mortality throughout all ages while RSV is generally a pathogen of age extremes, i.e. the young and old [2, 3], and is the leading cause of serious lower respiratory tract infection in infants worldwide [4-6]. Ninety percent of infants are estimated to be infected by their second year of life [7-10]. Despite an effective vaccine, influenza virus infects 3 to 5 million individuals each year, resulting in 250,000 to 350,000 deaths worldwide [11]. Mortality associated with RSV infection is lower compared to influenza virus, however morbidity is substantial where at least 120,000 infants are hospitalized annually, and there are >60,000 hospitalizations that occur annually in the elderly [12]. Combined, these respiratory viruses contribute to a majority of respiratory tract infections in the human population, worldwide [13].

Influenza viruses

Influenza A virus, a member of the *Orthomyxoviridae* family, was first isolated in 1933 [14]. There are 3 types of influenza viruses: Influenza A, B, and C. Influenza A is found

naturally in aquatic birds but also infects a wide variety of mammalian species including humans. Influenza B is strictly a pathogen of humans and has not been detected in other species. Influenza C has be isolated from the lungs of humans as well as swine populations [15]. Combined, this zoonotic pathogen causes infections in avian, human, swine, and equine species among other mammals including bats [16-18]. Influenza virus is a single-strand, negative-sense RNA virus with a segmented genome. There are eight gene segments that encode up to 12 different proteins: hemagglutinin (HA), neuraminidase (NA), matrix 1 (MI), matrix 2 (M2), nuclear protein (NP), non-structural protein 1 (NSI), non-structural protein 2 (NS2) also called nuclear export protein (NEP), polymerase acidic protein (PA), PA-X, polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and for some strains, polymerase basic protein 1-F2 (PB1-F2) [19, 20]. HA, NA, NP, PA, PB1, and PB2 are each encoded on their own RNA segment while M1 and M2 as well as NS1 and NS2 are alternatively spliced from the two remaining RNA segments [21, 22]. Not every strain of influenza virus encodes PB1-F2, but when it is present, it is spliced from the PB1 gene segment. The HA protein is located on the surface of the virus particle and serves as the attachment protein. The HA protein is also involved in mediating the release of viral RNA (vRNA) into the host cytoplasm [23]. The NA protein cleaves the bond between the host cell sialic acid residues and the HA protein of the virus, releasing progeny virus into the extracellular matrix [24]. The primary function of the M2 protein is to create an ion channel in the cellular endosome which reduces the pH inside the endosome ultimately leading to release of vRNA segments [25]. The M1 protein is the major structural protein and is involved in release of vRNA from the nucleus as well as recruitment of viral proteins to the apical surface of the host cell for assembly and release [26]. PA, PB1, PB2 and NP are components of the replication machinery needed to replicate vRNA [27, 28]. PB1-F2

has been associated with increased virulence in strains that possess the alternatively spliced protein [29]. NS1, the only non-structural protein encoded by the viral genome, has the ability to interact with host mRNA, vRNA and polyadenylated mRNA [30]. NS1 is involved in down-regulation of the host immune response. NS2 is critical for export of newly synthesized vRNA segments from the nucleus. The NS2 protein interacts with Crm1, a known export receptor, resulting in exportation of vRNA into the cytoplasm after transcription is complete [31, 32].

The HA protein is a trimeric type I integral membrane protein on the surface of the influenza virion [22]. HA is synthesized as HA0, but must be post-translationally cleaved in order to be active. Host trypsin-like proteases cleave HA0 into HA1 and HA2, allowing the virus to be infective [33, 34]. There are 17 distinct subtypes of HA: H1-H17 [16]. Sixteen of the seventeen subtypes can be found circulating in avian species [35]. The HA protein has two very important roles in the influenza virus lifecycle. First, the HA protein binds to sialic acids receptors on the surface of target host cells. HA binding to cells is in part linked to influenza virus tropism, however influenza virus can also replicate in epithelial cells lacking α2,3 sialic acid receptors (sias) [36, 37]. Human strains of influenza virus preferentially bind to alpha 2,6 sias, while avian influenza viruses preferentially bind to alpha 2,3 sias [38]. While the human respiratory tract expresses both alpha 2,6 and 2,3 saias, their distribution is not evenly dispersed throughout the lungs. Alpha 2,6 sias are located primarily in the upper respiratory tract, while alpha 2,3 sias are found in the lower respiratory tract [39]. This distribution may explain why avian stains do not readily infect humans, with the exception of avian H5 and H7 viruses [40]. Alpha 2,3 sias can be found in the digestive tract of avian species [41]. Influenza virus infection of avian species is generally mild having no detectable symptoms. However, infections may lead to decreased egg production, excessive lacrimation, edema of the head, diarrhea, neurological

issues, and death from highly pathogenic avian influenza (HPAI) [42]. The virus is transmitted via fecal-oral route in aquatic environments, but the virus can also be transmitted via direct or indirect contact.

Being that 16 of the 17 HA types, and all 9 NA types have been found in avian species, and humans interact with these animals, humans are often experience zoonotic infection with avian influenza viruses. Of these strains, infections with the H5N1 subtype are becoming increasingly more common particularly in Asia. The first reported case of H5N1 infection in humans was detected in 1997 [43], after which there have been many more cases reported with a mortality rate greater than 50% [44]. H5N1 infections may present with fever, cough, fatigue, malaise, sore throat, headache, abdominal pain, vomiting, and in some cases diarrhea has been reported [45-47]. Autopsy and blood work from patients infected with H5N1 have revealed characteristics such as low T cell counts, high proportions of macrophages in the lungs, and high levels of bronchial epithelial cell secretion of cytokines and chemokines [48, 49]. Although H5N1 viruses infect humans, the virus has not yet incurred mutations that would allow efficient human-to-human transmission [50]. On March 1, 2013, an outbreak of H7N9 influenza infections was reported in China [51] with >75% of reported cases coming from individuals that work directly with poultry [52]. Recent ferret studies suggest H7N9 can transmit via direct contact from ferret-to-ferret, but displayed limited ability to transmit after airborne exposure. Domestic pigs also show reduce susceptibility to airborne exposure to H7N9 influenza virus [53]. Currently, H7N9 has not acquired the appropriate mutations to effectively transmit person-to-person.

Influenza virus can cause robust infection in swine which are considered to be a mixing vessel for avian and human strains. This is in part because swine lung epithelium contains both

alpha 2,3 and alpha 2,6 sias which make swine susceptible to both avian and human strains of influenza virus [54]. Swine can also support highly pathogenic H5 and H7 subtypes [55]. If two different strains of influenza virus enter the same host cell, there can be reassortment of the viral genomes resulting in a completely novel strain of influenza virus, also known as antigenic shift [56, 57]. If the infected swine is able to transmit the reassorted virus to a susceptible human, there can be wide-spread transmission throughout the human population, termed a pandemic. Given that a reassorted virus has not previously circulated in the human population, there will be little to no pre-existing immunity to the virus. The latest influenza virus pandemic was a triple reassortant of avian, human, and swine origins [58]. The PB1 gene originated from human H3N2 viruses, which was initially from the avian population in 1968. PB2 and PA genes originated from North American avian viruses while NA and M genes stemmed from Eurasian avian viruses. The HA, NP, and NS genes, which are closely related to the 1918 human influenza virus strains, were derived from classical swine viruses [58, 59]. The first case of H1N1 was reported in February of 2009 in La Gloria, Mexico [60]. The virus spread rapidly throughout the world and was declared a pandemic by June of 2009 [61].

Prior to the 2009 H1N1 pandemic there were three other influenza virus pandemics in the 20th century. The most infamous and well-documented pandemic was the 1918 "Spanish flu". The 1918 Spanish Flu pandemic was responsible for the death of > 50 million people worldwide [62, 63]. The origin of the 1918 influenza virus was clarified after reconstruction of the virus concluded it was closely related to avian H1N1 viruses [64-68]. The virus was found to be extremely virulent in several animal models for influenza virus infection including mice, ferrets, and non-human primates [69]. Another factor associated with the fatality rate of the 1918 pandemic was secondary bacterial infection. Much of the death toll attributed to the 1918

pandemic is suspected to be due to bacterial infections for which antibiotics were not yet available [70-75]. Viruses containing segments form the 1918 pandemic strain continued to circulate for decades causing seasonal influenza virus infections [76]. The two pandemics that followed contained genes from the 1918 pandemic. The "Asian" pandemic arose in 1957 from a recombination event between an H2N2 virus that maintained the N, NP, PA, PB2, and NS genes of the 1918 pandemic stain. The HA, NA, and PB1 genes came from a newly emerged H2N2 virus [77, 78]. Compared to the 1918 pandemic, the 1957 pandemic caused significantly less mortality with around 2 million deaths [79]. The 1968 H3N2 "Hong Kong" pandemic followed the Asian pandemic. The 1968 virus also resulted from a recombination event between circulating H2N2 seasonal strains and an avian H3 virus [79, 80]. Like the 1957 pandemic, the 1968 pandemic retained five genes from the 1918 pandemic strain [81, 82]. There was also significantly less death associated with the 1968 pandemic, resulting in roughly 1 million deaths [79, 81]. These pandemics show how reassortant between influenza viruses of different species can crossover into humans and quickly spread in a susceptible population.

Another important function of the HA protein is to mediate release of the viral genome into the host cytoplasm. The HA proteins undergoes a conformational change under low pH conditions which exposes the fusion peptide component of the protein [22]. The fusion peptide fuses with the host endosome resulting in loss of the lipid membrane between the vRNA and the cells cytoplasm. Fusion of the viral envelope with the host endosome results in release of the vRNA into the cytoplasm of the cell [83]. The M2 protein aids in the acidification of the endosome by recruiting H+ ions into the endosome which accelerates the acidification process [22]. Release of the viral genome is critical for infection and propagation of the virus.

The neuraminidase (NA) glycoprotein is a type II integral membrane protein found on the surface of the influenza virus [22]. The primary function of the NA protein is to cleave the bond between the HA protein and the sialic acid receptors on the surface of the target cells [84]. If the NA protein is inactived, virions aggregate at the host cell surface. During infection the host develops antibodies against the NA protein. Antibodies directed against the NA protein that block the activity will lead to cell surface virus aggregation [85]. Aggregation of the virus leads to decreased shedding by the infected host. Selective pressure on the NA protein has been shown to lead to viral escape mutants [86, 87]. Mutations can occur in the surface loops surrounding the active site of the protein rendering host antibodies ineffective which leads virus detachment and propagation [24]. The NA protein has also been implicated in other early binding events. NA has been shown to bind and most likely remove components of the extracellular matrix such as mucins, cilia, and decoy receptors [88]. Elimination of objects that could interfere with viral binding increases the replication efficiency of the virus.

Influenza Replication

Influenza virus primarily infects airway epithelial cells in the upper and lower respiratory tracts of humans and swine, while infecting the gastrointestinal tract of birds [89]. Birds are the reservoir for influenza virus since 16 of the 17 types of HA and 9 types of NA are able to replicate in these animals [90]. Upon binding to sias via the HA protein, the virus enters the cell through receptor mediated endocytosis. Clathrin, caveolae, non-caveolae coated, non-clathrin coated, and micropinocytosis pathways have been identified as entry vesicles for influenza [22, 91]. Shortly after endocytosis, the acidic condition inside the host endosome causes the HA protein to undergo a conformational change which exposes the fusion component of the protein

[22]. This exposure allows the viral membrane to fuse with the endosome [92]. The M2 protein simultaneously forms an ion channel which recruits H+ ions into the endosome causing the pH inside the endosome to lower [22]. Once the viral and endosomal membranes have fused, the contents of the virus are released into the host cell cytoplasm. The eight RNA segments exit as a ribonucleoprotein (RNP) complex, with the NP, PB1, PB2, and P proteins surrounding the vRNA. The RNP complex must be transported to the nucleus to be transcribed .[22] NP harbors the nuclear localization signal (NLS) which allows the vRNP complex to be actively transported into the nucleus [22]. Karyopherin α, also known as importin α, recognizes the NLS of NP and recruits karyopherin β into the nucleus which allows transport of the influenza RNP complex across the nuclear membrane [22, 93]. Once inside the nucleus, viral transcription is catalyzed by the viral RNA dependant RNA polymerase (RdRP) consisting of the PB1, PB2 and P [22]. The RdRP produces positive sense RNA (mRNA) that will be transcribed into the 10 to 12 viral mRNA segments, at which time PB2 will snatch caps from host mRNA and place them on the 5' end of the viral mRNA [22]. Negative sense viral RNA (cRNA) is also transcribed so that it can be packaged into newly formed progeny virus. The positive and negative sense RNA exit the nucleus with the help of NS2 and MI. NS2 interacts with crm1, a nuclear export receptor, to facilitate RNA release from the nucleus [22]. mRNA gets translated by host translation machinery, while the viral RNP complex travels to the apical surface of the cell where budding occurs. Approximately 8 hours after infection, influenza progeny virus is released from the host cell surface, encapsidated in the host cell membrane [94]. The viral NA has to cleave the bond between the viral HA and the host sialic acid receptor in order for the virus to be released into the airway of the infected human [95]. At this point the virus is able to infect neighboring cells, or spread to other susceptible hosts via respiratory droplets.

Host Response to Influenza

A first line of defense in the host response to influenza is the innate immune response. The innate immune system is comprised of a network designed to detect and respond to pathogens using pattern recognition receptors (PRRs) [96]. PRRs are specialized, germ-line-encoded receptors that detect pathogen associated molecular patterns (PAMPs) expressed by pathogens [96, 97]. Toll-like receptors (TLRs) are very important members of the innate immune response. During influenza infection, TLR7 recognizes single stranded RNA [98]. Upon recognition of ssRNA, TLR7 in conjunction with retinoic acid inducible gene-1 (RIG-I) trigger the stimulation of the interferon (IFN) response leading to the induction of NF-κB which leads to the transcription of proinflammatory cytokines [98, 99]. Detection of vRNA in the cytoplasm of the cell can be difficult due to the presence of host RNA in the form of mRNA, rRNA, tRNA, microRNAs and other host sources of RNA. RIG-I detects vRNA through the 5'triphosphate present on vRNA [100]. Once vRNA activates RIG-I, there is a conformational change in the protein that exposes the CARD domain which then gets ubiquitinated, allowing RIG-I to interact with downstream IFN stimulated genes (ISGs) [101, 102]. RIG-I detection of influenza virus is independent of virus replication which allows the host immune system to detect the virus before replication can occur [103, 104]. Pro-inflammatory cytokines recruit vital innate immune cells to the site of infection. Alveolar macrophages (AM) are among those cells recruited to the site of infection by CCL2 expression. AM express CCL2R on the surface which binds to CCL2. After recruitment, AMs upregulate their expression of tumor necrosis factor-related apoptosis inducible ligand (TRAIL) which leads to the elimination of influenza infected cells [105]. NOD-like receptor associated inflammasomes have also been implicated in influenza virus detection.

Triggering of inflammasomes lead to the production of IL-1β, which is critical in the innate immune system/adaptive immune system interface [106].

Natural killer (NK) cells are part of the innate immune response and have a critical role in the elimination of influenza-infected cells. NK cells directly kill influenza infected cells, and secrete anti-viral IFNα and IFNγ in response to influenza infection [107-109]. The HA protein has been shown to interact with NKp44 and NKp46 on the surface of the NK cell, leading to apoptosis of the infected cell [109, 110]. NK cells also contribute to the clearance of influenza virus through the secretion of proinflammatory cytokines which contribute to limiting the spread of infection [107]. Neutrophils, also innate immune cells, limit influenza spread by expression of IFNγ, TNFα, MIP-1α and MIP-2 which aids in the recruitment of immune cells which reduces the spread of infection [111-113]. Increased immune cell recruitment can lead to increased inflammation in the lungs that can contribute to or mediate disease pathogenesis.

The IFN response is critical to controlling influenza infection. Type I and III IFNs can act in an autocrine or paracrine manner. Type I and III IFN stimulated through TLR-PAMP interaction acts in an autocrine manner by binding to IFNAR1 which induces the transcription of IFN-stimulated gene factor 3 (IRF3) [114]. IRF3 is then able to stimulate the production of ISGs involved in the antiviral response needed to control influenza infection [115]. Type I and III IFNs also act in a paracrine manner where it is secreted out of infected cells. Secreted IFNs binds to receptors on neighboring cells inducing an antiviral state and limiting the spread of the virus by eliminating the ability of the virus to infect neighboring cells. IFNs also facilitate the cytotoxic response in NK cells and cytotoxic T cells (CTLs) [116].

Dendritic cells (DC), also innate immune cells, provide a bridge between the innate and adaptive immune response. These specialized antigen presenting cells (APCs) are responsible for

the initiation of the cell mediated adaptive immune response [117]. DCs specialize in sampling the host environment, through phagocytosis and/or pinocytosis, and subsequently migrate to the nearest draining lymph node (LN) to initiate the immune response [118-120]. DCs upregulate surface markers such as CCR7 which allow entry into the LN [121-123], and inside the LN, present antigen to naive CD8+ and CD4+ T cells through MHC class I and MHC class II, respectively. Upon recognition of cognitive antigen in the presence of co-stimulation, CD8+ T cells mature and produce TNFα, IFNγ, perforin and granzymes and clonally expand into antigen-specific CTLs in the LN [124-126]. CD8+T cells upregulate the expression of sphingosin-1-phosphate receptor 1 (S1P1) allowing for leukocyte egress to the draining LNs into the periphery [127]. At the site of infection, CD8+ T cells recognize influenza virus infected cells and eliminate these cells through the secretion of perforin and granzymes [128]. CD8+ T cells can also induce apoptosis in influenza virus infected cells through Fas/FasL interaction [129]. DCs also present antigen to CD4+ T cells which help B cells become better secretors of antibody. CD4+ T cells stimulate B cells in the germinal center to undergo affinity maturation and class switching in order to produce a high affinity anti-influenza virus antibody [130]. These B cells then secret antibodies that are able to bind and neutralize the virus through the shared activity of complement [131]. During infection, CD4+ T cells also secrete IL-2, IFNγ, IFNα, and IL-10 to both promote and limit the inflammatory immune response [132, 133].

Influenza Pathogenesis

Influenza virus may counteract the host immune response using several different mechanisms. The first and most notable is via replication. Influenza virus replication machinery does not have a proof reading mechanism. Lack of proof reading allows for mutations to occur

during replication. These amino acid changes can contribute to immune evasion by influenza virus. “Antigenic drift”, which are subtle changes in HA and NA most often due to selective pressure applied by the host, constitutes a different mechanism of immune evasion by influenza virus [57]. Influenza virus can also undergo “antigenic shift” which is defined as a substitution of either the HA or NA from a strain that has not been circulating in the human population for an extended period of time [15, 56]. Pandemics can arise following antigenic shift due to the lack of herd immunity in the existing population. The 2009, 1957, and 1968 pandemics previously discussed all arose from recombination events.

The influenza virus NS1 protein has a multitude of features that facilitate influenza virus replication. NS1 can promote influenza virus replication from both the cytoplasm and the nucleus. Located on the NS1 protein is a RNA binding domain that binds dsRNA [134]. Host innate immune factors, such as PKR and OAS, bind dsRNA and trigger the innate immune response [135, 136]. The NS1 protein is able to bind to and compete for dsRNA, thus reducing the chances of being detected by the host [137]. NS1 has been shown to bind to PKR and inhibit activation of this protein [138]. PKR is a host defense mechanism which recognizes dsRNA in response to viral infection [139]. Activation of PKR eventually leads to apoptosis of the infected cell. Inhibition of PKR activation can lead to prolonged survival of the infected cell allowing the virus to replicate to higher titers [140]. RIG-I, as mentioned above, has a critical role in controlling influenza virus replication [141, 142]. NS1 interferes with RIG-I activation by binding to and interfering with TRIM25 [142]. TRIM25 is important for optimal activation RIG-I [101]. By interfering with RIG-I activation, the influenza virus NS1 protein inhibits activation of the host interferon response which is pivotal in controlling influenza virus replication and shedding.

In the nucleus, NS1 can interfere with host mechanisms involved in exporting host mRNA to the cytoplasm. NS1 can inhibit the trafficking of host mRNA by interfering with processing of the mRNA [143]. The cleavage and polyadenylation specificity factor (CPSF) is required for full maturation of host mRNA by processing the 3 end of pre-mRNA and preparing the pre-mRNA for polyadenylation [143]. NS1 bind to CPSF and inhibit the ability of CPSF to completely process host mRNA, thereby leading to retention of host mRNA in the nucleus. Since influenza virus is not reliant on host CPSF for maturation of vRNA, the vRNA is processed and released into the host cytoplasm where it is then translated into protein [143]. The retention of host mRNA in the nucleus impairs the Type I and Type III IFN response and the activation of ISG, which leads to increased influenza virus replication. Furthermore, NS1 has been shown to interact with host nuclear export machinery to reduce the ability of the host to export mRNA into the cytoplasm [144]. Taken together, the influenza virus NS1 protein can bind and interfere with several host mechanisms involved in triggering the host IFN response, leading to increased viral proliferation and shedding, increasing the overall fitness of the virus

Another protein, PB1-F2, has also been associated with increased influenza virus pathogenesis though not all influenza viruses possess this protein [29]. PB1-F2 is spliced from the PB1 gene [22]. This 80 amino acid protein has been implicated in the increased virulence and pathogenesis of influenza viruses including the 1918 Spanish flu pandemic [145, 146]. PB1-F2 is believed to enhance influenza virus virulence by interfering with mitochondrial antiviral signaling protein (MAVS) in the mitochondria which are important in activating the IFN response [147]. PB1-F2 binds to the mitochondria, both the inner and outer membrane, and disrupts the membrane potential which leads to cellular death [148, 149]. In addition to binding to MAVS, PB1-F2 binds to adenine nucleotide translocator-3 (ANT3) and voltage-dependent

anion channel 1 (VDAC1) through the C-terminal domain [150]. PB1-F2 has also been shown to interact with PB1 late during the influenza virus replication cycle and enhances replication [151]. Taken together, these properties influence the pathogenesis of influenza viruses that possess this protein.

Influenza Vaccines

Currently, there are three FDA approved influenza virus vaccines available in the United States - a live attenuated influenza virus vaccine, a trivalent inactivated influenza virus vaccine, and a quadrivalent inactivated influenza vaccine. The efficacy of the annual vaccine is based on a protective influenza virus hemagglutination inhibition (HI) titer of at least 1:40 which has been shown to confer protection [152]. However, antigenic drift associated with virus mutation often leads to immune evasion [153], thus influenza vaccines must be prepared annually. This is a cumbersome production process requiring 6-to-8 months for preparation [154]. Additionally, production of the vaccine requires selecting vaccine strains based on worldwide surveillance coordinated by the Institution of the Global Agenda for Influenza virus Surveillance and Control. Pending surveillance, circulating strains are chosen for incorporation into the annual influenza virus vaccine. If there is a discrepancy between the vaccine strains and circulating strains the vaccine may not sufficiently provide protection. This was recently highlighted in the 2009 influenza virus pandemic, where the pandemic strain was not available for inclusion into the annual vaccine and the population was unprotected [155, 156]. Typically there are two subtypes of influenza A (H1N1 and H3N2) and one subtype of influenza B incorporated into the trivalent annual vaccine [61] and two subtypes of influenza A (H1N1 and H3N2) and two subtypes of influenza B (one from each lineage) incorporated into the quadrivalent annual vaccine. The introduction of the new quadrivalent inactivated influenza vaccine, including 2 influenza B

viruses, was approved by the FDA in February 2013 [157]. Production of embryonated hen eggs must be timely. The annual vaccine is heavily dependent on the poultry industry for embryonated chicken eggs required for expansion of the vaccine. The influenza A vaccine strains are reassorted with an A/PR8/34 master strain which grows to high titers in embryonated chicken eggs [20, 154]. There is no seed or master strains for influenza B and the seed strain is chosen from field isolates. After the vaccine seed strain are prepared, the stains are sequenced for the presence of the vaccine HA and NA surface glycoproteins and the absence of the master strains HA and NA. The vaccine seed strains are then shipped to vaccine producers where the growth kinetics and antigenicity are evaluated, then inactivation and purification occur [61]. Reactogenicity issues can also arise during influenza virus vaccine production. Use of detergents used to inactivate/split the vaccine can interfere with the antigenicity of the viral proteins and increase the amount of antigen required to achieve protection [158]. Additionally, viruses such as HPAI H5N1 often kill the embryo before substantial viral titers can be obtained [159]. Taken together, this process takes a substantial amount of time from selection, to manufacture, to distribution. Although the production process is tedious with many steps that could compromise efficacy, the end-product vaccine often approaches 100% efficacy in health adults [160, 161].

The live-attenuated vaccine is only approved for people 5-49 years of age [162]. In this vaccine, viruses displaying the HA and NA from the donor strain are selected while seed strain maintains the six internal genes. A cold adapted stain, A/Ann Arbor/6/60 H2N2, is used as the backbone strain for the live attenuated vaccine. Anti-H2N2 serum is used to eliminate strains containing the donor surface glycoproteins [22]. Those strains not displaying the donor surface glycoproteins are sequenced and sent to vaccine distributors similar to the inactivated vaccine.

In order to reduce the dependence on embryonated chicken eggs for the production of the annual vaccine, many groups have evaluated mammalian cell-based culture methods for influenza vaccine development. One of the more studied cell lines is Madin-Darby canine kidney cells (MDCK). Influenza virus readily infects MDCK cells and the virus isolated from these cells tends to retain the glycosylation pattern of the isolate, whereas viruses propagated in chicken eggs can have more heterogeneous glycosylation and readily adapt for growth in avian species [163, 164]. Chicken egg amniotic cells contain both α2,6 and α2,3 sias whereas allantoic cells contain specifically α2,3 linkages which could result in selection pressure to change the receptor specificity of the passaged virus [165], therefore vaccine production in MDCK cells may result in decreased selective pressure for human viruses. Other cell types shown to retain the glycosylation pattern of the original isolate include LLC-MK2, WI-38, MRC-5, African green monkey kidney cells (Vero), and chicken and guinea pig kidney cells [166-170]. Vero cells have been approved as a safe vaccine platform for influenza virus vaccine production [171, 172]. A commercially available cell line, i.e. PER.C6 human fetal retinoblast cell line has been shown to support influenza virus replication similar to that produced in allantoic fluid [173]. HEK293 cells, human embryonic kidney cells, have also been investigated for large scale production of influenza virus. These cells have been shown to produce high levels of influenza virus and could be suitable for large scale virus production [174]. Taken together, the vast amount of research directed toward cell based vaccine production highlights the need for development of mammalian cell-based methods that may provide a more effective platform for the annual influenza virus vaccine.

Along with cell culture based influenza vaccine platforms, the use of viral vector vaccine platforms has also been investigated. Vaccines expressing a single protein alone or a

combination of influenza virus proteins have been tested for efficacy against influenza virus infection. These viral vectors are chose for a variety of reasons including, but not limited to, prevalence in the human population, immunogenicity, and replication characteristics. One such viral vector exploited for its immunogenicity and ease of manipulation is vaccinia. This virus has been used to successfully express influenza virus proteins that are immunogenic and protective against influenza virus challenge [175-178]. The drawback for using vaccinia as a vial vector is it is extremely immunogenic, and neutralizing antibodies are readily produced in response to infection. Neutralizing antibodies pose a problem for boosting requirements as well as any additional vaccines that would be required for other influenza virus subtypes due to the facts that antibodies directed against vaccinia are long-lived [179]. Adenovirus is another virus that has been examined as an influenza virus vaccine vector. Adenoviruses have been shown to immunogenic and induce protection from various strains of influenza virus [180-184]. One major issue associated with the use of adenovirus vectors is the prevalence of adenoviruses in the human population [185, 186]. Therefore, like vaccinia, the issue of overcoming pre-existing immunity in the human population becomes a problem. To get around pre-existing immunity, viruses such as Newcastle disease virus (NDV), baculovirus, and vesicular stomatitis virus (VSV) have been examined. These viruses have been shown to elicit both humoral and cell mediated immunity to influenza virus infection [187-190].

As an alternate strategy to control influenza virus, several antiviral drugs have been licensed and shown to have a level of efficacy to alleviate the disease burden caused by influenza virus. There are two families of drugs: the neuraminidase inhibitors and the M2 ion channel inhibitors. The M2 ion channel inhibitors, including amantadine and rimantadine, function by interfering with the ability of M2 to form the ion channel inside the endosome [191]. This

channel is necessary to lower the pH inside the endosome which causes a conformational change in the HA protein to expose the fusion component. Blocking M2 function essentially halts the release of RNPs from virions. Since 2003, resistance has emerged and virtually all seasonal H3N2 stains are resistant to amantadine and rimantadine [192]. The neuraminidase inhibitors, including oseltamivir and zanamivir, act by occupying the active site of the NA protein that is responsible for cleavage of neuraminic acid, thereby inhibiting the ability of the NA to cleave HA protein from the host sialic acid receptor [193, 194]. To date, there is resistance to oseltamivir due to a point mutation at position 274 in which a histidine residue has been replaced with a tyrosine residue [195]. This point mutation completely eliminates the antiviral effect of oseltamivir, but does not affect the antiviral properties of zanamivir. Blocking NA activity causes aggregation of progeny virus at the apical surface of the cell and inhibits virus release and spread.

Respiratory Syncytial Virus

RSV is the most common cause of serious lower respiratory tract disease in infants and young children. Greater than 70% of children are infected with RSV during their first year, and nearly 90% infected by their second year of life [8, 10, 196]. More than 80% of children infected in their first year of life are re-infected in their second and third years of life [197-199]. RSV, a member of the *Paramyxoviridae* family, was first discovered in 1956 after a colony of chimpanzees developed a sudden respiratory illness that quickly spread throughout the colony [199-202]. RSV is a single-stranded, negative sense RNA virus with a non-segmented genome. There are two strains, RSV A and RSV B, and these strains are serologically differentiated [22]. The strains are further divided into different lineages based on nucleotide sequence of the

attachment (G) proteins as well as restriction mapping of the N protein [203]. The genome encodes eleven proteins: the fusion protein (F), attachment protein (G), matrix protein (M), matrix 2-1 protein (M2-1), matrix 2-2 protein (M2-2), small hydrophobic protein (SH), nucleoprotein (N), phosphoprotein (P), nonstructural protein 2 (NS1), nonstructural protein 1 (NS2), and the polymerase protein (L) [22]. The primary function of the F protein is to mediate fusion of the viral envelope with the host cell membrane. The protein is also responsible for the formation of syncytia that gives this virus its name. [204]. The syncytia are formed when there is a loss of cellular border between neighboring infected cells, where infected cells appear as a single giant multi-nucleated cell. The G protein acts synergistically with the F protein in mediating attachment [205]. The function of the SH protein is not yet fully understood, but it is speculated that it serves as an ion channel or it enhances cell to cell contact [22]. The matrix protein interacts with the viral ribonucleoproteins (RNPs) to terminate transcription so that the genome can be packaged into virions [206]. M2-1 is a transcription factor that is essential for viral viability and it allows the polymerase to read through intergenic junctions in the viral RNA [207]. M2-2 has multiple functions, including increasing viral replication, regulating RNA synthesis, and providing a balance between replication and transcription [208]. N, P, M2-1, and L together make up the RNA dependent RNA polymerase (RdRP) which transcribes vRNA, polyadenylates mRNA, and adds the necessary 5’ mRNA cap so translation can occur [209]. The virus also encodes two nonstructural proteins, NS1 and NS2, that are only found in infected cells and are not packaged into the actual virion [199]. NS1 and NS2 together inhibit the translocation of Interferon regulatory factor 3 (IRF3) into the nucleus by blocking phosphorylation [19]. Without this, IRF3 cannot enter the nucleus and activate transcription of IFNα/β antiviral genes [22].

The RSV F protein has many of the same basic features of other Paramyxoviridae family members including Newcastles Disease (NDV), parainfluenza virus 3 (PIV3), and parainfluenza virus 5 (PIV5) [210]. The F protein is synthesized as an F0 precursor that must be post-transcriptionally cleaved by furin-like proteases at two sites in the trans-golgi network, removing a 27 amino acid fragment (p27), resulting in F1 and F2 subunits that are then disulfide bonded together [211]. Cleavage of F0 is required for a fully functional protein [212]. Cleavage of F0 places the hydrophobic fusion peptide at the N terminus of the F1 subunit in order to interact with the host cell membrane and initiate fusion [213]. The receptor for the F protein has been proposed to be the host receptor, nucleolin, which was recently implicated in F protein binding [214]. Nucleolin is a host protein expressed in the cell nucleus, cytoplasm, and plasma membrane [215]. The primary function is to assist in ribosomal biosynthesis and maturation and also it plays a role in cellular growth [216, 217]. In recent studies, soluble nucleolin was shown to prevent RSV infection, and RSV viral titers were also decreased if cells were treated with anti-nucleolin antibodies, or if nucleolin expression was knocked down through the use of siRNA directed against nucleolin [214]. The F protein induces antibody as well as cell-mediated responses against RSV through the production of neutralizing antibodies and F protein-specific CD8+ T cells [22].The F protein is divided into distinct antigenic sites: antigenic site I, II, IV, V, and VI [218, 219]. Antigenic site II, also called antigenic site A, encompasses residues 255-275 [220]. Antigenic site IV, also called antigenic site C, encompasses residues 422-438 [220]. Antigenic sites II, IV, V, and VI are located on the F1 subunit near the cysteine rich region, while antigenic site I is located inside the cysteine rich region [221]. Monoclonal antibodies against the F (and G) protein have been shown to confer protection when given prophylactically or shortly after RSV infection [222-224]. Specifically, two monoclonal antibodies directed

against the F protein, Palivizumab and Motavizumab, have been shown to decrease RSV replication *in vivo* [218, 225]. Palivizumab binds to antigenic site II (site A) which includes residues 255-275 and is approved for preventative treatment of children who are at risk of developing RSV (Fix) [226-229]. Other monoclonal antibodies including 101F and MAb19 bind to antigenic site IV (site C) which includes residues 422-438 [226, 230], while 131-2A binds to antigenic site I [231]. Adoptive transfer of RSV F protein-specific CD8+ T cells into immunologically naïve mice has been shown to facilitate viral clearance although there was increased lung histopathology [232, 233]. The combined antibody and cell mediated protection afforded by the RSV F protein make this protein an ideal candidate for future vaccination platforms.

RSV Replication

RSV infects ciliated cells in the respiratory tract causing the cells to slough off into the airway leading to airways obstruction [234, 235]. RSV replication is initiated by the G and F proteins binding to receptors on the surface of ciliated cells [236]. The F protein bind to nucleolin while G binds to glycosaminoglycans (gag), in particular, heparin sulphate, on the surface of the host cell and mediate viral entry [22, 214, 237]. Other receptors such as ICAM-1, Rho-A, annexin II, and CX3CR1 have also been implicated in RSV entry into host cells [22]. Upon binding, the fusion protein mediates fusion of the viral membrane with the host membrane resulting in release of the viral genome into the cytoplasm of the host cell [238]. The RdRP, composed of the N, P, and L proteins, but M2-1, is also required for efficient transcription. Transcription occurs from the 3´ to 5´ the direction and transcription occurs in a gradient fashion based on proximity of the gene to the promoter [239], therefore the NS1 protein is the most

abundant protein expressed because it is the first gene at the 3´ end of the RNA segment. N, P, M, and F are the only proteins essential for RSV replication, however replication is greatly diminished in the absence of the G protein [240]. vRNA can be detected approximately 4hrs post infection and peaks 24 hrs post infection. The bidirectional RdRP transcribes the vRNA into mRNA as well as negative sense RNA that will be packaged into new viruses. RSV depends on host actin and profilin machinery for efficient viral replication and assembly of infectious virions [237]. After transcription and translation, the viral proteins and viral RNP complex accumulate at the apical cell surface and bud, retaining the host cell plasma membrane as its viral envelope [22].

Host Response to RSV

Both innate and adaptive immune mechanisms are involved in control of RSV infection. The TLRs of the innate response are critical in alerting to infection by RSV. RSV infection has been shown to trigger TLR2, TLR3, and TLR4 [241-243]. Triggering of TLR4 by RSV increases the TLR4 expression on the surface of respiratory epithelial cells [244]. TLR3 has also been implicated in having a role in RSV infection. It has been shown that mice infected with RSV lacking the TLR3 receptor have increased secretion of Th2-type cytokines, increased eosinophil recruitment, and increased mucus production in the lungs [245]. TLR activation leads to the activation and translocation of NF-κB into the nucleus where it facilitates the transcription of anti-viral genes [246, 247]. Among these genes IL-6, IL-8, IL-10, and IFNβ are expressed [241, 248]. IFNβ is expressed and initiates a more general type I IFN response in the infected cells in a paracrine fashion affects neighboring cells and initiates their IFN response to limit virus susceptibility and spread [249]. IFN stimulation of IFNAR results in STAT-1 and STAT-2

activation leading to the translocation of IRF-9 into the nucleus initiating the transcription of other antiviral genes and there function [250]. RSV infection of mice deficient in STAT-1 and STAT-2 signaling display increased pro-inflammatory production in the lungs, increased lung pathology and increased eosinophil infiltration [249]. IFN-α/β and IFN-γ knockout mice also exhibited increased pulmonary eosinophilia while displaying decreased leukocyte trafficking to the lung following RSV infection [251]. RSV also can bind and activate PRK, which also leads to the activation NF-κB and MAPKs resulting in activation of the innate immune response and antiviral functions similar to TLR activation [242, 252]. Taken together, innate immune system recognition of RSV is critical for initiating and activating key antiviral activities needed to recruit the correct cell types to the site of infection to combat and eliminate RSV from the lungs.

Cytokine expression is important in the host immune response. Cytokine expression facilitates the activation and recruitment of immune cells to respond to sites of infection. Respiratory epithelial cells express cytokines such as CXCL10, CCL5, CCL3, MIP-1α, MIP-1β, MCP-1, IP-10, and type I IFNs in response to RSV infection [253-255]. Expression of these cytokines may recruit neutrophils, eosinophils, monocytes, and leukocytes to the site of infection resulting in a pro-inflammatory response [256-258]. Some of these pro-inflammatory cytokines contribute to virus clearance directly, or through induction of cytotoxic T cell responses, but over-exuberance of cytokine secretion may also lead to increased lung pathology. Studies have shown that blocking certain pro-inflammatory cytokines, such as RANTES, or knocking out TLR3 leads to decreased lung pathology [245, 254]. RSV infected mice lacking MIP-1α exhibited decreased lung pathology, but interestingly there was no difference in RSV viral titers [259]. Therefore the immune response to RSV infection can have an unfavorable outcome on the host and leave viral titers unaffected. Despite the possibility of enhanced tissue damage,

cytokines are absolutely critical for cellular trafficking to affected tissue for the eventual elimination of virally infected cells.

In response to RSV infection, epithelial cells up-regulate the expression of adhesion molecules to attract and bind to effector cells [260]. Epithelial cells also up-regulate the expression if MHC class I molecules in order to present antigen to effector CD8+ T cells [261, 262]. Up-regulation of adhesion and MHC class I molecules are critical for not only directing immune cells to the site of infection, but for alerting cells such as CD8+ T cells and NK cells to their infection and eventual elimination [22]. DCs are found throughout the respiratory tract and capture antigen at the site of infection. Upon uptake of antigen, DCs down-regulate their phagocytic capabilities and up-regulate the expression of MHC class I and class II molecules as well as co-stimulators CD80 and CD86 molecules [263]. After maturation, DCs travel to the draining LN to present antigen to T cells [264]. Two important subsets of DCs involved in RSV detection and clearance are myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). mDCs secrete IL-12, a feature that stimulates both CD4+ and CD8+ T cells, and polarizes the immune system towards a Th1-type response which is important for the elimination of RSV infection [265]. pDC are potent secretors of IFNα [249], and IFNα has an important role as an antiviral cytokine during RSV infection [22]. RSV has the ability to infect and replicate in DCs [266], where infection down-regulates the expression CCR7, and prevents DCs from effectively presently antigen to T cells and reducing IFNα production [266-270]. Mice that are deficient in pDCs exhibit increased lung pathology [271]. The increased pathology may be attributed to the decrease in IFNα associated with the decrease in pDCs, and the absence of IFNα could also lead to polarization toward a Th2-type response. The inefficient activation of RSV-specific T cells leads to a diminished adaptive immune response in the host. CCR7 expression is

directly linked to the ingress into the LN where antigen presentation takes place. Inefficient up-regulation of CCR7 may be linked to the prolonged persistence of RSV-specific DCs in the lungs which have been observed for several weeks post-infection [272, 273]. This may enable RSV to evade detection as well as establish a more robust infection.

There are other innate immune cells involved in the response to RSV infection. Alveolar macrophages are recruited to the site of infection, and have an important role in limiting virus spread. In response to infection, these cells express TNFα, IL-6, IL-8 and MIP-1α leading to the recruitment of leukocytes [274, 275]. NK cells are also important innate cells that have the capacity to eliminate RSV infected cells through ADCC thereby limiting virus replication [276]. The inappropriate expression of pro-inflammatory cytokines can result in tissue damage, and subsets of T cells, i.e. Tregs, express IL-10 to inhibit or decrease the host pro-inflammatory immune response [277, 278].

The adaptive immune response is important for eliminating RSV from the infected host. The cell-mediated arm of the adaptive immune response is initiated in the draining LNs where antigen is presented to RSV-specific T cells by DCs [279]. In the LN, CD4+ and CD8+ T cells are activated and proliferate in response to the viral infection [280, 281]. Specific cytokines are known to contribute to T cell activation and maturation and these include IL-1, IL-2, IL-6, IL-12, and IFNγ [282]. Subsequently, RSV-specific CD4+ and CD8+ T cell express CD62L, CD44 and other adhesion molecules [283] that allow them to exit the LN, and migrate to the site of infection via high endothelial venules [284], and via chemotaxis, to track, identify, and eliminate infected cells [256]. CD8+ T cells are capable of recognizing and attacking cells expressing sequences from F, M, M2, NS2 proteins, but have little to no recognition of G, NS, or P proteins [285]. Recognition of cognate antigen by CD8+ T cells results in the release perforins and

granzymes into the target cell resulting in loss of osmotic retention and apoptosis [286]. CD8+ T cells can also induce apoptosis of virally infected cells by binding to the Fas receptor on infected cells [287]. Following RSV infection, respiratory epithelial cells up-regulate Fas expression and because CD8+ T cells express FasL, the Fas-FasL interaction leads to death signals in the infected call and apoptosis [288]. Interestingly, approximately 40% of the CD8+ T cells directed against RSV recognize the M2 protein [289, 290], and only one half of the M2-specific T cells express IFNγ or produce perforin and granzymes [291]. The M2-specific T cells also exhibit diminished capacity to control secondary RSV infection [292]. RSV-specific CD8+ T cells have also been shown to produce decreased amounts of granzymes B, perforin, and IFNγ in response to primary and secondary infection [293]. Granzyme B, perforin, and IFN-γ are important for the elimination of infected cells and contribute directly to the functionality of CD8+ T cells [294, 295]. It has been shown that exogenous IL-2 can repair the activity of dysfunctional T cells, and restore their effectiveness against RSV-infected cells [292]. The inefficiency of CD8+ T cells to respond to secondary RSV challenge may explain, in part, why individuals are re-infected throughout their lifetime.

Both CD4+ and CD8+ T cell responses are essential for RSV clearance. Mice deficient in both cell types exhibit prolonged RSV replication in the lungs [296]. Upon activation, CD4+ T cells can initiate a Th1- or Th2-type cytokine response characterized by the expression of IL-2, IFN-γ, and TNF-α (Th1-type) , or IL-4, IL-5, IL-6, IL-10 and IL-13 (Th2-type), respectively [297]. A Th1-type response, or a balanced Th1/Th2-type response, is preferable for clearance of RSV infection [298-300]. RSV has been shown to initiate both Th1 and Th2-type immune responses [301-303]. The Th1-type response required for elimination of RSV has been linked with increased lung pathology associated with a robust influx of immune cells at the site of

infection [301]. A Th2-type cytokine response to RSV infection has been linked with inappropriate recruitment of immune cells and increased lung tissue damage [304]. It has been shown that during RSV infection, CD4+ T cells that recognize the F protein are biased toward Th1 cytokine profiles, while CD4+ T cells that recognize the G protein elicit a Th2 cytokine profile [305]. The priming of the initial immune response is important for the memory response that will be established. In a BALB/c mouse model, RSV has been shown to establish a Th2-type CD4+ T cell memory response, and upon reinfection, the memory CD4+ T cells express IL-4 and IL-5 further polarizing the immune system towards a Th2-type response [304]. Transfer of CD4+ T cells from soluble G primed mice have been shown to predispose RSV challenged mice to increased disease and lung pathology [306]. This enhanced pathology was found to be dependent on IL-5 and IL-13 while independent of IL 4 [305]. Therefore, the type of immune priming, as well as the subsequent cytokine environment drives cytokine bias in the immune response to RSV infection.

The humoral immune response to RSV is directed at the major surface glycoproteins, F and G, and can provide protection from infection [307]. Antibodies directed at the F protein can either be against F0 or F1 and F2, which are linked via a disulfide bond and represent the mature and infective form of the protein [226, 230]. The immune system can recognize and produce antibodies in equal concentration to both forms of the protein [308], a feature that could lead to incomplete neutralization of the virus. The G protein can also provide protection against RSV infection; however, antibodies targeting several epitopes appear to be required for effective neutralization because the pattern of glycosylation differs depending on the type of cell infected [309-313]. The anti-G protein antibody response can also be directed to the central conserved loop region comprising the CX3C region, though rare during infection [314, 315]. Since the

virus produces a soluble form of the G protein (Gs) [316], it is possible that the virus can circumvent the host immune response by deploying Gs as a decoy to prevent neutralization. Since the G protein is so divergent among the different subtypes and strains [317], antibodies directed at the G protein would be less likely to offer cross-protection, while the F protein is more highly conserved among the different subtypes and strains [318], and provides a better level of cross-protection against heterosubtypic challenge [319, 320]. There have been conflicting reports in the literature as to the significance of RSV antibodies and protection. Studies have shown that prior RSV infection resulted in decreased disease severity upon reinfection, while other studies have shown that prior RSV infection had little to no effect on disease severity [197, 321-323]. Of note, these studies were done in rodent or non-human primate animal models, thus these models may contribute to the discrepancies.

RSV Immune Evasion and Disease Pathogenesis

RSV NS1 and NS2 are the two most copious proteins expressed due to their proximity to the promoter [324], and both NS1 and NS2 may facilitate RSV replication through inhibition of the IFN response [325-329]. NS1 and NS2 have different roles antagonizing IFN where NS1 is the more potent inhibitor [329]. *In vitro* NS1 and NS2 proteins have been shown to inhibit the ability of human epithelial cells to produce type I and III IFNs [329]. NS2 is a suppressor of the type I IFN response by affecting the degradation of STAT leading to decreased protein in the cell [326, 327]. The NS1 protein contains an elongin C and cullin-2 binding sites which enable the protein to act as a ECS E3 ubiquitin ligase thereby resulting in proteasomal degradation of STAT2 [330]. Decreased STAT2 expression leads to decreased activation of ISGs in response to infection, and an overall diminished immune response [326]. NS1 and NS2 have also been

shown to interfere with the translocation of IRF-3 into the nucleus [331]. Recombinant NS1 and NS2 deletion mutants exhibited decreased ability to block IRF-3 activation and translocation into the nucleus to enhance the transcription of antiviral genes [331], and inhibition of IRF-3 translocation was shown to be mediated by NS2 interaction with RIG-I [332]. Thus, NS1 and NS2 are capable of delaying the host immune response by decreasing STAT2 expression and IRF3 translocation into the nucleus, and antagonizing the IFN response.

RSV NS proteins have also been implicated in suppressor of cytokine signaling (SOCS) activation [333]. RSV NS deletion mutants have been shown to be deficient in activation SOCS1 expression compared to the wild type RSV indicating the NS1 is an inducer of SOCS1 expression [334]. RSV NS1 and NS2 mutant viruses have been shown to have attenuated replication in BALB/c mice, as well as chimpanzees [335, 336]. NS1 has also been shown to have a role in affecting cellular apoptosis [337]. Both NS1 and NS2 have been implicated in reducing the ability of the host cell to activate pro-apoptotic pathways in an IFN-independent manner [337], therefore not only do the RSV NS proteins inhibit the host antiviral response by interfering with nuclear translocation of IRF3 and degrading STAT2, but these proteins also interfere with the ability of the host cell to activate pro-apoptotic pathways that would lead to cell death.

The RSV G protein has been associated with immune evasion. While the primary function of the G protein is to mediate attachment of the virus to the membrane of target cells [309], the virus also produces a truncated version of the protein (soluble G; Gs) lacking the transmembrane domain [316, 338]. During RSV infection, Gs has been shown to be expressed on the apical side of RSV infected cells [316, 339], while membrane bound G protein (mG) remains in the infected cell. Both forms of G protein contain a CX3C chemokine motif located in

the central conserved region [314] that has been shown to be similar to fractalkine [223, 314]. Fractalkine (CX3CL) is the only known CX3C chemokine and is responsible for leukocyte migration and adhesion [340]. The CX3C motif is located between residues 182-186 of the RSV G protein [341]. CD4+ and CD8+ T cells, as well as certain subsets of NK cells, express the fractalkine receptor, CX3CR1 [223]. The CX3C motif in the central conserved cysteine noose region of G protein has been shown to be important for development of formalin inactivated RSV (FI-RSV) vaccine associated disease and production of substance P [342]. Pulmonary leukocyte trafficking, particularly of RSV specific IFNγ producing cells, has been shown to be affected the G protein CX3C motif expression [341]. The G protein CX3C motif has been shown to affect the Th cytokine response [341]. As Gs is produced early during infection, typically before the membrane form can be detected [339], it may have a role in delaying or confounding CD4+ T cells, CD8+ T cells, and NK cells which bear CX3CR1. The Gs protein has also been shown to antagonize TLR-2, TLR-4, and TLR-9 mediated immune responses, and Gs was shown to inhibit the production of cytokines initiated through TLR signaling [331]. Thus, RSV has the ability to control the anti-inflammatory response by decreasing pro-inflammatory cytokine production. Therefore, RSV employs different methods for modifying the host immune response.

G protein expressed by a vaccinia virus vector was shown to be able to reduce the viral burden in the lungs of RSV challenged mice [343]. Passive transfer of antibodies against the RSV G protein has also been shown to reduce the viral burden in RSV infected mice [344, 345]. Despite the protective effect attributed to anti-G protein antibodies, the G protein has been linked to vaccine enhanced disease [346, 347]. In particular, CD4+ T cells specific for the G protein have been shown to predispose toward a Th2-type immune response upon RSV challenge which leads to increased disease pathogenesis [348]. Immunization with Gs, in the absence of mG, has

been associated with increased IL-5 production, increased pulmonary eosinophil infiltration, and overall increased immunopathology [348]. Immunization with Gs was shown to be associated with FI-RSV-like vaccine enhanced disease symptoms [348]. Transfer of CD4+ T cells from Gs-primed mice predisposed RSV challenged mice to increased disease and lung pathology [306]. This enhanced pathology was found to be dependent on IL-5 and IL-13 while independent of IL-4 [305]. The RSV F protein has been shown to bind and signal through TLR4 in a similar mechanism as LPS [241, 349]. Binding and signaling are dependent on the presence of M2-D, a protein associated with the ectodomain of TLR4 [349]. Studies have shown that individuals who have polymorphism in this receptor have difficulty clearing RSV from the lungs [350-352]. Mice deficient in the TLR4 receptor exhibit decreased IL-12 production and reduced NK cells trafficking to the lungs [353]. Decreased NK cell trafficking was shown to correlate with reduced elimination of RSV infected cells and an overall delay in RSV clearance [353]. In humans there are two TLR4 single nucleotide polymorphisms (SNPs) that exist in the ectodomain, Asp299Gly and Thr399Ile [354]. The polymorphisms in TLR4 have been associated with decreased sensitivity to LPS in the epithelial cells of the human airway. The Asp299Gly mutation specifically has been shown to interfere or interrupt the LPS signaling cascade through TLR4 [354]. Infants that possess either of these SNPs are more susceptible to RSV infection [352], and a direct correlation between the presence of Asp299Gly and Thr399Ile mutations in high risk premature infants and RSV disease has been shown [355]. In addition, decreased signaling of the RSV F protein through TLR4 receptors containing this polymorphism has been described [356]. Decreased TLR4 sensitivity in infants containing this polymorphism can result in reduced translocation of NF-κB into the nucleus and an overall reduced IFN response enabling the virus to establish a more robust infection in infants with underdeveloped immune systems. However,

other studies have not found a correlation between RSV disease and TLR4 polymorphism. For example, one study examining the link between TLR4 SNPs and severe RSV disease showed that there was no correlation between disease severity and TLR4 polymorphism [357]. Although there are conflicting studies relating to the relevance of the TLR4 polymorphisms and disease severity, it can be inferred that the innate immune response in individuals containing these SNPs is diminished and potentially impaired allowing a more severe RSV infection.

Lung surfactants have also been shown to affect RSV disease. Surfactants are phospholipids responsible for reducing the surface tension at the air-liquid interface in the respiratory tract [358]. While these proteins reduce surface tension, they also have a role in the innate host defense. There are four surfactant proteins, i.e. surfactant protein-A (SP-A), surfactant proteins-B (SP-B), surfactant protein-C (SP-C), and surfactant proteins-D (SP-D) [359]. SP-A and SP-D are not essential for normal lung function, but are important for host defense mechanism [360]. SP-A and SP-D both modulate the immune response by binding to TLR4 and reducing NF-κB induction through this signaling pathway [361, 362]. Attenuation of TLR4 signaling reduces the pro-inflammatory immune response in the lung and reduces the cellular recruitment that follows TLR4 activation. SP-$A^{-/-}$ mice challenged with RSV exhibit impaired virus clearance and increased inflammatory response compared to wild type mice; however, when these mice were administered exogenous SP-A, the phenotype was restored to that of wild type mice [363]. SP-D has been shown to bind to both RSV G and F proteins [364, 365]. SP-D-/- mice also exhibited reduced viral clearance due to the inability of cells to phagocytize the virus [364]. When SP-D deficient mice were administered exogenous SP-D, there was an 80% decrease in RSV infection [365]. There are single amino acid substitutions that are known to occur in the SP-A allele, but they are not regarded as mutations due to their

frequencies in the population [360]. There are three polymorphisms associated with the SP-D allele, i.e. Met11Thr, Ala160Thr and Ser270Thr [366]. The Met11Thr SNP has been linked to higher risk of developing a more severe RSV disease progression [367]. Infants that are heterozygous for the Ala160Thr polymorphism are at higher risk for developing severe RSV infections [368]. Taken together, RSV can take advantage of these naturally occurring mutations in the human population to enhance infection and transmission.

RSV Vaccines

Currently, there is no FDA-approved vaccine for RSV. There have been numerous attempts to create a safe and effective RSV vaccine, however none have been successful despite >50 years of effort. Perhaps the most well-known RSV vaccine study, and unfortunately the least successful, was the FI-RSV vaccine study [342, 369-371]. In the late 1960s, naïve children were intramuscularly vaccinated with FI-RSV. Although there was minimal reactogenicity to vaccination, upon natural RSV infection, 80% of the vaccinated children were hospitalized compared to only 5% in the unvaccinated control group and two vaccinees died from complications [372, 373]. Analysis of the serum from the FI-RSV vaccinated children revealed non-neutralizing antibodies to the RSV F protein [374, 375]. In addition, pulmonary eosinophilia, increased CD4+ T cells, and a skewing of the immune response from a Th1 to Th2 response was observed [232, 256, 283, 369, 376-378]. Immunohistochemistry of lung sections from two vaccinated children who died following RSV infection revealed deposition of immune complexes in the airways [379]. Over the decades, the mechanisms contributing to vaccine enhanced disease associated with the FI-RSV have been investigated. For example, the G protein has been implicated extensively in the vaccine enhanced pathogenesis associated with the FI-

RSV vaccine [380]. Blocking the G protein during infection with 131-2G, a monoclonal antibody (mAb) reactive against several RSV strains, reduced FI-RSV associated disease [381, 382]. Priming mice with live RSV followed by FI-RSV decreased Th2-type cytokine mRNA, granular cells, and decreased CD4+ to CD8+ T cell ratios [383]. The G protein CX3C motif has been associated with increased recruitment of IL-4 producing T cells and decreased pulmonary cell trafficking of IFNγ producing T cells [341]. The FI-RSV enhanced disease was not associated with a single component, but rather a multitude of events and mechanisms leading to detrimental outcomes, but was indeed related to G protein CX3C-CX3CR1 interaction in mice [342].

Protein-based subunit vaccines consisting of RSV F protein alone or in combination with G have also been examined for efficacy in animal models [384-390]. For example, a chimeric vaccine comprising the extracellular and signaling domains of the F protein fused to the extracellular domain of the G protein expressed by a baculovirus vector and used to infect cotton rats and was extremely effective at producing neutralizing antibodies to RSV and was able to reduce viral burden following challenge [390]. Other viral vectors including parainfluenza virus, Newcastle virus, Sendai virus, and adenovirus have been utilized as possible delivery mechanisms for RSV antigens [391-395]. Vaccines composed entirely of the F proteins have also been evaluated for clinical use. The purified F protein (PFP) 1, PFP-2, and PFP-3 vaccines have all been evaluated in both animal and human models [385, 396-398]. While these vaccines were shown to be immunogenic and elicited neutralizing antibodies, these vaccines were not approved for use against RSV infection because there was no statistically significant reduction in the occurrence of lower respiratory tract infection, possibly due to the small number of randomized trials [384, 396, 399-401], emphasizing that antibodies against F protein are in

sufficient to protect from disease. Live-attenuated vaccine strategies have also been evaluated for RSV vaccine development, but all have failed because of over- or under-attenuated. This is not surprising given that natural RSV infection is insufficient to provide durable protection from disease. Most notably are the cold-passaged temperature-sensitive mutants (cpts). These viruses were passed in cell culture at lower temperatures to create mutations that would attenuate the virus. These vaccines have been tested in adults, seropositive and seronegative children [402, 403]. Although these cpts mutants were found to be safe in humans, the attenuation rendered the mutant non-immunogenic and therefore unable to elicit enough of an immune response to confer protection from RSV infection [404].

There are currently three FDA approved therapeutics for treatment of severe RSV infection. Ribavirin is a synthetic nucleoside analogue drug that has been shown to be effective against severe RSV infection [405]. Treatment of RSV infected children with the drug has been shown to reduce the viral burden [406]. Despite being approved by the FDA, Ribavirin has been shown to have toxic side effects and is very expensive [407]. The drug can be harmful to children with preexisting heart conditions because it can build up inside of red blood cells [408]. This drug is not typically recommended for treatment of RSV infection.

Palivizumab is a FDA approved treatment for severe RSV infection. This is a monoclonal antibody directed against the RSV fusion protein. The antibody is approved for prophylactic use in infants with preexisting health conditions including premature birth, heart defects, or defective lung function [407]. Despite having neutralizing activity against the F protein, palivizumab is not recommended for an RSV infection once it is established. It can only help prevent RSV infection in infants at higher risk of falling ill to infection. The antibody is designed to neutralize the virus by blocking the binding and fusion activity of the RSV F protein [229]. Motavizumab, a mAb

similar to palivizumab, has the same mechanism of action as palivizumab but has higher neutralizing activity [409]. RSV immunoglobulin (RSV-IVIG), developed by RespiGam, is the last FDA-approved RSV therapy. It is a human blood product used for the treatment of preterm infants or children diagnosed with congenital heart disease (CHD) [410]. Treatment with this regimen was shown to reduce RSV associated hospitalizations up to 41-63% [411], however there was a possibility that morbidity and mortality was associated with children with CHD [412]. Because of the risk of increased morbidity and mortality in CHD children, and because this treatment is derived from human blood and carries a risk of introducing blood borne infection, this drug treatment has not been used since 2003. An RSV infection would place even more burden on the underdeveloped immune system of these children. Once the RSV infection is established, the only defense is the body's immune system and possibly Ribavirin depending on the overall health condition of the child. Motavizumab has since been rejected by the FDA due to concerns with testing and increased incidence of hypersensitivity in children [413].

There is a desperate need for the development of an RSV vaccine that is immunogenic, safe, easy to produce, and effectively protects against infection and reinfection with minimal adverse side effects. Since immune compromised individuals, such as infants and the elderly, are the primary population affected by RSV, the vaccine will need to focus on eliciting a robust and efficient primary and memory response. The ability of RSV to reinfect throughout the lifetime of an individual makes eliciting an efficient memory response a key factor.

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CHAPTER 3

A NOVEL HA-F PROTEIN SUBUNIT VACCINE AGAINST INFLUENZA AND RESPIRATORY SYNCYTIAL VIRUS[1]

Turner TM, Jones LP, Tompkins SM, Tripp RA (2013) A Novel Influenza Virus Hemagglutinin-Respiratory Syncytial Virus (RSV) Fusion Protein Subunit Vaccine against Influenza and RSV. Journal of Virology 87: 10792-10804. Reprinted with permission from publisher.

Abstract

Influenza A virus and respiratory syncytial virus (RSV) cause substantial morbidity and mortality afflicting the ends of the age spectrum during the autumn through winter months in the United States. The benefit of vaccination against RSV and influenza using a subunit vaccine to enhance immunity and neutralizing antibody was investigated. Influenza hemagglutinin (HA) and RSV fusion (F) protein were tested as vaccine components alone and in combination to explore the adjuvant properties of RSV F protein on HA immunity. Mice vaccinated with HA and F exhibited robust immunity that when challenged had reduced viral burden for both influenza and RSV. These studies show an enhancing and cross-protective benefit of F protein for anti-HA immunity.

Introduction

Influenza virus and respiratory syncytial virus (RSV) may cause respiratory tract illness in individuals of all ages, but have particular impact on the young and elderly (4, 41, 76, 77). Annually, 3 to 5 million people worldwide are infected by influenza virus with more than 250,000 of those cases resulting in death (61). Influenza, a member of the *Orthomyxoviridae* family, is a single-stranded negative sense RNA virus with a segmented genome encoding 10 - 11 proteins from 8 different RNA segments. RSV is a member of the *Paramyxoviridae* family, and is a single-stranded negative-sense RNA virus having a non-segmented genome encoding 11 different proteins. Clinically, influenza infection can present with high fever, headache, chills, nausea, vomiting, cough, fatigue, and other symptoms (26, 76, 77), while RSV, a leading cause of hospitalization of infants and children under two years of age (14, 24), is associated with the development of bronchiolitis and pneumonia (25). Additionally, severe infection of the lower respiratory tract has been associated with asthma and recurrent wheezing in RSV infected children (32, 59). Notably, both viruses may co-circulate during the autumn through winter months in the United States (41, 51, 67), where peak influenza infections occur between November and April, while peak RSV infections occur between late September and April (8, 15, 22, 52). Since both of these viruses cause substantial morbidity and disease, having a vaccine that protects against both would be valuable.

The influenza hemagglutinin (HA) and RSV fusion (F) proteins have similar viral functions. The HA serves as a viral receptor influencing host cell specificity as well as cell entry via binding to sialic acid receptors and mediating virus-host interaction (19). HA is also a major antigenic glycoprotein on the surface of the virus, and antibodies raised against HA are neutralizing. The F protein is involved in viral attachment and fusion of the viral envelope with

the host cell (79). The F protein binds to glycosaminoglycans (GAGs), and putatively nucleolin on host cells (75), and like HA is a major viral glycoprotein where antibodies to F protein neutralize RSV (29). One such antibody directed against RSV F protein is palivizumab (Synagis) that is used to treat premature infants and those at high-risk for developing severe RSV infection (64, 72).

There is currently no licensed RSV vaccine. An early attempt using formalin-inactivated RSV (FI-RSV) vaccination was associated with vaccine enhanced disease in vaccinees naturally infected with RSV (9, 27, 48, 49, 58) where 80% of FI-RSV vaccinated children were hospitalized compared to only 5% in the control group (37). Vaccinees developed a Th2-type response, had poor neutralizing antibody responses (46), and there were increased cases of pulmonary eosinophilia (31). Subsequently, there has been considerable effort to develop safe and immunogenic RSV vaccines, but unfortunately none have been successful (7, 38, 60, 62, 94). For example, several temperature-sensitive RSV mutants vaccine candidates were evaluated and found to be either over- or under-attenuated, and in some cases reverted back to wide type (12, 36, 46, 56, 91). In addition, RSV subunit vaccines were evaluated, specifically purified F protein (PFP) vaccine which consisted of F protein alone (23, 55), as well as RSV F and G protein vaccines (5). The RSV PFP subunit vaccine candidates showed clinical promise (3, 18, 45, 54, 84), but ultimately were not licensed because there was no substantial decrease in the incidence of lower respiratory tract infection evident in the vaccinees (57, 66).

Currently, the annual influenza vaccine is designed to protect against three of the most prominent circulating influenza virus strains which are identified before vaccine production begins each year (20). Each virus strain is produced separately and later combined into one vaccine, thus millions of chicken eggs are required to produce the vaccine. The antibodies

induced by vaccination are generated predominantly to HA, and most target the globular head of HA (10). Recent studies of the 2009 pandemic H1N1 vaccine showed that there is a dose-dependent antibody response to HA where vaccinees receiving a 30 μg HA dose had higher geometric mean titers than those receiving a 15 μg HA dose, in both young and old age groups (16). This and other studies indicate that HA can be inadequately immunogenic and adjuvants are needed to enhance the immune response (73). Historically, adjuvants such as MF59, Type I interferon, cholera toxin, and oil-in-water emulsions have been used to increase the immunogenicity of the HA protein (2, 28, 44, 74), but these adjuvants have issues with reactogenicity and immunogenicity (30, 85).

Molecular adjuvants, such as regions on microbial antigens referred to as pathogen associated molecular patterns (PAMPs), offer novel approaches as adjuvants to vaccination. One potential molecular adjuvant is the RSV F protein which has been shown to trigger Toll-like receptor 4 (TLR4) and CD14 (39). The use of the F protein in a vaccine platform would have two distinct advantages. First, the F protein through TLR4 activation would trigger the innate immune response at the site of immunization, and second, the F protein would act as an antigen capable of inducing neutralizing antibodies to RSV thus facilitating a RSV vaccination strategy (17, 21, 56, 83, 89).

In this study, we examined the efficacy of a dual vaccine composed of influenza HA and RSV F protein that was used to induce immunity to both viruses. The role of F protein as a molecular adjuvant to facilitate the immune response to HA was addressed, and the findings showed that F protein enhances the overall immune response to HA, and vaccination with HA and F protein induce robust immunity that reduces both influenza and RSV disease burden in mice.

Materials and Methods

Mice, cell lines and viruses

Six-to-eight week old C57BL/6, C57BL/10ScNCr (TLR4 $^{-/-}$), and C57BL/10ScCr (TLR4 $^{+/+}$) mice were purchased from National Cancer Institute (NCI) (Raleigh NC), or the Jackson Laboratory (Bar Harbor, ME), and housed at the Animal Health and Research Center at the University of Georgia. The mice were fed *ad libitum* and experiments conducted in accordance with The Institutional Animal Care and Use Committee (IACUC).

Vero E6 (ATCC, Manassas, VA) cells were cultured in DMEM containing 5% FBS (Hyclone, Logan UT), Freestyle^{TM} 239-F cells (239-F cells, Invitrogen, Carlsbad CA) were cultured according to the manufacture instructions, A549 cells (ATCC) were cultured in DMEM containing 5% FBS, and Madin-Darby canine kidney (MDCK) cells (ATCC) were cultured in 5% FBS (Hyclone, Logan UT) for expansion, but were used serum-free for virus infection assays.

A/Puerto Rico/8/34 [H1N1] (PR8), A/HK/X31 [H3N2] (X31), A/Udorn/307/72 [H3N2] (Udorn), A/Philippines/2/82-X79 [H3N2] (X79), A/New York/55/04 [H3N2] (NY), A/Wisconsin/67/05 [H3N2] (Wisconsin), A/Illinois/02860/09 [H1N1] (Illinois), and A/California/04/09 [H1N1] (California) were propagated in nine-day old embryonated hen eggs and allowed to incubate for three days at 35°C (35). The allantoic fluid was harvested 48 or 72 hrs post inoculation and the tissue culture infectious dose ($TCID_{50}$/mL) as well as the plaque forming units (PFU/mL) were determined as previously described (70, 78). RSV A2 and RSV B1 viruses was propagated in Vero E6 cells and viral titers were determined using methods previously described (33, 48).

Vaccine formulations, transfection and purification.

The HA gene of influenza X31 was cloned into the pCDNA3.1 cloning system as previously described (86) to generate pCDNA-HA (43). Freestyle^{TM} 239-F cells were cultured according to manufacturer's protocol (Invitrogen) and allowed to reach a density of 1 x 10^6 cells/mL before transfection**.** Freestyle^{TM} 239-F cells were transfected with pCDNA-HA using 293fectin according to manufactures protocol (Invitrogen) and incubated for 5 days. The supernatant was harvested on day 5 and purified in a two-step process using AKTA fast protein liquid chromatography (FPLC) (Amersham Biosciences, Bath UK). Supernatant containing HA protein was brought to pH 7.5 using 2M Tris, pH8. The starting buffer for the first run consisted of 100mM NaCl, 50M 7.5 Tris. The elution buffer consisted of was 50mL of pH7.5 2M imidazole plus 150mL of the starting buffer. The supernatant was run over a His-Trap FF 5x5 column (GE Healthcare, Pittsburgh PA). The column was equilibrated with 4 column volumes (CV) of start buffer and supernatant run over the column at a flow rate of 4mL/ min for the first run and 1ml/min for the second run. The column was then washed with 10 CV of start buffer to wash off unbound protein. The bound protein was eluted with elution buffer in 2ml fractions. Fractions containing HA protein were collected and dialyzed against the start buffer for the second purification run. The start buffer for the second purification run was 500mM NaCl, 50M pH7.5 Tris and 12.5mL of pH7.5 2M imidazole. The elution buffer for the second purification run was 25mL of pH7.5 2M imidazole plus 175mL of the second start buffer. The FPLC run for the second purification was the same as the first with the exception of the injection flow rate being 1ml/min. After the supernatant was purified a second time, again using the His FF column, the eluted material was dialyzed against 1X PBS overnight and stored at -80°C until needed. F protein purification used in all experiments was extracted as previously described (53, 80). The

purified RSV F protein was endotoxin tested via limulus amebocyte lysate kit (Lonza, MD) and stored at -80°C until needed.

Protein conjugation

HA was conjugated to the F protein using chemical cross linkers 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Sigma, St. Louis MO). The HA protein was incubated in coupling buffer consisting of 0.1M 2-(N-morpholino) ethanesulfonic acid (MES) (Sigma, St. Louis MO) and 0.5M NaCl at pH 6 overnight at 4°C. A 100X mixture of coupling reagents (Sulfo-NHS and EDC) was made by adding 40mg of EDC and 110mg of Sulfo-NHS dissolved in 1ml of coupling buffer at pH 6. A volume of 3µl of the 100X coupling reagent mix was added to the HA protein, vortexed and incubated at room temp (RT) for 15 minutes. After the 15 minute incubation, sodium carbonate buffer was added to the HA reaction to neutralize the pH. The F protein was immediately added to the HA protein to initiate the conjugation process to limit the production of HA-HA or F-F. The combination of the HA, F and cross linkers was allowed to incubate for 2h at RT. Lastly, following the 2hr incubation, the proteins were again dialyzed against PBS overnight and stored at -80°C until needed.

Protein verification

The HA and F proteins were purified. To ensure the recombinant HA (rHA) protein produced in 293 freestyle cells had the same sequence as HA found on the surface of X31, the plasmid encoding the HA protein was sequenced. To generate anti-HA hyperimmune serum, mice were infected with 5×10^4 $TCID_{50}$ of influenza X31 virus 3 times over a 6 week period.

The hyperimmune serum was then used to detect rHA via western blot described below (Fig. 3.1A). Native purified F protein was also confirmed via western blot using the monoclonal antibody (MAb) 131-2A. The HA-F conjugation product was detected using hyperimmune serum from X31 infected mice (data not shown) as well as a MAb (131-2A) against the RSV F protein (Fig. 3.1B).

Western blot

The recombinant HA protein was compared to viral protein using Western blot for conformational similarity. The purified proteins were run on a Criterion gel (Bio-Rad Hercules, CA) at 125V for 1h and 30 minutes to create separation between the bands of the ladder and transferred to a PVDF membrane and blocked for 1h at RT. The membrane was removed from the transfer apparatus and blocked with 5% milk in Tris-buffered saline-tween (TBS-tween) for 1h prior to being probed with mouse hyperimmune serum from either X31 or 131-2A at 1:200 and 1:1000 dilutions for 1h, respectively. The blot was washed 3 times with TBS 0.5% Tween. An anti-mouse AP conjugated antibody was used as the secondary antibody (Thermo Scientific, Rockford IL), and membranes were developed with ECF (GE Healthcare, Pittsburgh PA).

Vaccination and infection

C57BL/6, C57BL/10ScNCr (TLR4 $^{-/-}$), and C57BL/10ScCr (TLR4 $^{+/+}$) mice were intramuscularly (i.m.) vaccinated with either HA only (HA), F only (F), HA combined with F at equimolar concentration (HA+F), HA directly conjugated to F (HA-F), X31, PR8, RSV A2, RSV B1, or PBS. Two doses for each vaccine were evaluated: a) one dose at 15 μg proteins, single or combined, and b) one dose at 30 μg proteins, single or combined. Mice were pre-bled

from the tail vain and vaccinated on day 0. At 21 days post-prime vaccination, mice were bled from the tail vain and their antibody titers were determined by ELISA where HA or F were coated on the plates for detection as previously described (68). At day 28 post-prime, mice were i.m. boosted with 30 μg of either HA, F, HA+F, HA-F or 1 x 10^6 PFU/100μL of X31, PR8, RSV A2 or RSV B1. Subsequently, at day 35 post-boost, mice were anesthetized with Avertin intraperitoneally (i.p) and challenged intranasally (i.n.) with 240 HA units (HAU) of X31 ($10^{6.3}$ $TCID_{50}$/50μL), 10 HAU of PR8 ($10^{3.6}$ $TCID_{50}$/50μL), or 10^6 PFU/50μL of RSV A2. Mice were sacrificed at day 3 post-influenza challenge, or at day 5 post-RSV challenge, to determine lung viral titers as previously described (82). The lungs were homogenized, centrifuged, and the supernatant collected and stored at - 80° C until use.

For purified F protein vaccination, C57BL/6 mice were immunized at day 0 and boosted at day 28 by i.m. injection with 30 μg of the RSV F protein or saline. Seven days post-boost, mice were challenged i.n. with either X31 (1 x 10^5 PFU/50μL), A/Udorn/307/72 (5 x 10^4 PFU/50μl), or A/Philippines/2/82 (X79) (1 x 10^2 PFU/50μL). Three days post challenge, mice were sacrificed and viral titers determined via $TCID_{50}$ as previously described (71).

Analysis of HA and F protein-specific IgG in serum samples

Serum samples were collected from vaccinated mice at days 21 and 35 post-boost. HA and F protein-specific IgG antibodies were detected by ELISA using 96-well high binding plates (Costar, Corning NY) coated with 4 μg/ml HA from X31, 0.2 μg/mL of HA from A/Wisconsin/67/05 (eENZYME, Gaithersburg, MD), or F protein in PBS. Sera were added to plates in serial dilutions. HA and F protein-specific antibodies were detected with alkaline phosphatase conjugated antibodies specific for mouse IgG (Thermo Scientific, Rockford IL)

followed by addition of *p*- Nitrophenylphosphate (KPL, Gaithersburg, MD**)** for 20 min. Endpoint titers were determined as the last sample dilution that generated an OD_{405} reading of greater than 0.2.

Competition ELISA

High binding 96-well ELISA plates (Costar, Corning NY) were coated with 50ug/mL of X31 virus and allowed to adhere overnight at 4°C. Plates were blocked with 1%BSA in PBS for 1h at RT. Anti-F protein serum was incubated with serial dilutions of RSV F peptides (New England Peptide, Gardner MA) at a constant dilution of 1:300 for 1h at RT. The mixture of anti-F protein serum and serially diluted peptides were added to wells coated with X31 virus for 1h at RT. Plates were washed 3x with KLP (Gaithersburg MD) and F protein-specific antibodies detected with horseradish peroxidase conjugated antibodies specific for mouse IgG (Thermo Scientific, Rockford IL) followed by addition of TMB ELISA substrate (Thermo Scientific, Rockford IL) for 20 minutes. The ELISA was stopped with 2N H_2SO_4 and read at 450nm. Reduction in binding was determined to an irrelevant peptide form human metapneumovirus (HMPV) N protein.

Influenza $TCID_{50}$

MDCK cells were cultured at 4 x 10^4 cells per well in a 96-well plate overnight. The cells were washed 2 times with PBS and infected with lung homogenate supernatant as previously described (71).

Influenza micro-neutralization Assay

Serum from mice bled on day 35 post-priming (7 days post boost), or anti-F protein hyperimmune serum generated in mice vaccinated two or three times with 30μg of F protein/vaccination was used to evaluate neutralizing antibody titers. Serum was diluted 2-fold in serum-free DMEM containing 1% antibiotic/antimycotic. 100 $TCID_{50}$/100 μL of A/Puerto Rico/8/34 [H1N1] (PR8), A/HK/X31 [H3N2] (X31), A/Udorn/307/72 [H3N2] (Udorn), A/New York/55/04 [H3N2] (NY), A/Wisconsin/67/05 [H3N2] (Wisconsin), A/Illinois/02860/09 [H1N1] (Illinois), or A/California/04/09 [H1N1] (California) was added to serially diluted antibody and incubated at 37°C for 1h in the presence of 1 μg/mL TPCK trypsin. MCDK cells cultured at 4 x 10^4 cells/well were washed twice with PBS. 100 μL of the antibody/virus/trypsin combination was added to MDCK cells and incubated at 37°C. Day 3 post infection, 50 μL of the antibody/virus/trypsin combination was added to 50 μL of 0.5% chicken red blood cells and incubated for 1h and the HI titer was determined as previously described (71).

RSV plaque assay

Lungs harvested from RSV infected mice were homogenized (Miltenyi Biotec, Cambridge, MA) and centrifuged at 12,000 x g for 5 minutes as previously described (71). The clarified supernatant was used to infect Vero E6 cell monolayers as previously described (81).

RSV micro-neutralization assay

Serum from mice bled 35 days post-vaccination (7 days post boost) was used to determine neutralizing antibody titers. Serum was serially diluted (10-fold) in serum-free DMEM containing 1% antibiotic/antimycotic. 100PFU/100μL of RSV A2 was added to the serially diluted antibody and incubated for 1h at 37°C. Vero cells plated at 3 x 10^5/ well in 24 well flat-

bottom plates (Costar, Corning NY) were washed twice with PBS. Two hundred μL of the antibody/virus mixture was added to Vero cells and incubated for 2h at 37°C. From this point, the plates are treated as plaque assays and follow the same method as described above (42).

Passive antibody purification and transfer

Serum collected from mice was purified for IgG using Pierce Protein G Agarose beads (Thermo Scientific, Rockford IL) according to manufactures protocol. Briefly, serum samples were incubated with protein beads for 2h at RT. IP buffer (25 mM Tris, 150 mM NaCl; pH 7.2) was added to slurry and centrifuge for 2-3 minutes at 2,500 × g. This step was repeated 3 times. IgG was eluted by incubating slurry in elution buffer (0.2 M glycine•HCl buffer, pH 2.5-3.0) for 5 minutes and then centrifuged for 3 minutes at 2,500 × g. The supernatant containing the IgG was collected. This step was repeated 3 times. 10 μL of neutralization buffer (1 M Tris, (pH 7.5-9)) was added to the purified IgG for every 100μL of elution buffer. Purified antibody was dialyzed against PBS at 4°C overnight. Naïve recipient mice were injected i.p. with two doses of 250ug of purified IgG from either X31 challenged mice, RSV F protein vaccinated mice, or naive IgG from naïve mice 48h. Mice were then challenged 48h later with 2.5 x 10^3 PFU/0.05mL of X31 virus. Three days p.i., lungs were harvested and lung viral titer determined via $TCID_{50}$.

Histopathology

The lungs from 3 mice per/group of vaccinated and challenged mice were collected in 10% buffered formalin (Fisher, Fairlawn NJ) for histological examination. Formalin-fixed tissue was embedded in paraffin wax, sectioned (4 to 5 μm thick), and mounted on glass slides. Tissues

were stained using hematoxylin and eosin and examined by a board certified pathologist in the College of Veterinary Medicine at the University of Georgia.

Cross reactive epitopes and vaccination

The protein sequences of HA and F were evaluated using the Immune Epitope Database (IEDB) Analysis Resource. This software predicts B cell epitopes based on several characteristics of the amino acid sequence such as polarity, hydrophobicity, flexibility, exposed surface, accessibility, secondary structure, and turns among other properties. From this analysis, four peptides were made and evaluated for potential cross- reactivity to both the influenza HA and the RSV F protein. These four peptides, two from X31, and two from RSV A2, were conjugated to KLH and used to vaccinate mice, specifically RSV20F: FCFASG-NITEEFYQSTOH, RSV431: YVSNKGMDTVSVGNTLYYOH, X31HA10: IFCLALGQDLPGNDNSTOH, and X31HA428: YVEDTKIDLWSYNOH (Table S2).

Statistics

Data was analyzed via Student's T tests, One-way ANOVA, and two-way ANOVA in GraphPad Prism version 5 (GraphPad Software; La Jolla, CA). Statistical tests performed and noted in the appropriate figure legends.

Results

RSV F protein enhances the antibody response to influenza HA protein during vaccination.

To test the ability of the F protein to enhance the anti-HA antibody response to X31 influenza virus, mice were intramuscularly (i.m.) vaccinated with 15 or 30 μg of HA, HA

combined with F in equal molar concentrations (HA+F), HA conjugated to F (HA-F), F protein alone (F), or 10^6 PFU of X31, PR8, RSV A2, or RSV B1. Antibody titers were determined for X31 HA reactivity, or to RSV A2 F protein. The initial finding showed that data from the 30 μg studies were best, thus this was antigen concentration was further pursued. At day 21 post-vaccination, mice receiving 30 ug HA, HA+F, HA-F, or X31 virus had detectable antibodies against purified X31 HA protein (Fig. 3.2A). F protein directly conjugated to HA (HA-F) significantly (**$p<0.0005$**) increased IgG titers compared to HA alone vaccinated mice. There was no detectable increase in the HA antibody response when F protein was given in equal concentration with HA (HA+ F) compared to HA vaccinated mice. As expected, there was an antibody titer to HA in X31 vaccinated mice (Fig. 3.2A). Twenty-eight days post-immunization, the mice were i.m. boosted with the appropriate vaccine, i.e. X31, PR8, HA, F, HA+F, or HA-F. Seven days post-boost (day 35), the boosted mice had substantially higher HA antibody titers (Fig. 3.2A). Mice vaccinated with HA+F had significantly (**$p<0.0005$**) higher antibody titers compared to HA vaccinated mice, and HA-F vaccinated mice had significantly (**$p<0.005$**) increased titers compared to HA alone. These findings indicate that F protein enhances the humoral IgG response to HA protein when co-delivered during vaccination.

The antibody response to F protein was also evaluated (Fig. 3.2B). Mice vaccinated with RSV A2, RSV B1, F, HA+F, and HA-F had detectable antibody titers against F protein at day 21 post-vaccination, however seven days post-boost (day 35 post-vaccination), antibody titers against F protein increased 60-fold in mice vaccinated with F protein, and 20-fold in mice vaccinated with HA+F, HA-F and RSV A2 compared to the day 21 response (Fig. 3.2B). Mice vaccinated with RSV B1 had low levels of anti-F antibody that cross-reacted with RSV A2, and there was no anti-F IgG antibody detected in mice vaccinated with HA only as expected. Mice

vaccinated with HA+F and HA-F had lower anti-F antibody titers relative to mice vaccinated with the F protein only. It is possible that epitopes in the globular head of the F protein (83) could have been masked by the conjugation process (20, 26); however, as mice responded equivalently to dual vaccination with HA+F, HA-F, or to RSV A2, it is likely that purified F protein generates more robust antibody responses perhaps because of antigen availability, processing and/or presentation.

RSV F protein enhances the neutralizing antibody response to influenza

The neutralizing antibody response to vaccination was examined (Table 3.1). Mice receiving HA, HA+F, HA-F, or X31 had similar but significantly ($p<0.01$) higher neutralizing antibody titers against X31 compared to mice vaccinated with PBS, F, or PR8 (Table 3.1). Mice vaccinated with HA+F or HA-F had approximately 4-fold higher neutralizing IgG antibody responses against X31 compared to mice vaccinated with HA alone showing that F protein potentiates the neutralizing antibody response to HA. Mice vaccinated with HA from X31 to neutralize PR8 was also examined; however, only mice vaccinated with PR8 had a neutralizing antibody response against PR8 (Table 3.1). As antibodies to F protein have been shown to neutralize RSV (18, 23, 45, 66), it was not unexpected that mice vaccinated with the RSV A2, F, HA+F, and HA-F were able to significantly ($p<0.05$) neutralize RSV A2 compared to PBS vaccinated mice (Table 3.1).

HA and F vaccination provides influenza and RSV immunity.

To determine if the vaccine candidates generated protective immunity to influenza, mice were vaccinated with X31, PR8, HA, F, HA+F and HA-F and subsequently intranasally (i.n.)

challenged with X31 or RSV (Fig. 3.3). Mice vaccinated with HA, HA+F, HA-F, and F had significantly ($p<0.05$, $p<0.005$, $p<0.005$, and $p<0.01$ respectively) reduced X31 lung titers at day 3 pi compared to PBS control mice, and F protein significantly **(**$p<0.05$**)** enhanced X31 clearance in HA+F and HA-F vaccinated mice compared to mice vaccinated with HA alone (Fig. 3.3A). There was no detectable cross-reactivity to PR8 (H1N1) for mice vaccinated with any candidate containing H3 HA as expected (6). X31 lung titers were also evaluated at 6 and 8 days post infection to determine if addition of the F protein further affected viral clearance. Mice vaccinated with HA+F and HA-F had significantly ($p<0.005$ and $p<0.01$) less virus detectable in the lungs at day 6 relative to mice vaccinated with PBS and HA alone respectively (Fig. 3.3B). Mice vaccinated with PR8, and having antibodies against PR8, were refractory to PR8 challenge as expected having no detectable lung virus titers compared to the PBS control (data not shown). Mice vaccinated with RSV A2, RSV B1, HA, F, HA+F, or HA-F were challenged with RSV A2 (Figure 3.3C). Mice vaccinated with RSV A2, F, HA+F, or HA-F, had significantly ($p<0.005$, $p<0.005$, $p<0.01$, and $p<0.01$ respectively) reduced RSV A2 lung titers (Fig. 3.3C). Although mice vaccinated with HA-F or HA+F had to some extent higher lung viral titers compared to mice vaccinated with F protein only, the difference in titers was not significant ($p<0.1$) between these groups (Fig. 3.3C).

Vaccination is not linked to substantial lung pathology following virus challenge

To determine if vaccination with any of the candidates affected lung pathogenesis, lung histopathology from mice immunized with X31, RSV A2, or PR8 was evaluated (Fig. 3.4). Lungs were collected 3 days post-challenge for X31 and PR8 and 5 days post-challenged for RSV A2 and scored for histopathology using a 0 to 4 scale where a score of zero is normal, a

score of one indicates minimal pathology, and a score of four indicates maximal tissue damage and associated cellular infiltration (Fig. 3.4A) (69). No substantial differences in histopathology scores were evident in any of the vaccinated mice challenged with X31(Fig. 3.4B) or PR8 (data not shown). Also, there was no evidence of vaccine-enhanced lung pathology in any vaccinated mice challenged with RSV A2 (Fig. 3.4C).

RSV F protein adjuvants HA through TLR4

C57BL/10ScNCr (TLR4 $^{-/-}$) mice were used to determine if F protein could adjuvant HA in the absence of the TLR4. TLR4 $^{-/-}$ or TLR4$^{+/+}$ mice were vaccinated with PBS, HA, HA+F, or HA-F and the serum antibody titers to HA determined at day 21 post vaccination, and 7 days post-boost, i.e. day 35 (Fig. 3.5). Antibody titers in TLR4 $^{-/-}$ vaccinated mice were generally lower than TLR4$^{+/+}$ mice. There was no significant ($p<0.01$) difference in antibody titers between HA and HA-F in TLR4 -/- mice 7 days after boost, i.e. day 35; however antibody titers in mice vaccinated with HA+F were significantly ($p<0.05$) higher than HA alone (Fig. 3.5A). In contrast, TLR4$^{+/+}$ mice vaccinated and boosted with HA+F or HA-F had significantly ($p<0.001$ and $p<0.01$, respectively) higher antibody titers than mice vaccinated and boosted with HA alone suggesting an F protein adjuvant effect through TLR4 (Fig. 3.5B). TLR4 $^{-/-}$ mice vaccinated with PBS, HA, HA+F, or HA-F, and challenged with X31, had reduced X31 lung titers at day 3 post-challenge compared to the PBS control (Fig 3.6A). Despite the increased HA-specific antibody response in HA+F immunized mice, there was no detectable increase in virus clearance attributable to F protein as was observed in wild type (TLR4$^{+/+}$) mice (Fig. 3.3A). Viral titers were also evaluated at 6 and 8 days p.i. to determine if mice vaccinated with HA+F and HA-F exhibited increased viral clearance. At day 6 pi, wild type mice vaccinated with HA+F and HA-F

had enhanced lung virus clearance compared to HA alone X31 virus replication at day 6 when compared with mice vaccinated with HA alone (Fig. 3.3B), and all mice cleared virus by 8 days pi (data not shown). In contrast, at day 6 pi in the absence of TLR4 (day 6 KO), HA, HA+F and HA-F lung tiers were equivalent indicating that RSV F protein mediates an HA adjuvant effect in the presence of TLR4 (Fig. 3.6B). Neutralizing antibody titers in these vaccine groups were evaluated against X31 (Table 3.2). Mice vaccinated with HA, HA+F or HA-F had neutralizing antibodies against X31; however, there was no significant difference in titer between the groups, and neutralizing titers in the absence of TLR4 were lower compared to $TLR4^{+/+}$ mice.

F protein provides cross-protective antibody epitopes

The finding that mice vaccinated with F protein alone had reduced X31 lung titers relative to the control (Fig. 3.3A), suggested that F protein may induce cross-reactive antibodies to HA. To address this possibility, antibody epitope analysis was performed comparing the F and HA proteins based on properties that included polarity, hydrophobicity, flexibility, exposed surface, accessibility, secondary structure among others (63). Four putative regions near the membrane stalk of the F protein were predicted to compliment the stalk region in HA (data not shown). Mice were immunized and boosted with 30 μg of these peptides conjugated to KLH, or with 30 μg of HA or F proteins on days 21, 42, and 63. Despite multiple boosts, at day 63 post-vaccination, the mice did not raise a detectable HA or F protein antibody response by ELISA. After X31 or RSV A2 challenge, no substantial differences were detected in lung virus titers for any peptide vaccination compared to HA or F vaccinated mice (data not shown). These results suggest that the peptides to the four complimentary regions between F and HA were not able to generate cross-reactive antibodies most likely because they consisted of linear epitopes and both

HA and F protein are typically homotrimeric membrane glycoproteins, and thus antibodies may likely be generated to discontinuous epitopes. Anti-F antibodies however are able to neutralize influenza virus (Table 3.1), and bind influenza HA (Figure 3.7). An ELISA assay was used to determine the reactivity of purified anti-F, anti-X31(positive control), or naïve (negative control) antibody against HA (Fig. 3.7A). The results show that anti-F antibodies are cross-reactive to HA across several dilutions compared to naïve serum (Fig. 3.7A). Purified HA from A/Wisconsin/67/05 was used for detecting cross reactive anti-F antibodies against other H3N2 influenza as shown in Table 2. The results showed that the anti-F protein antibodies were cross-reactive against A/Wisconsin/67/05 HA protein (Fig. 3.7B), and support the earlier observations that antibodies generated against the RSV F protein cross-react with the H3N2 viruses examined, a feature that can inhibit replication of these viruses.

In an effort to identify the region(s) responsible for cross-reactive binding between the F protein and HA protein, a series of overlapping peptides were generated against the RSV F protein (Table 3.3). Peptides overlapping by 10 amino acids, starting at 1 through residue 160, were used in a competition ELISA to determine if these peptides could block anti-F antibodies from binding to influenza X31 virus. Of the peptides tested, peptide 120-140 was able to significantly ($p<0.0001$) block binding by 42.8 % ± 2.57% at the highest concentration tested, i.e. 10μg (Fig. 3.8). There was a dose-dependent reduction of anti-F antibody binding to X31 with decreasing concentrations of the peptide (Fig. 3.8). Although residues 120-140 do not completely block binding to influenza X31 virus, it does reduce the binding of cross-reactive antibodies, indicating that this epitope has a role in cross-reactivity.

As F protein vaccinated mice reduced X31 lung titers (Fig. 3.3A), and F protein vaccination was associated with the induction of neutralizing antibodies against X31(H3N2) but

not PR8 (H1N1) (Table 3.1), the propensity of F protein to induce neutralizing antibodies against other influenza strains was examined. Anti-F hyperimmune serum generated against F protein was evaluated for reactivity against H3N2 virus strains A/New York/ 55/04 (NY), A/Wisconsin/67/05 (Wisconsin), or H1N1 virus strains A/Illinois/02860/09 (Illinois) and A/California/04/09 (California). Anti-F hyperimmune serum was capable of neutralizing H3N2 strains X31, NY, and Wisconsin with titers of 480, 1280, and 1280, respectively (Table 3.4). Consistent with the HA and F protein vaccination findings, anti-F antibody was unable to neutralize H1N1 strains (Table 3.1, Table 3.4); however, a 1:3200 dilution inhibited H3N2 X31, NY, and Wisconsin replication (Table 3.4). Together, these findings supports our earlier data showing that antibodies to RSV F protein cross-react and inhibit H3N2 replication *in vitro,* and increasing the anti-F antibody titer facilitates neutralization of H3N2 influenza viruses.

Passive transfer of RSV F antibodies inhibits influenza virus replication in vivo

To determine if antibodies directed against the RSV F protein were in part responsible for the reduction in X31 viral titer observed in F protein vaccinated mice, naïve mice received passive antibody transfer of purified IgG isolated from hyperimmune serum from mice vaccinated with F protein, infected with X31 virus, or naïve serum was collected and purified for IgG. Naïve recipients were received two doses of 250μg of IgG 48h and 24h prior to infection with X31. X31 viral titers were similar between PBS treated mice and mice receiving antibodies from naïve mice (Fig 3.9). However mice receiving anti-X31 antibodies and anti-F antibodies had significantly ($p<0.001$ and $p<0.01$ respectively) reduced X31 lung viral titers relative to control mice (Fig. 3.9). This data demonstrates that IgG from RSV F protein vaccinated mice can reduce influenza virus replication *in vivo*.

RSV F vaccination protects against H3N2 influenza virus challenge

To determine if F protein vaccination induced antibodies that protected against H3N2 virus challenge, mice were vaccinated with F protein and challenged with H3N2 X31, X79, or Udorn viruses (Fig 3.10). F protein vaccinated mice had significantly ($p<0.005$) decreased X31 lung virus titers compared to PBS vaccinated controls as was observed in previous studies (Fig. 3.2A and Fig 3.10). Similarly, F protein vaccinated mice challenged with Udorn had significantly ($p<0.05$) reduced lung titers compared to the PBS control group (Fig 3.10), while F protein vaccinated mice challenged with X79 did not affect lung virus replication - a finding consistent with neutralization data for X79 where anti-F serum was unable to reduce virus replication *in vitro* (data not shown). This finding may be explained by the highly virulent nature of X79 which is mouse-adapted and known to rapidly replicate rand be pathogenic in mice (40, 92). These findings indicate that F protein vaccination can induce cross-reactive antibody responses to some H3N2 viruses providing a level of protection from lung virus replication.

Discussion

There is a considerable need for a safe and effective RSV vaccine platform, and for a means to facilitate influenza vaccination. In this study, we examined the utility of a dual vaccine composed of the HA protein of influenza and the F protein of RSV. There have been various RSV vaccine studies, but hurdles remain in RSV vaccine development that include the need to immunize infants who may respond inadequately to vaccination, and to confer the presence of maternal antibody to RSV in very young infants. To date, no safe and efficacious RSV vaccine is available despite decades of effort. The most notable RSV vaccine failure occurred in the late 1960's where children receiving a formalin-inactivated RSV (FI-RSV) vaccine who were later

naturally infected by RSV developed vaccine enhanced disease which resulted in the death of two children (9, 27, 48, 49, 58). From the literature, it appears that more than one type of vaccine may be needed to prevent RSV disease in the populations at risk that include the very young and old as well as the immune compromised. Clinical trials with purified F protein (PFP) subunit vaccines and live attenuated vaccines have been evaluated (3, 18, 21, 34, 55, 56). PFP vaccines were promising vaccine candidates for the elderly and for RSV-seropositive children with underlying pulmonary disease because vaccination studies in the rodent model showed that the F protein vaccination was associated with decreased viral titers, overall reduced disease burden, and minor pathology following RSV challenge (5, 47, 90). However, PFP vaccines did not provide sufficient RSV neutralizing antibodies in seronegative chimpanzees that received three doses, thus the studies were halted (11). Similarly, studies with live cold-passaged, temperature-sensitive RSV vaccine candidates that were primarily targeted for use in young infants were shown to induce inadequate protection being under- or over-attenuated in a variety of animal models (12, 13, 56). However, it is clear that anti-RSV F protein antibody responses are effective at neutralizing RSV (24, 87, 88), and this is further supported by studies examining the efficacy of Palivizumab, which is a humanized antibody specific to the F protein and is which has been approved for prophylactic use in children with certain risk factors (29, 64, 65).

The RSV F protein has been shown to be a TLR4 and CD14 agonist (39). Thus, it was hypothesized that the F protein, through TLR4, would act as a molecular adjuvant to enhance the immune response to HA protein to confer enhanced immunity to influenza virus challenge. As the F protein is a major target in the host immune response upon RSV infection (29, 93), and RSV F protein vaccine candidates have shown a level of efficacy based on neutralizing properties (50, 55), it was also hypothesized that the F protein would not only serve as a

vaccine adjuvant for HA, but also as a vaccine antigen to RSV. Mice vaccinated with the RSV F only, HA+F or HA-F were able to mount antibody titers that neutralized RSV viral titers, and addition of the HA component as a dual vaccine did not hamper immunity to F protein. Importantly, RSV or influenza virus challenge of mice vaccinated with F, HA+F, or HA-F was not associated with lung pathogenesis highlighting the safety and immunogenicity of a dual HA and F vaccine platform. However, it was observed that mice vaccinated with HA-F or HA+F had lower antibody titers than mice vaccinated with F only, a feature that may be explained by potential epitope masking attributed to the HA component. Interestingly, mice vaccinated with HA protein directly conjugated to the F protein (HA-F), or administered in equal concentrations with the F protein (HA+F), induced substantial enhancement of the anti-HA antibody response at day 21 and 35 post-vaccination compared to HA alone vaccinated mice. There was also a significant increase in HA neutralizing antibody titers in mice vaccinated with HA+F and HA-F compared to HA alone. These findings indicate that RSV F protein acts to adjuvant the HA response, an effect measurable by the overall antibody response as well as neutralizing antibody titer to HA. Importantly, the increase in the neutralizing antibody HA titer correlated with decreased X31 lung viral titers in HA+F and HA-F vaccinated mice compared to the HA vaccinated group.

The mechanism by which the F protein adjuvants HA appears linked to TLR4, as the F protein adjuvant effect was reduced in TLR4 -/- mice where mice vaccinated with HA-F or HA+F who had anti-HA antibodies titers lower than $TLR4^{+/+}$ mice. Dual HA and F vaccinations also resulted in reduced X31 virus challenge titers at day 6 pi in $TLR4^{+/+}$ mice relative to TLR4-/- mice supporting a role of the RSV F protein as an adjuvant. Although anti-HA antibodies

were higher in HA+F vaccinated mice, this increase did not correlate with a significant decrease in X31 lung viral titers as mice vaccinated with HA, and HA-F had similar lung virus titers.

Unpredictably, vaccination with F protein induced cross-protective immunity to X31, a feature that reduced X31 lung virus titers and raised neutralizing antibodies to X31. The possibility of cross-reactive B cell epitopes predicted by Chou and Fasman algorithms (1) was examined for four epitope regions that shared sequence homology between HA and F proteins. Vaccination with these KLH-peptides was unable to induce cross-reactive antibodies or reduce lung X31 lung titers. As these peptides did not generate detectable cross-reactive immunity, peptides generated against the stalk region of the F protein were evaluated for their ability to block anti-F antibody binding to X31. The findings from this study indicated that the 120-140 aa peptide region is antigenic as it adsorbed much of the anti-F protein antibody that was cross-reactive toward X31. The inability of linear peptides to mimic proteins to induce cross-reactive antibodies is not unexpected as it is likely F and HA proteins share a conformational, discontinuous B cell epitope given both proteins are typically homotrimeric membrane glycoproteins.

To further investigate the level of cross-protective immunity afforded by F protein, the anti-F antibody response to other H1N1 and H3N2 viruses was determined. It was found that anti-F antibody inhibited several H3N2 virus isolates in vitro, but was unable to neutralize H1N1 isolates. It is likely that anti-F antibody recognizes an epitope on H3 that is not present on H1. An alternate explanation that precludes HA is that anti-F antibody may recognize an epitope on the neuraminidase (NA) of one virus but not the other. However, the ELISA results indicate that anti-F antibodies are reactive against the purified HA protein from X31 and A/Wisconsin/67/05. Of note, anti-F antibody was also capable of neutralizing X31 or Udorn infection in mice, but

there was no protection from X79 challenge. This is most likely due to the highly pathogenic nature of this mouse-adapted virus, and this finding was consistent with the inability of anti-F protein serum to neutralize X79 *in vitro.* To clarify the role of anti-F antibodies in protection, mice were passively transferred anti-F antibodies and challenged with X31. The findings showed that anti-F antibodies could reduce X31 lung virus titers, thus F protein not only adjuvants HA responses but by itself provides a level of cross-protection for some H3N2 influenza viruses. Taken together, the findings from this study show that a dual HA and F vaccine approach can be used to enhance the immune response to HA, is safe and efficacious, and vaccination is associated with increased neutralizing antibody titers to RSV and influenza A viruses.

Acknowledgements

This research was supported by funding from the National Institute of Health, R01-1021RR211294, and HHSN266200700006C, and the Georgia Research Alliance. We thank Elizabeth W. Uhl for her help with the hematoxylin and eosin staining of lungs, and Patricia Jorquera, Mary Hauser, and Kate Oakley for their review.

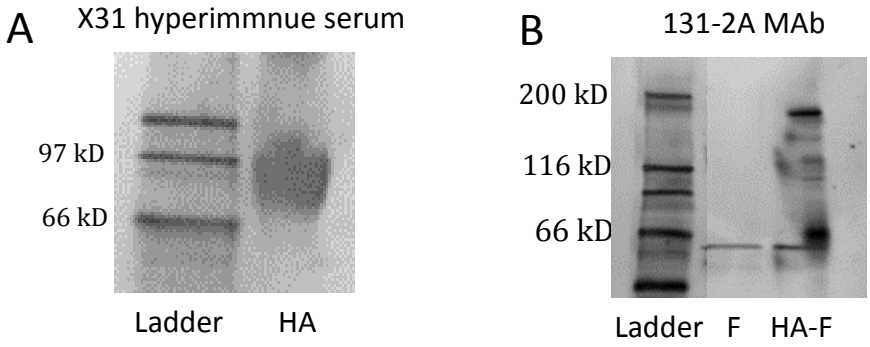
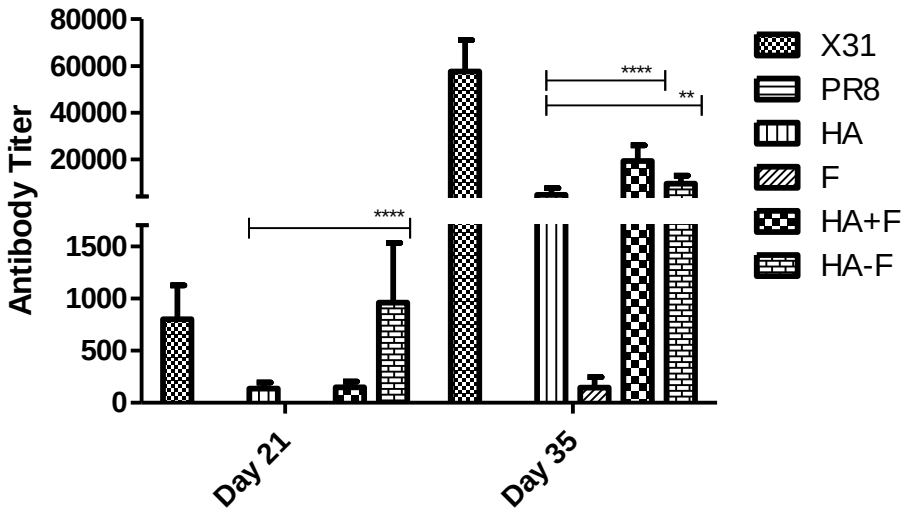


Figure 3.1: Western blot detection of recombinant HA, F and HA directly conjugated to F protein (HA-F). (A) Hyperimmune serum generated in mice to X31 was used to detect recombinant HA protein; (B) Native F protein purified from RSV A2 infected Vero E6 cells was detected with MAb 131-2A.

A


HA Antibody Titers
Antibody Titer
80000
60000
40000
20000
1500
1000
500
0
****
****
**
Day 21
Day 35
X31
PR8
HA
F
HA+F
HA-F

B

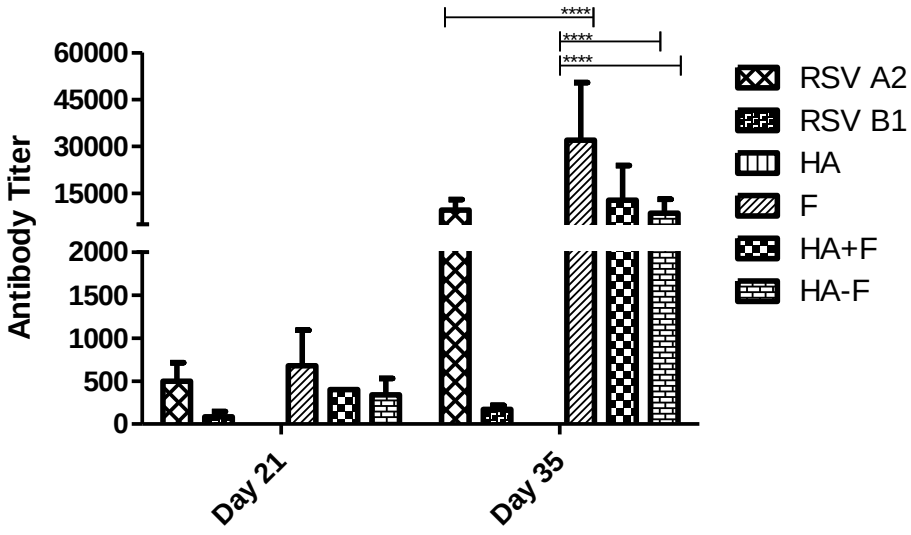

Anti-F antibody titers
Antibody Titer
60000
45000
30000
15000
2000
1500
1000
500
0
****
****
****
Day 21
Day 35
RSV A2
RSV B1
HA
F
HA+F
HA-F

Figure 3.2: Serum anti-HA and anti-F protein IgG antibody responses following vaccination. Mice were vaccinated then boosted at day 28 post-vaccination. Serum was collected on days 21 and 35 post-vaccination to determine antibody titers. (A) Serum collected from mice vaccinated with PBS, HA only, HA+F, HA-F, X31, F only, or PR8 was analyzed for X31 HA-specific IgG via ELISA. (B) Serum collected from mice vaccinated with PBS, HA, F, HA+F, HA-F, RSV A2, or RSV B1 was analyzed for RSV A2-specific IgG antibodies via ELISA. Representative data from two independent experiments with 5 mice per group is shown. Two way ANOVA performed (******p<0.0005 and, **p<0.01**).

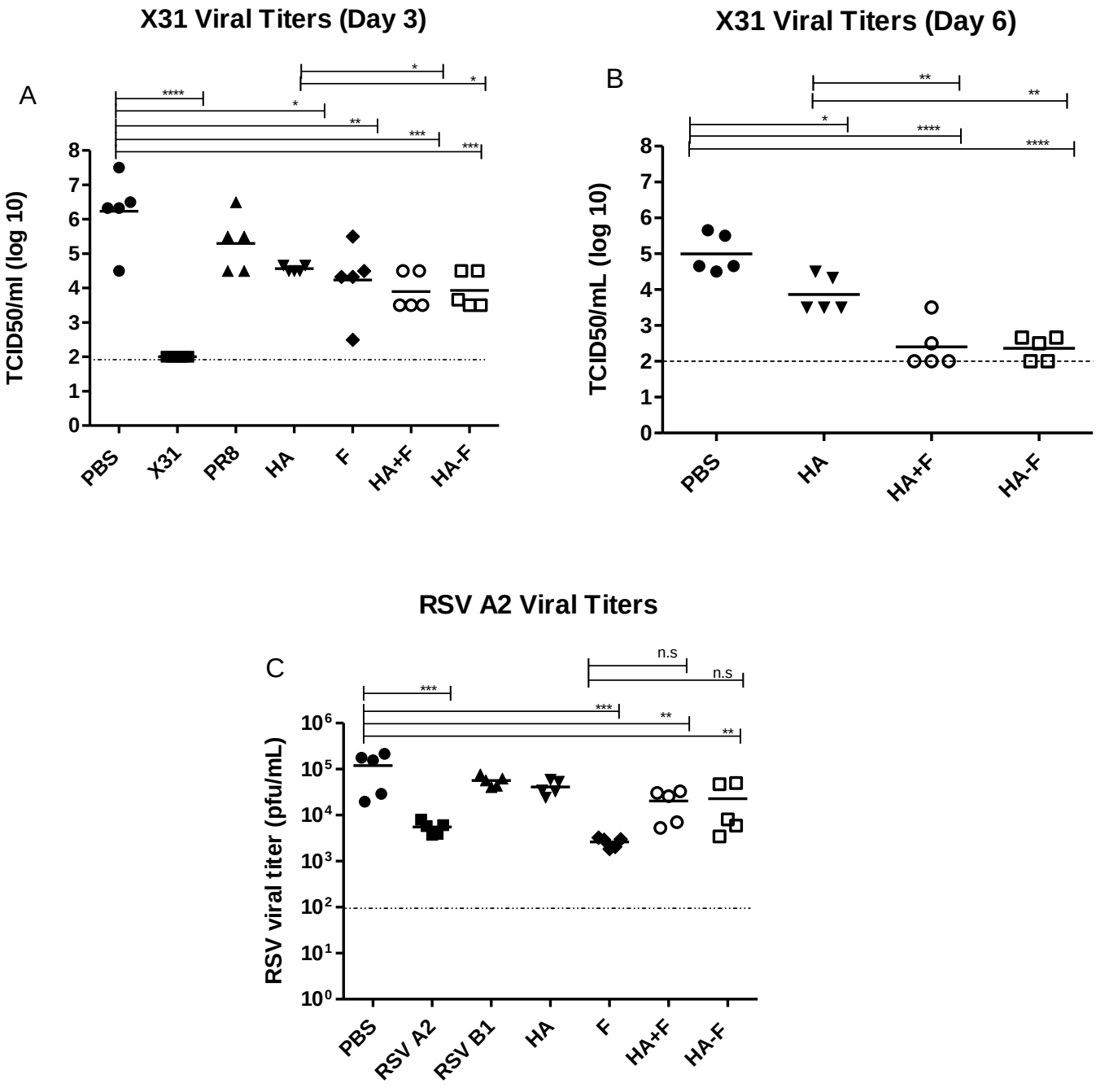
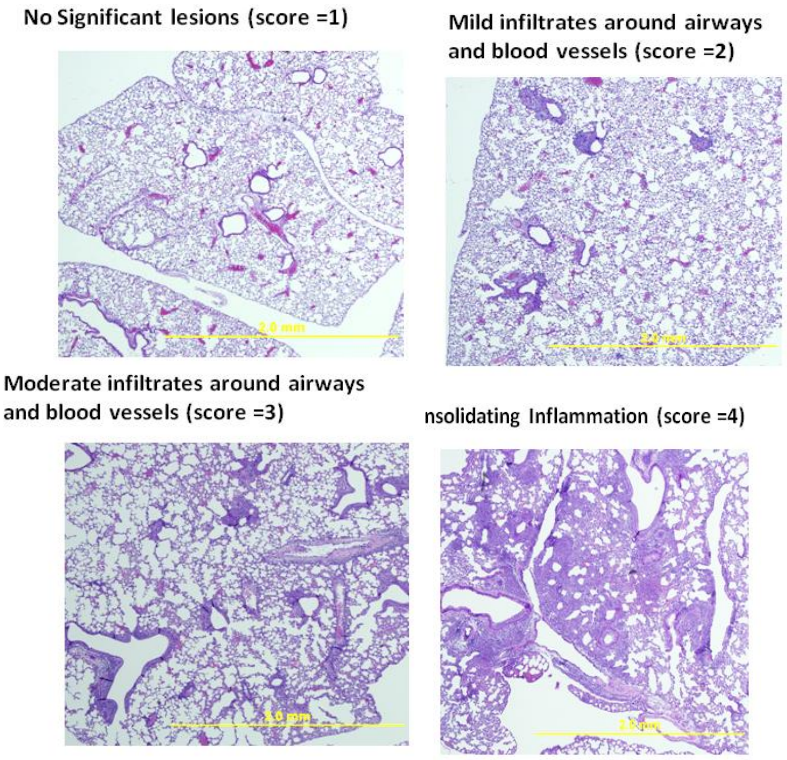
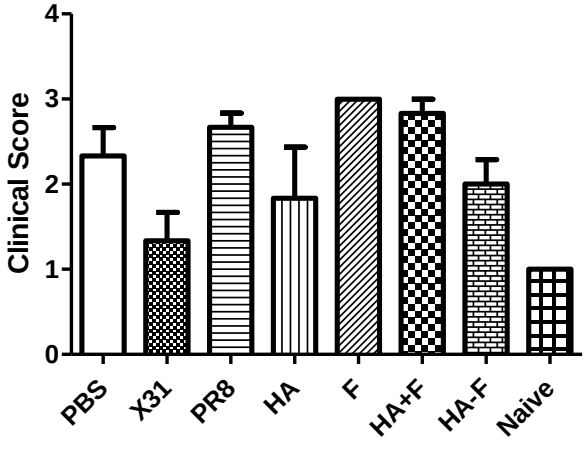

X31 Viral Titers (Day 3)
A
TCID50/ml (log 10)
PBS
X31
PR8
HA
F
HA+F
HA-F
X31 Viral Titers (Day 6)
B
TCID50/mL (log 10)
PBS
HA
HA+F
HA-F
RSV A2 Viral Titers
C
RSV viral titer (pfu/mL)
n.s
PBS
RSV A2
RSV B1
HA
F
HA+F
HA-F

Figure 3.3: Lung viral titers in vaccinated mice challenged with X31 or RSV. Mice vaccinated with PBS, HA, HA+F, HA-F, X31, F, PR8, RSV A2, or RSV B1 were homologous challenged with X31 or RSV A2 to determine vaccine efficacy. Mice vaccinated with PBS, HA only, HA+F, HA-F, X31, F, or PR8 was challenged with X31 and lung viral titers determined as $TCID_{50}$ at (A) three and (B) six days post infection. (C) Mice vaccinated as in (A) were challenged RSV A2, and viral titers determined via plaque assay. Representative data from two independent experiments with 5 mice per group is shown. Dashed line represent limit of detection. One way ANOVA statistical test performed in GraphPad Prism version 5. (******p<0.0001, ***p0.005, p<0.01, *p<0.05**).

A


No Significant lesions (score =1)
Mild infiltrates around airways and blood vessels (score =2)
Moderate infiltrates around airways and blood vessels (score =3)
nsolidating Inflammation (score =4)

B


X31
Clinical Score
0
1
2
3
4
PBS
X31
PR8
HA
F
HA+F
HA-F
Naive

C

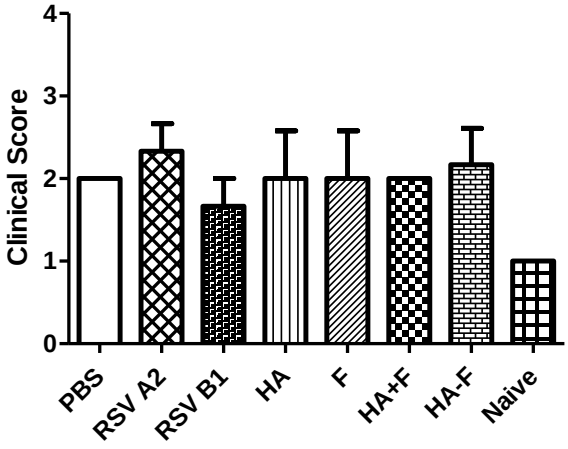

RSV A2
Clinical Score
0
1
2
3
4
PBS
RSV A2
RSV B1
HA
F
HA+F
HA-F
Naive

Figure 3.4: Histopathology in vaccinated and challenged mice. (A) The lungs from vaccinated and challenged mice were scored for histopathology using a 1 -4 scoring system where 1 = minimal pathology and 4 = maximum pathology. (B) Mice vaccinated with PBS, HA, HA+F, HA-F, X31, F, or PR8 was challenged X31, or (C) Mice vaccinated with PBS, RSV A2, RSV B1, HA, F, HA+F, or HA-F were challenged with RSV A2. Three or five days post challenge, respectively; the lungs were sectioned, stained, and scored. Representative data from two independent experiments with 3 mice per group are shown. Student's t test and one way ANOVA statistical test performed in GraphPad Prism version 5.

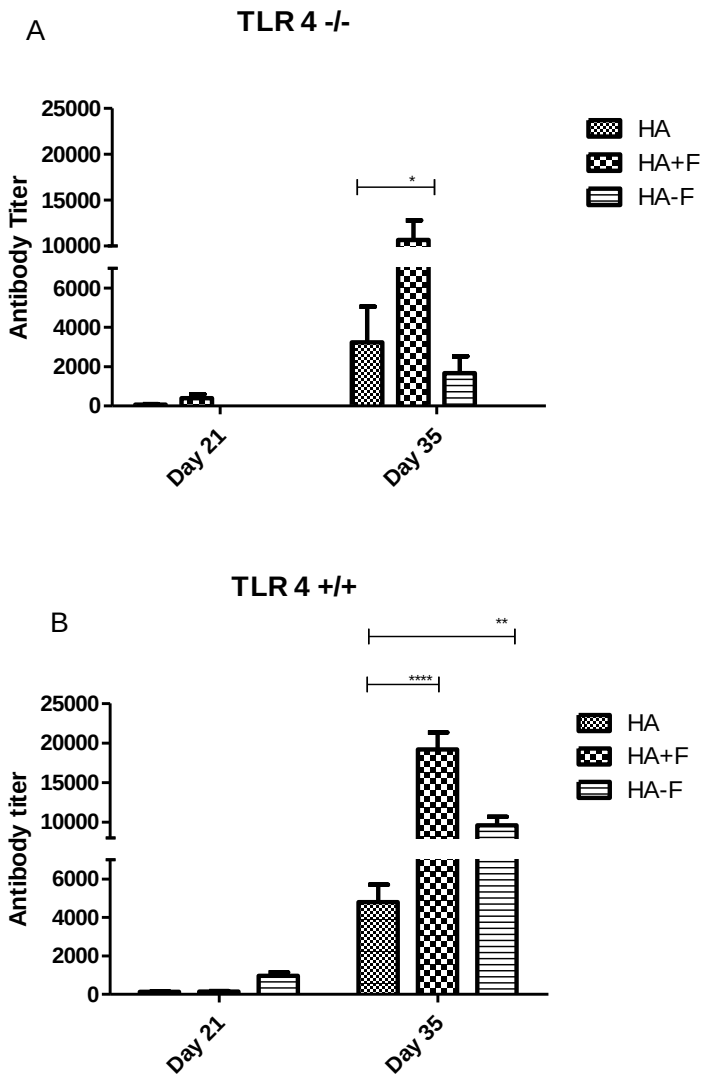


Figure 3.5: Anti-HA antibody titers from TLR4$^{-/-}$ and TLR4$^{+/+}$ mice. C57BL/10ScNCr (TLR4 knockout) and C57BL/6 mice were vaccinated on days 0 and 28 with HA, HA+F, or HA-F. Serum was collected on day 21 post-vaccination and day 7 post-boost. (A) Serum from TLR4$^{-/-}$ mice was evaluated for anti-HA antibodies by ELISA using X31 HA protein, and (B) and serum from TLR4$^{+/+}$ was similarly evaluated. The graph is from representative data from two independent experiments with three mice per group. Two-way ANOVA was performed in GraphPad Prism version 5; (******p<0.001, **p<0.01, *p<0.05**).

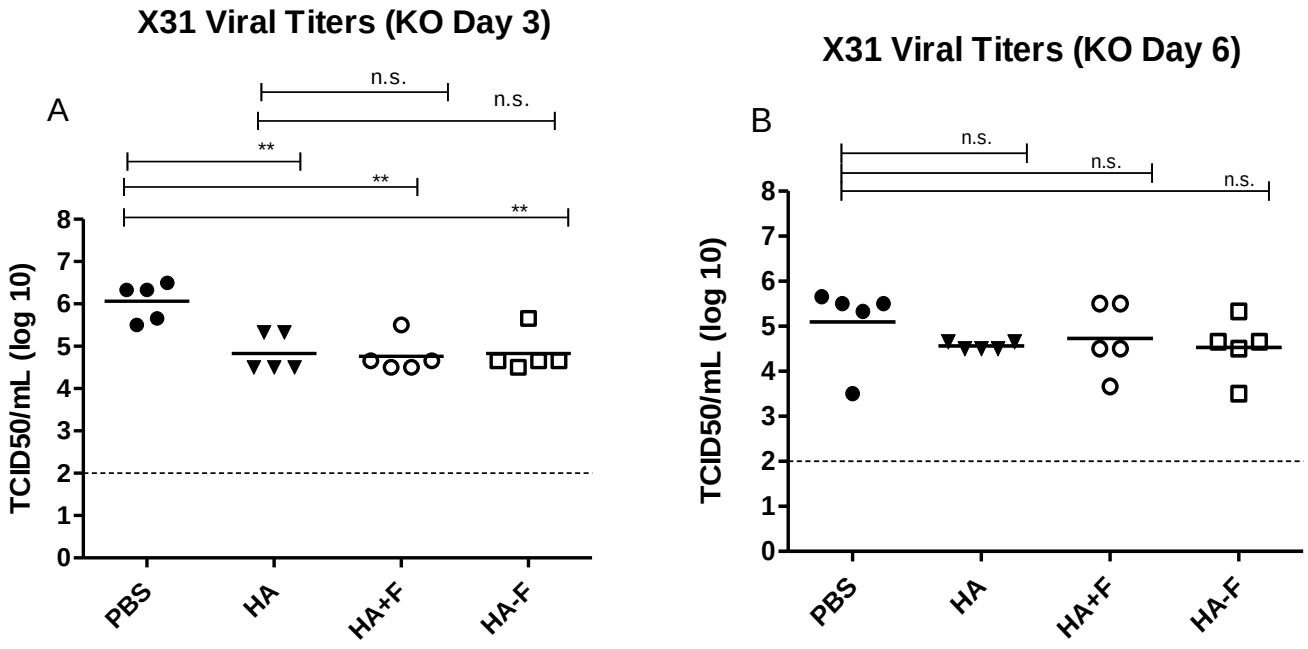


Figure 3.6: Influenza virus lung titers in TLR4-/- mice. C57BLScNCr (TLR4 -/-) mice were vaccinated on days 0 and 28 with PBS, HA, HA+F, or HA-F and challenged with X31. (A) Three and (B) 6 days post-challenge lung viral titers were determined in TLR4-/- mice by $TCID_{50}$. Dashed line represent limit of detection. Representative data from two independent experiments with 5 mice per group is shown. One-way ANOVA performed in GraphPad Prism version 5 (****p<0.01**).

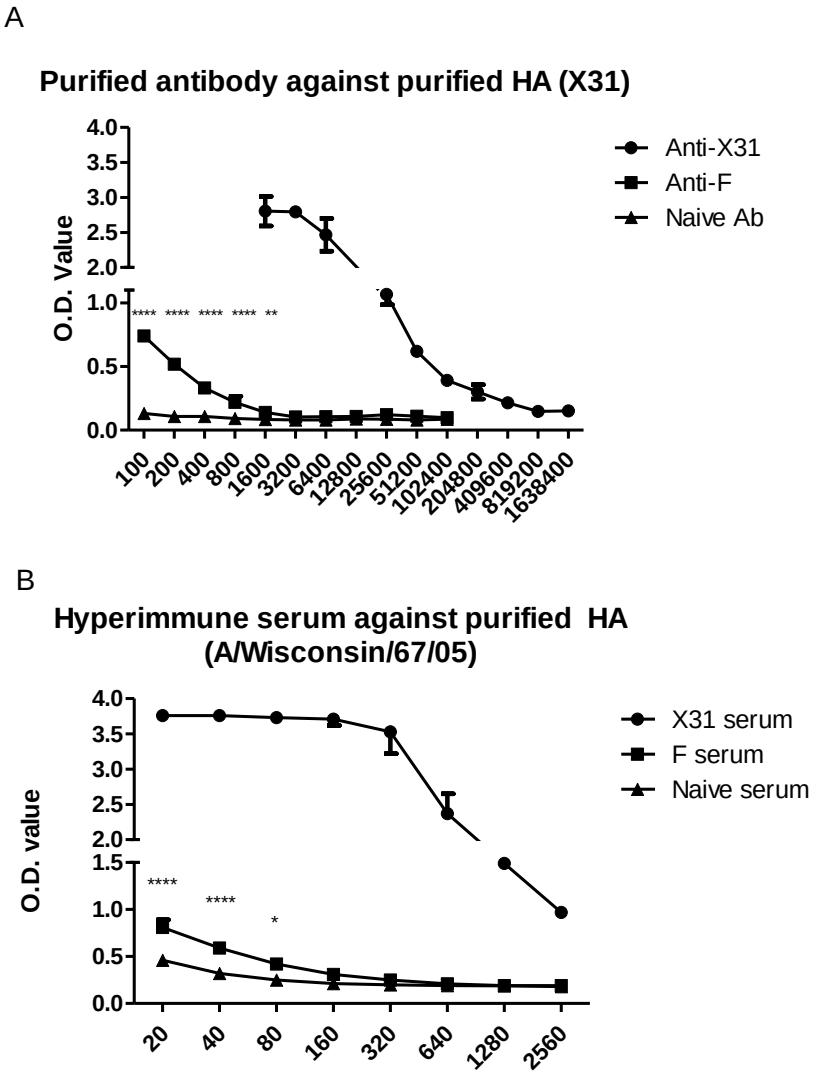


Figure 3.7: Reactivity to influenza HA protein. (A) Purified antibody generated to X31 virus and RSV F protein was tested for the ability to bind to purified HA protein from X31 (B) Recombinant HA from A/Wisconsin/67/05 was used determine cross reactivity of anti-F serum. (*) indicates significance between naïve and anti-F serum. Results are from representative data using three serum specimens per group. Two way ANOVA performed in GraphPad Prism version 5;(******p<0.0005, **p<0.01, *p<0.05).**

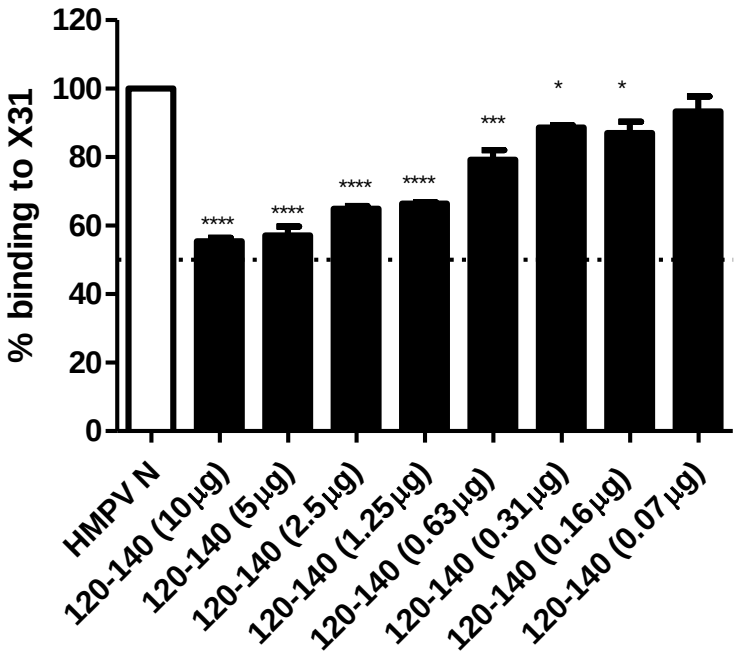


Figure 3.8: RSV F peptide 120-140 reduces binding of anti-F antibodies to influenza X31 virus. Anti-F serum was incubated with peptide 120-140 at decreasing concentrations to block binding of anti-F serum to influenza X31 virus. Results are representative of 3 independent experiments. One way ANOVA performed in GraphPad Prism version 5; (******p<0.0005, ***p<0.005, *p<0.05**).

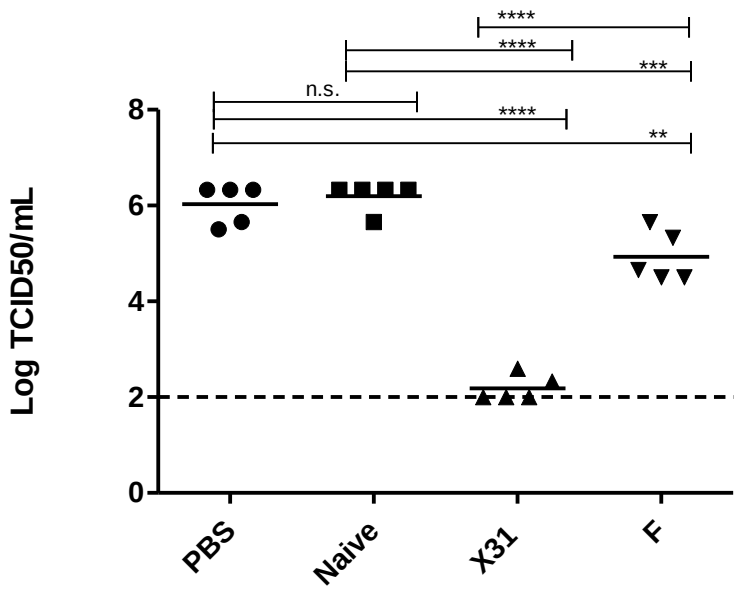


Figure 3.9: Passive antibody transfer and challenge with X31. Mice were injected with 250μg of purified antibody from X31 infected mice, RSV F protein vaccinated mice, and naïve antibody 48h and 24h prior to infection with X31. Three days post infection mouse lungs were harvested and lung virus titer determined via $TCID_{50}$. Dashed line represent limit of detection. Results are representative of 3 independent experiments. One way ANOVA performed in GraphPad Prism version 5; (******p<0.0005, ***p<0.005, *p<0.05**).

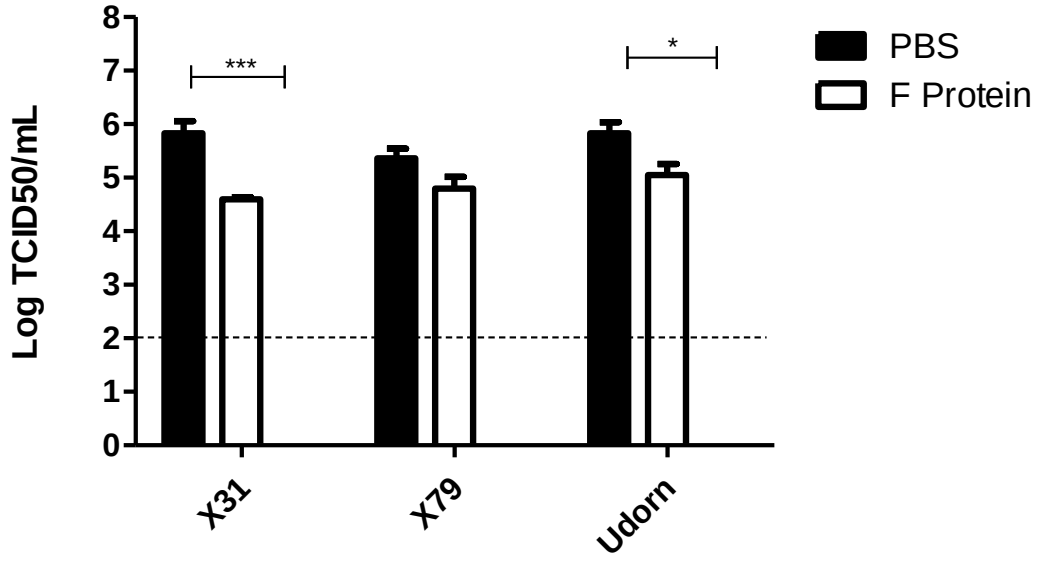


Figure 3.10: Lung viral titers in RSV F protein vaccinated mice challenged with H3N2 influenza viruses. Mice were vaccinated with F protein only on day 0 and boosted on day 28 post-vaccination. Thirty-five days post-vaccination mice were challenged with X31, X79 or Udorn. Three days post-challenge, mice lung viral titers were determined by $TCID_{50}$. Dashed line represent limit of detection. Representative data from two independent experiments with 5 mice per group is shown. Student's t test performed in GraphPad Prism version 5 (*****p<0.005, *p<0.05**).

Table 3.1: Neutralizing antibody titers day 35 post vaccination

Neutralization Titers : Day 35 post-vaccination			% Plaque Reduction Day 35 post-vaccination
Vaccination Groups	X31	PR8	RSV A2
PBS	>10	>10	0
HA	1024 ± 324	>10	3
HA+F	3093 ± 1587*	>10	25*
HA-F	3328 ± 1203 *	>10	15*
X31 Virus	3712 ± 627 *	>10	--
F	844 ± 420	>10	29*
PR8 Virus	832 ± 301	992 ± 327	--
RSV A2	--	--	31*
RSV B1	--	--	5

* Significantly different ($p<0.05$) compared to HA; one-way ANOVA test.

Table 3.2: Neutralizing antibody titer in TLR4$^{-/-}$ mice

Neutralizing Antibody Titers from TLR4$^{-/-}$ mice: Day 35 post-vaccination	
PBS	<10
HA	204 ± 15
HA+F	169 ± 16
HA-F	177 ± 31

Serum collected from TLR4$^{-/-}$ mice 35 days post-vaccination

Table 3.3: RSV F peptide from the stalk region of the RSV F protein

Peptides from the Stalk region of the RSV F protein	
1-20	MELPILKANAITTILAAVTF
10-30	ITTILAAVTFCFASSQNITE
20-40	CFASSQNITEEFYQSTCSAV
30-50	EFYQSTCSAVSKGYLSALRT
40-60	SKGYLSALRTGWYTSVITIE
50-70	GWYTSVITIELSNIKENKCN
60-80	LSNIKENKCNGTDAKVKLMK
70-90	GTDAKVKLMKQELDKYKNAV
80-100	QELDKYKNAVTELQLLMQST
90-110	TELQLLMQSTPAANNRARRE
100-120	PAANNRARRELPRFMNYTLN
110-130	LPRFMNYTLNNTKKTNVTLS
120-140	NTKKTNVTLSKKRKRRFLGF
130-150	KKRKRRFLGFLLGVGSAIAS
140-160	LLGVGSAIASGIAVSKVLHL

Peptides were generated from the F protein sequence and used to block binding of anti-F serum to X31 virus

Table 3.4: Neutralizing antibody titers of influenza H3N2 and H1N1 viruses

Neutralization of H3N2 and H1N1 Influenza Viruses		
Neutralizing Antibody Titers		
	Anti-F hyperimmune sera (1:1600)	Anti-F hyperimmune sera (1:3200)
A/Aichi/2/68 (H3N2)	480±277	1280±308
A/NY/55/04 (H3N2)	1280±0	2560±0
A/Wisconsin/67/05 (H3N2)	1280±0	1706±738
A/PR/8 (H1N1)	<10	<10
A/Illinois /02860/09 (H1N1)	<10	<10
A/California/04/09 (H1N1)	<10	<10

Serum generated against the RSV F protein was used to neutralize H3N2 and H1H1 influenza viruses

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CHAPTER 4

RESPIRATORY SYNCYTIAL VIRUS (RSV) FUSION PROTEIN ACUTATES THE IMMUNE RESPONSE TO INFLEUNZA VIRUS

Tiffany TM, Jorquera PA, Jones LP, and Tripp RA. To be submitted to the Journal of Virology

Abstract

Respiratory syncytial virus (RSV) fusion (F) protein induces strong cell-mediated and humoral immune responses following vaccination or natural infection. Purified F protein (PFP) vaccines have been evaluated in clinical trials and are immunogenic and were well-tolerated. RSV F protein has been shown to be recognized by the shared components of MyD88 and Toll-like receptor (TLR4), a feature that modulates the immune response. In this study RSV F protein was used to actuate the immune response to influenza virus as a novel strategy to facilitate the immune response to influenza virus vaccination. The results show that RSV F protein priming boosts both innate and adaptive immune response to influenza virus infection.

Introduction

Respiratory syncytial virus (RSV) is a ubiquitous virus affecting the young and old and causing significant morbidity and mortality in immune compromised individuals, young infants and the elderly [1-4]. Importantly, RSV is a leading cause of hospitalizations of infants and children under the age of two [2,5]. RSV, a member of the *Paramyxoviridae* family, is a single-stranded negative-sense RSV virus with a non-segmented genome. The genome encodes 11 different proteins. Currently there is no vaccine available for the prevention of RSV. This is related to our incomplete understanding of the mechanisms of immunity and disease, and because of substantial vaccine failure, in particular a formalin-inactivated RSV vaccine trial in children. In this study, children receiving the inactivated vaccine developed vaccine enhanced disease upon natural RSV infection resulting in the death of two children [6-9]. The current RSV vaccine strategies still focus on generating immunity to the F and attachment (G) surface glycoproteins of RSV, as these proteins can induce neutralizing antibodies [10-14]. Subunit vaccines such as purified F protein vaccine-1 and -2 (PFP-1 and PFP-2) have been tested in clinical trials; however, these vaccines were not approved for use due to a lack of reduction in incidence of lower respiratory tract infection (LRI) in study cohorts [15,16]. Additionally a third PFP vaccine (PFP-3) was tested in phase II clinical trials, however the vaccine failed to lower the incidence of RSV infection after vaccination in children with cystic fibrosis [17,18]. It is likely that vaccines targeting both the F and G proteins can be used to provide more robust protection from disease by generating neutralizing antibodies and antibodies that inhibit the immune modulatory activities of the G protein [19-23].

Influenza virus is a major cause of worldwide morbidity and mortality despite the availability of an effective vaccine. Millions of individuals are infected annually with more than

250,000 of those cases resulting in death in the United Sates [24]. Influenza virus, a member of the *Orthomyxoviridae* family, is a single-stranded negative-sense segmented RNA virus. The eight RNA segments encode between 10 to11 proteins. During initial infection, both the innate and adaptive responses are critical for recognition and control of infection [25-27]. Innate cell types such as macrophages, DC, and NK cells recognize, kill and phagocytize viral particles and infected cells while secreting cytokines and chemokines to attract leukocytes to the site of infection [25,28,29]. This is important as CD4+ and CD8+ T cells help to direct and are critical in facilitating the immune response to infection while B cells and antibody bind to virus and stop spread [26,27,30]. Specifically, CD4+ T cell are responsible for initiating a Th1-type profile during influenza infection characterized by the production of Th1 cytokines IFNγ, TNFα, and IL-12 [31]. These cytokines activate T cells and mediate proliferation as well as recruitment of other effector cell types which aid in virus elimination [30-32]. CD8+T cells are the primary cell type responsible for eliminating influenza infected cells via Fas/FasL and perforin/granzyme mediated mechanisms [33]. For example, in C57Bl/6 mice ($H2^b$), the CD8+ T cells recognize the immunodominant influenza virus epitopes NP, PA, and PB1 in the presence of MHC class I [34-36]. CD4+ T cells also provide co-stimulation for B cells enabling these cells to undergo somatic hypermutation and class switching for the development of specific high affinity antibodies [37,38]. Together, the innate and adaptive immune responses are efficient at eliminating influenza viral infection.

The RSV F protein is one of the major surface glycoprotein responsible for fusing the viral membrane with the host cell membrane [39,40]. Antibodies directed against the F protein are able to effectively neutralize viral entry and decrease viral spread [12,41]. Palivizumab (Synagis), a monoclonal antibody directed against the F protein is prophylactically administered

in the United States to high-risk infants. This treatment contributes to a reduction in RSV-induced hospitalizations [42-46]. In addition, the F protein has also been shown to trigger Toll-like receptor-4 (TLR4) and CD14 through the shared activities of MyD88, activating the innate immune response by driving NF-κB-mediated cytokine expression [47]. Countering this response, the F protein has also been shown to modulate the host response by increasing the expression of suppressor of cytokine signaling proteins-1 and -3 (SOCS1 and SOCS3), and decreasing the expression of type 1 interferons [48]. Given the evidence for molecular activation of the TLR4 pathway [47,49], immune sensitization using purified RSV F protein was explored as a means to augment the host response to influenza infection.

Methods and Materials

Mice, Cell Lines and Virus

Six-to-eight week old C57Bl/6 mice were purchased from National Cancer Institute (NCI) (Raleigh NC) and housed at the Animal Health and Research Center at the University of Georgia. The mice were fed and experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC).

A/HK/x31 [H3N2] (X31) was propagated in eleven day old embryonated hen eggs and allowed to incubate for three days at 35°C [50]. The allantoic fluid was harvested 48 or 72 h post-inoculation and the 50% tissue culture infectious dose (TCID50/mL), as well as the plaque forming units (PFU/mL), was determined. RSV A2 virus was propagated in Vero E6 cells (ATCC Manassas, VA) cultured in DMEM containing 2% FBS. Seventy two hours post-infection the cell pellets were collected and disrupted by sonication 3 times for 5 seconds to release cell-bound virus. The PFU/mL was determined using methods previously described

[6,51]. The RSV F protein was purified from Vero E6 cells infected with RSV A2 as previously described [48,51].

Protein purification

RSV F protein was purified from RSV A2 infected Vero E6 cells (ATCC Manassas, VA) F protein purification used in all experiments was extracted as previously described [48,51]. The purified RSV F protein was endotoxin tested via limulus amebocyte lysate kit (Lonza, MD) and stored at -80°C until needed.

Western Blot

The purified F protein was run on a Criterion gel (Bio-Rad Hercules, CA) at 125V for 1h and 30 minutes and transferred to a PVDF membrane and blocked for 1h at RT. The membrane was removed from the transfer apparatus and blocked with 5% milk in Tris-buffered saline-tween (TBS-tween) for 1h prior to being probed with 131-2A at 1:1000 dilutions for 1h. The blot was washed 3 times with TBS 0.5% Tween. An anti-mouse AP conjugated antibody was used as the secondary antibody (Thermo Scientific, Rockford IL), and membranes were developed with ECF (GE Healthcare, Pittsburgh PA).

Immune actuation and virus infection

Six-to-eight week old C57Bl/6 mice were intramuscularly (i.m.) immunized with 30μg of purified RSV F protein or administered carrier, i.e. PBS. Seven days later, mice were i.n. infected with $5x10^5$ PFU/0.05mL X31. Subsequently, mice were sacrificed at 2, 4, 6, 8, and 10 days post-infection. Bronchoalveolar lavage (BAL), lungs, and/or spleens were harvested. To

determine if F protein actuation modified lung viral titers, lungs were collected without manipulation and titer determined as previously described [52].

Influenza TCID50

MDCK cells were cultured at 4 x 10^4 cells/well in a 96-well flat-bottom plate (Costar, Corning NY) overnight. The cells were washed twice with PBS and infected with lung homogenate supernatant as previously described [52].

IFN-γ and IL-4 ELISPOT

IFN-γ and IL-4 ELISPOT assays were performed using nitrocellulose-lined 96-well microplates (Millipore, Billerica, MA) using a mouse IFN-γ and IL-4 ELISPOT kit (R&D Systems) according to the manufacturer's instructions with minor modifications. Briefly, plates were coated overnight with antibody to mouse IFN-γ or IL-4 and washed three times. Spleen cells were stimulated for 48h with either 5 μg/ml of purified RSV A2 F protein, HA 211-225 (YVQASGRVTVSTRRS), HA 441-455 (AELLVALENQHTIDL) or eGFP 200-208 (HYLSTQSAL) for 48 h at 37°C and 5% CO2. Cells were then removed and plates were developed with anti-mouse IFNγ or IL-4 IgG (biotinylated) and streptavidin-alkaline phosphatase. RSV and influenza-specific ELISPOT numbers were determined from triplicate wells/cell population by subtracting the mean number of ELISPOTs from wells stimulated with GFP

Flow cytometery

At days 4 and 10 post-X31 challenge, individual BAL or lung specimens were harvested and cell suspensions resuspended in PBS containing 1% BSA. For MHC tetramer analyses, cells were stained with PE-Cy7-conjugated anti-CD4, PerCP-Cy5.5-conjugated anti-CD8α an optimized amount of PE-conjugated MHC class I H-2K^b tetramer complexes bearing PB1 (701-711), PE-conjugated MHC class I H-2D^b tetramer complexes bearing NP (366-374), or APC-conjugated MHC class I H-2D^b tetramer complexes bearing PA(224-233) (NIH Tetramer Core Facility Emory, Atlanta, GA). To determine NK cells in the BAL, cell suspensions were stained with FITC-conjugated anti-CD49b (DX5) and purified anti-CD16/CD32 (BD Biosciences). The distribution of cell surface markers was analyzed on a BD LSRII flow cytometer using FACSDiva software (BD Biosciences) from 100,000 events.

Luminex

MILLIPLEX mouse cytokine/chemokine Magnetic Bead Panel kits, were purchased from Millipore (Billerica, MA) and used to detect IFNγ, TNFα, RANTES, eotaxin, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-13, IL-15, and IL-17 according to manufactures protocol. Samples were run on Luminex 200 System (Austin, TX). Mouse chemokine 6plex FlowCytomix multiplex kit was also used for the detection of GM-CSF, MIP-1α, MIP-1β, MCP-1, MCP-3, and RANTES according to manufactures protocol (eBiosciences San Diego, CA).

Results

To determine if immune potentiation occurred following RSV F protein treatment, the immune response to influenza virus infection was evaluated. Specifically, the cell-mediated

immune response in the lung was determined at day 10 post-infection which corresponds to the peak of the influenza T cell response in the C57Bl/6 (B6) mouse strain [36]. At day 10 post-X31 infection, PBS treated mice (PBS/X31) had similar numbers of total BAL cells compared to F protein treated and X31 challenged mice (F protein/X31; Fig. 1A). These two groups also had similar numbers of BAL CD8+ T cells recruited to the lung at day 10 pi (Fig. 1B). There was no substantial response to F protein or PBS treatment followed by PBS challenge in total BAL cells (Fig 1A) or in the total number of BAL CD8+ T cells (Fig. 1B). To determine if F protein actuated the influenza-specific CD8+ T cell response, BAL cells were stained with NP, PA, and PB1 positive tetramers (Fig. 1C and 1D). There was no difference in the number or percent of NP, PA, or PB1 influenza-specific CD8+ T cells between F proteins treated or PBS-treated mice following X31 challenge (Fig. 1C and 1D). As expected, there was also no difference between mice treated with F protein and challenged with PBS or for mice PBS-treated who received a PBS challenged.

F protein treatment did not affect total BAL cell numbers or percentages of CD8+ T cells 10 days post infection. The cytokine profile was evaluated to determine if F protein sensitization affected the effector function of T cells activated following infection with X31 (Fig. 2). At day 10 post-X31 infection, the number of IFNγ and IL-4 ELISPOTs was determined following in vitro restimulation of splenocytes with CD8+ T cell peptide $HA_{441-455}$, CD4+ T cell peptide, $HA_{211-225}$ [53], or RSV F protein. There was no significant ($p>0.05$) difference in IFNγ expression by splenocytes from unsensitized and F protein sensitized mice challenged with X31 and restimulated with either F protein, $HA_{441-455,}$ or $HA_{211-225}$ peptide (Fig. 2A). In contrast, there were significant ($p<0.05$) differences in IL-4 expression by T cells from PBS-treated and F protein sensitized mice challenged with X31 and restimulated with either F protein, or $HA_{211-225}$

peptide, but no difference in CD8+ T cells stimulated with $HA_{441-455}$ peptides between PBS treated and F sensitized mice (Fig. 2B). These findings suggest that RSV F protein sensitization modifies the CD4+ Th2-type response to influenza infection 10 days post infection.

To determine if F protein sensitization modulated the bronchoalveolar (BAL) T cell response to X31 challenge at an earlier time post infection, BAL was collected and evaluated for total number of cell recruited to the lung at day 4 days pi (Fig. 3A), the total number of CD4 and CD8 T cells (Fig. 3B), and the total number of NP, PA or PB1 tetramer positive CD8 T cells (Fig. 3C) were evaluated. In addition, the total number of NK cells in the BAL was determined (Fig. 3D). F protein sensitized mice had significantly ($p<0.01$) more cellular recruitment to the lungs day 4 post-X31 infection compared to PBS treated mice (Fig. 3A). F sensitized mice also had significantly ($p<0.01$) higher numbers CD8+ T cells recruited to the BAL day 4 post-X31 infection compared to PBS treated mice (Fig. 3B). There is an indication that CD4+ T cells may also be sensitized by F protein as there were more CD4+ T cells found in the BAL of F immunized compared to PBS treated mice, however it was not significant ($p>0.05$) (Fig. 3B). NP, PA, and PB1 MHC class I tetramers were utilized to quantify the influenza virus-specific CD8+ T cells response (Fig. 3C). Although there were higher number of NP-, PA-, and PB1-specific CD8+ T cells in the BAL from F protein/X31 challenged mice, the numbers were not significantly ($p>0.05$) different. The number of NK cells, NK T cells, and NKp46+ NK cells in the BAL were also examined (Fig. 3D). NK T cells are classified as a subset of T cells that share common receptors with NK cells, such as NK1.1 and DX5 receptors [54]. NKp46+ NK cells contain a natural cytotoxicity receptor (NCR) [55] which has been implicated in clearance of influenza virus infection [56,57]. Interestingly, of the cell types recruited to the lung the F protein sensitized mice had significantly ($p<0.005$) higher numbers of NK cells present in the

BAL compared to PBS treated mice (Fig. 3D). There were similar number of NK T cells and NKp46+ NK cells recruited to the BAL ay day 4 post-X31 between PBS treated and F protein sensitized mice (Fig. 3D). Taken together, the data suggests that F protein sensitization modifies the immune response to enhance the innate and adaptive cellular response in the BAL following influenza virus challenge.

RSV F protein has been shown to modulate the host immune response following infection through the modification of suppressor of cytokine signaling 1 and 3 (SOCS1 and SOCS3s) proteins [48,58], thus it was important to determine if F protein sensitization modulated the chemokine or cytokine response following influenza infection. Chemokines RANTES, MCP-1, MCP-3, MIP-1α, MIP-1β, and GM-CSF were evaluated. BAL cells from F protein sensitized mice challenged with X31 had significantly ($p<0.005$) higher RANTES expression at 12h pi relative to PBS/X31 challenged mice (Fig. 4A). However, by 48h post infection, F protein/X31 mice had significantly ($p<0.01$) less expression of RANTES present in the BAL relative to PBS/X31 challenged mice (Fig. 4B). At 48h post infection there was significantly ($p<0.005$, $p<0.05$) higher expression of MIP-1α and MCP-1 in F protein/X31 challenged mice relative to PBS/X31 challenged mice, respectively. There was no significant difference in the expression of GM-CSF, MCP-3, or MIP-1β at any time point tested between the F protein sensitized and challenged groups relative to PBS treated and challenged mice (data not shown). Together, this data demonstrates that prior exposure to the RSV F protein modulates the chemokine response during influenza virus infection resulting in increased cellular trafficking to the lung (Fig. 3B and D).

The cytokine response was also evaluated between the immunized groups. Again, mice were immunized with either the F protein or treated with PBS followed by X31 challenge.

Several cytokines were evaluated including IFNγ, IFNλ, TNFα, IL-1α, IL-1β, IL-2, IL-6, IL-12p40, IL-4, IL-5, IL-10, IL-15, and IL-17 at 12, 24, and 48 hrs post infection. We found that there was no difference in the level of these cytokines between PBS/X31 challenged and F protein/X31 challenged mice at 12 and 24 hrs post infection (data not shown). However by 48 hrs post infection mice sensitized with the F protein and challenged with X31 had significantly higher levels of TNFα ($p<0.05$), IFNγ ($p<0.01$), IL-12p40 ($p<0.005$) and IL-1α ($p<0.05$) in the BAL when compared to PBS/X31 challenged mice (Fig. 5A-D). However PBS/X31 challenged mice had significantly ($p<0.005$) higher expression of IFNλ relative to F protein/X31 challenged mice indicating that the F protein may modulate type II and type III interferon response to a subsequent influenza virus infection (Fig. 5E). Taken together, this data indicates that prior sensitization to the RSV F results in an altered or modified cytokine response to influenza virus infection.

To determine if the increased immune response seen in F protein/X31 challenged mice correlated with a decrease in influenza X31 viral titer, mice were sensitized with the RSV F protein 7 days prior to infection. At days 2, 3, 4, 6, and 8 days post infection mice were sacrificed and lung viral titers determined. Mice sensitized with the RSV F protein had significantly lower X31 viral titers 3 and 4 days post infection ($p<0.005$ and $p<0.05$ respectively) (Fig. 6). The reduction in X31 viral titers at days 3 and 4 post infection correlates with the increased NK cell and CD8+ T cell trafficking to the lungs at 4 days post infection in F protein sensitized mice, indicating that F protein sensitization reduces influenza X31 viral burden.

Discussion

There is currently no approved vaccine for RSV; however there have been great strides in developing a safe and immunogenic vaccine. Initially, the FI-RSV developed in the 1960s was

associated with increase vaccine enhanced disease. It is believed that the formalin used to inactive the vaccine cause distortion of important immune epitopes, resulting in generation of antibodies that were non-neutralizing [59,60]. In the decades following the FI-RSV vaccine, there have been attempts to generate vaccines based on the surface glycoproteins F and G. These major surface glycoproteins, F and G, have been shown to modulate the immune response to a subsequent RSV infection through the up regulation of SOCS1 and 3 and the down regulation of type I IFN response [12,48,61]. An initial RSV infection occurring before 4 months of age biases the immune response to a TH2 phenotype upon subsequent RSV infection [62,63]. This bias towards a TH2 response to subsequent RSV infection results in increased immunopathology and airway hyperresponsiveness [64]. We hypothesize prior exposure to the RSV F protein would modulate the immune response to a subsequent viral infection as seen with prior infection with RSV or vaccination with the glycoproteins. Here the immune response to influenza X31 virus was evaluated at early and late time points after infection.

The immune response to influenza is well characterized in the literature [26,30,31,36,65-68]. Optimal clearance of virus is reliant on both the humoral and cell mediated arms of the immune response. Antibodies are responsible for binding and eliminating free floating viruses and limiting the spread and transmission of the virus. Antibodies can also bind to antigen present on the surface of infected cells and trigger elimination through antibody dependent cellular cytotoxicity (ADCC). The cell mediated arm is responsible for eliminating virally infected cells. Either arm can control infection in the absence of the other. Here we evaluated the ability of the RSV F protein to modulate the immune response to influenza X31 strain. Initially the immune response was evaluated at day 10, which is the height of the T cell response during influenza infection [36]. There was no difference in the overall total BAL cell count between

PBS/X31challenged mice when compare to F protein/X31 challenged mice. There was also no significant difference in the overall CD8+ T cell response between PBS/X31challenged mice and F protein/X31 challenged mice. More specifically, PBS/X31challenged mice and F protein/X31 challenged mice had similar numbers of influenza specific NP, PA, and PB1 CD8+T cells recruited to the lungs 10 days post infection indicating that the magnitude of the influenza specific CD8+ T cell response was similar between PBS/X31challenged mice and F protein/X31 challenged mice at later stages of influenza virus infection.

The phenotype of influenza specific and RSV F protein specific T cells were evaluated 10 days post influenza infection from splenocytes. There was no detectable difference in the expression of IFNγ from splenocytes between PBS/X31 challenged mice and F protein/X31 treated mice in response to stimulation with whole F protein, $HA_{441-455}$ or $HA_{211-225}$. The IL-4 expression pattern was modified between F protein sensitized and PBS treated mice following influenza X31 infection. F protein/X31 challenged mice had higher frequencies of IL-4-secreting T cells than PBS/X31 challenged mice following stimulation with the RSV F protein or $HA_{211-225}$ peptide, thereby promoting a TH2 phenotype. There was no difference in IL-4 expression between F protein/X31 challenged and PBS/X31 challenged mice following stimulation with the $HA_{441-455}$ peptide. Previous reports have shown that RSV glycoproteins can skew the immune response towards a TH2 type response which could exacerbate disease outcome [69]. It is important to note that the increased IL-4 expression was seen in T cells from the spleen 10 days post infection and not from the lungs. There was no detectable difference in the expression of IL-4 in BAL fluid between F protein sensitized mice and PBS treated mice earlier during influenza virus infection. Here report here that exposure to the F protein prior to

influenza X31 infection, promotes a more balanced TH1/TH2 response in stimulated splenocytes.

To further elucidate and characterize the immune response following F protein sensitization, BAL was collected 4 days post influenza infection. F protein/X31challenged mice had significantly more overall cellular recruitment to the lungs than PBS/X31 challenged mice, indicating that the RSV F protein modulates the immune response in a manner that causes more cellular recruitment to the lungs. Of the innate cell types recruited to the lungs, F protein/X31 challenged mice had significantly more NK cells recruited to the BAL when compared to PBS/X31 challenged mice. There was also an increased presence of NK T cells and NKp46+ NK cells in mice sensitized with the F protein, though not significant when compared to mice treated with PBS and challenged with X31. NK cells play a significant role in influenza virus elimination. The NKp46 receptor on activated NK cells can bind directly to influenza HA on infected cells and lyse these cells, thereby reducing the spread of infection [70]. The increased presence of NK cell can be correlated with increased chemokine production, specifically RNATES and MCP-1 (Fig. 4A and D), at 12 and 48hrs pi respectively. RANTES and MCP-1 are important in the recruitment of NK cells to the site in infection [71,72]. NK cells are also important in the elimination of infected cells through ADCC [73]. Earlier findings reported by our laboratories indicate that there are cross reactive antibodies between the RSV F protein and influenza X31 virus [74]. It is possible that NK cells are recognizing the Fc receptor of these cross reactive antibodies bound to influenza infected cells and eliminating these cells through ADCC.

RANTES and MCP-1 are also critical for the regulation and recruitment of CD8+T cell [75,76]. Here we report that CD8+T cell numbers were also increased during X31 infection with

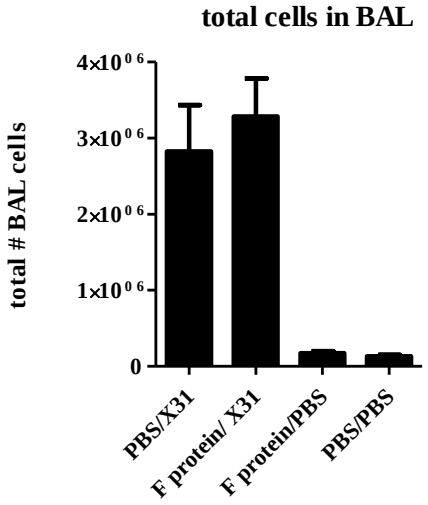
prior exposure to the RSV F protein. There was significantly more CD8+ T cell recruited to the BAL and increased, though not significant, numbers of CD4+ T cells recruited to the BAL 4 days post infection in F protein/X31 challenged mice relative to mice given treated with PBS and challenged with X31. Upon examination of the influenza specific CD8+ T cells response to the immunodominant peptides NP(366-374), PA(701-711), and PA(224-233), we found F protein/X31 challenged mice had increased levels of influenza specific CD8 T cells compared to PBS/X31 challenged mice, though not significant. An increased presence of influenza specific CD8+ T cells enhances the elimination of virally infected cells. Therefore this observation supports the conclusion that sensitization with the RSV F protein prior to influenza infection increases the influenza specific CD8+T cell response.

The increased presences of NK cells and CD8+T cells 4 days post infection in F protein sensitized mice led us to evaluate the viral burden following X31 challenge. Viral titers were determined at 2, 3, 4, 6, and 8 days post infection. Prior sensitization with the RSV F protein resulted in decreased viral titers at 3 and 4 days post infection. The decreased viral titers at earlier time points pi can be correlated back to the increased presence of NK cells and CD8+T cells present in the BAL. The data represented here demonstrates that prior sensitization to the RSV F protein potentiates the immune response to a subsequent influenza virus infection through the increased expression of chemokines RANTES and MCP-1, which resulted in increased trafficking of NK and CD8+T cells to the lungs. The increased presence of NK cells and CD8+T cells in the BAL fluid resulted in decreased viral burden through the elimination of virally infected cells. Taken together, RSV F protein sensitization enhances the immune response to a subsequent influenza virus infection.

Acknowledgements

This research was supported by funding from the National Institute of Health, R01-1021RR211294, and HHSN266200700006C, and the Georgia Research Alliance. We thank the NIH Tetramer Core Facility at Emory University for graciously providing influenza specific tetramers for the completion of this study.

A


total cells in BAL
total # BAL cells
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2x10^06
1x10^06
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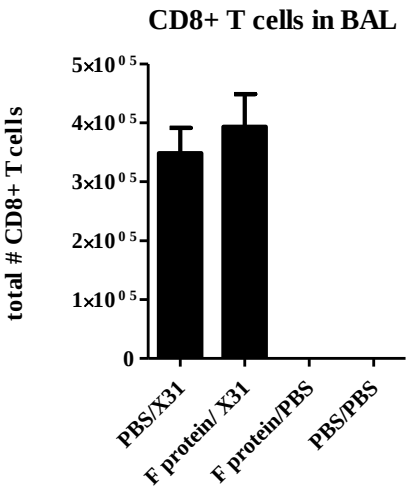
PBS/X31
F protein/ X31
F protein/PBS
PBS/PBS

B


CD8+ T cells in BAL
total # CD8+ T cells
5x10^05
4x10^05
3x10^05
2x10^05
1x10^05
0
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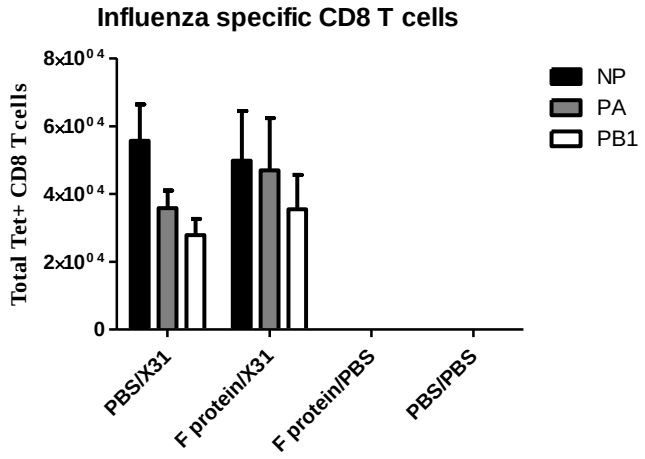
F protein/ X31
F protein/PBS
PBS/PBS

C


Influenza specific CD8 T cells
Total Tet+ CD8 T cells
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6x10^04
4x10^04
2x10^04
0
PBS/X31
F protein/X31
F protein/PBS
PBS/PBS
NP
PA
PB1

D

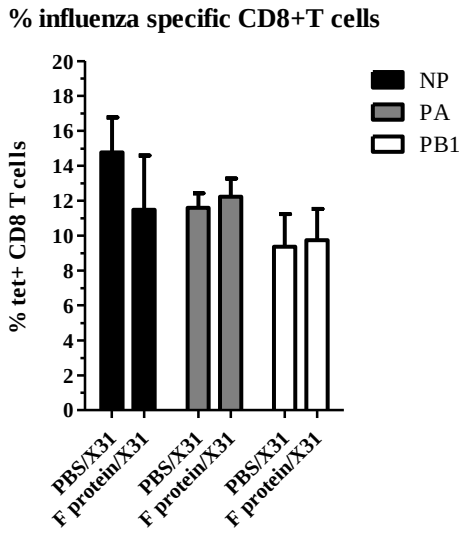

% influenza specific CD8+T cells
% tet+ CD8 T cells
20
18
16
14
12
10
8
6
4
2
0
PBS/X31
F protein/X31
PBS/X31
F protein/X31
PBS/X31
F protein/X31
NP
PA
PB1

Figure 4.1: Anti-influenza immune response 10 days post infection. Mice were immunized with 30μg of F protein and 7 days later challenged with influenza X31. BAL was collected 10 days post infection and cell populations determined via flow cytometry. (A) BAL collected 10 days post infection was counted for total cell count. (B) BAL collected 10 days post infection was analyzed for the total CD8+T cell population. (C) The total CD8+T cell population was probed for influenza specific CD8+T cells. (D) Percentage of influenza specific CD8+T cell. Results are representative of 3 independent experiments. One way ANOVA performed in GraphPad Prism version 5.

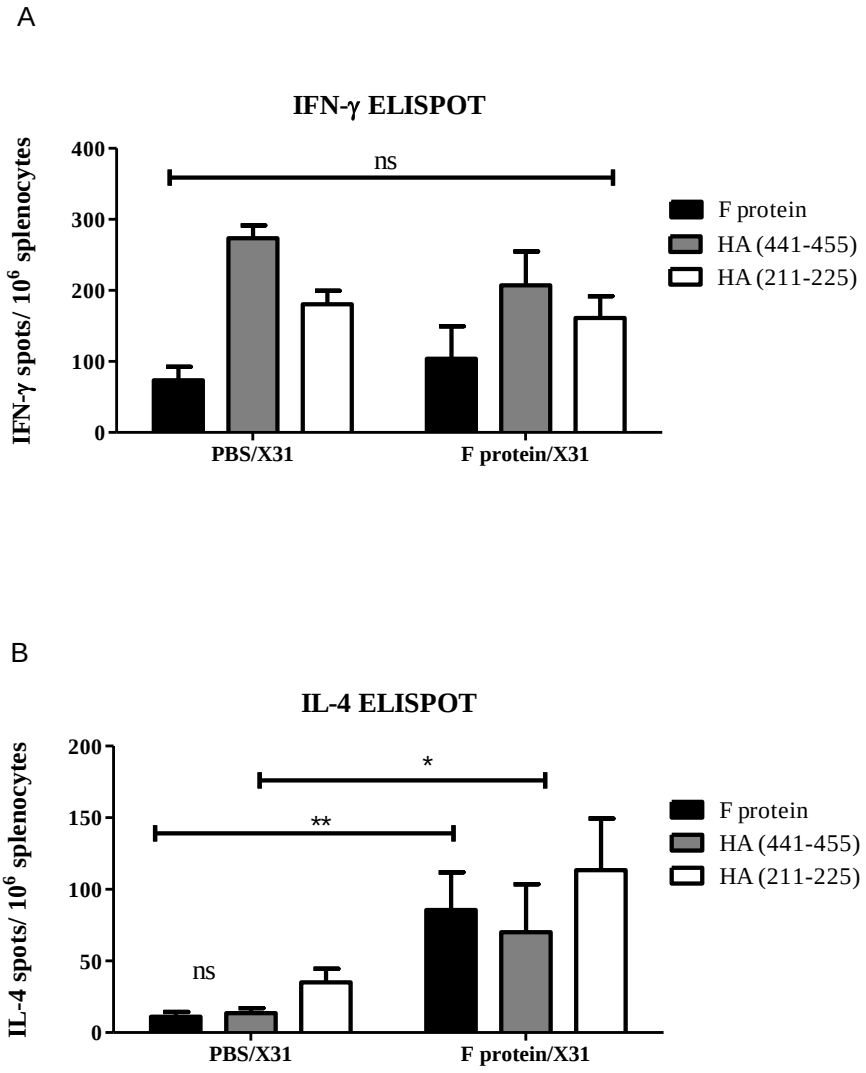


Figure 4.2: Phenotype of influenza specific T cells 10 days post infection. Mice were immunized with 30 μg of RSV F protein 7 days prior to infection with influenza X31 virus. (A) Splenocytes from influenza infected mice were harvested 10 days post infection and IFNγ spots determined. (B) Splenocytes from influenza infected mice were harvested 10 days post infection and IL-4 spots determined. Results are representative of 3 independent experiments. One way ANOVA performed in GraphPad Prism version 5; (**$p<0.01$, *$p<0.05$).

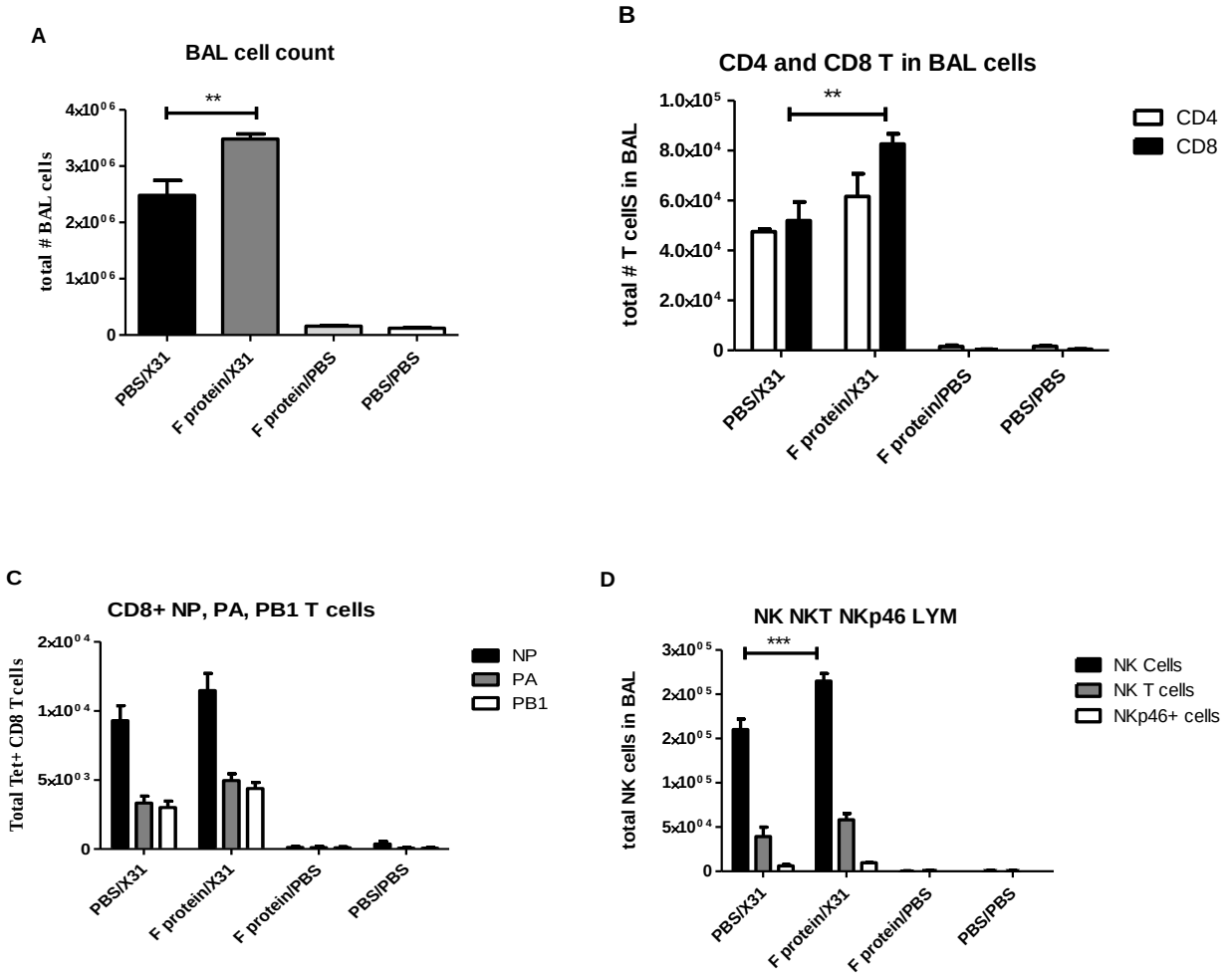


Figure 4.3: Anti-influenza immune response 4 days post infection. Mice were immunized with 30μg of F protein and 7 days later challenged with influenza X31. BAL was collected 4 days post infection and cell populations determine via flow cytometry. (A) BAL collected 4 days post infection was counted for total cell count. (B) The total BAL population was analyzed for the total CD8+T and CD4+ T cell populations. (C) The total CD8+T cell population was probed for influenza specific CD8+T cells. (D) BAL collected 4 days post infection was analyzed for NK cell population subsets. Results are representative of 3 independent experiments. One way ANOVA performed in GraphPad Prism version 5; (***$p<0.005$, **$p<0.01$).

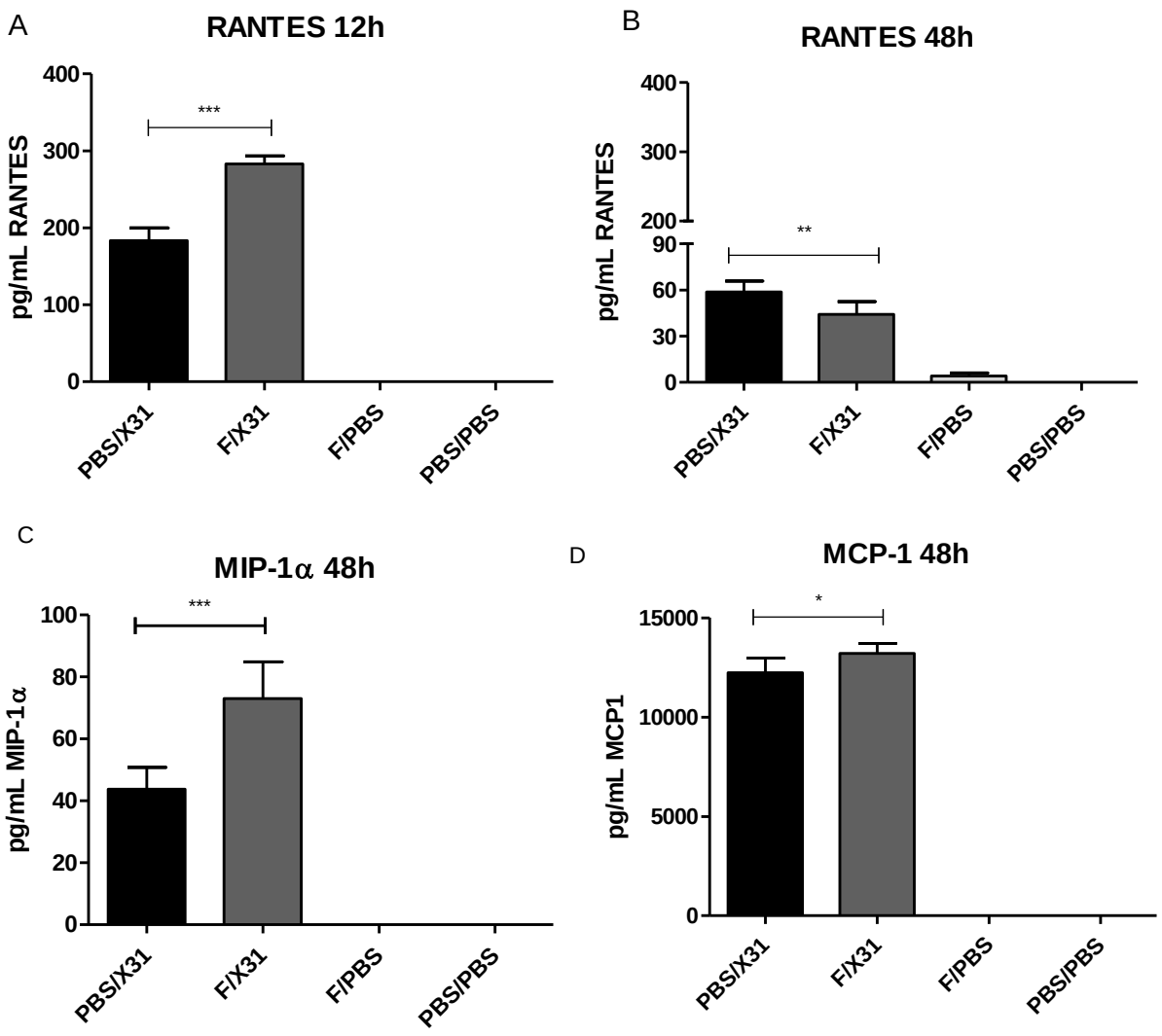
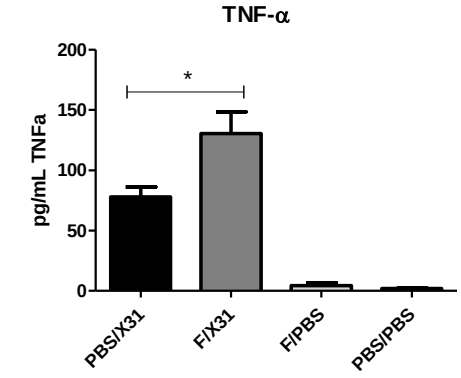


Figure 4.4: Chemokine response 48h post infection. Mice were immunized with the RSV F protein 7 days prior to influenza X31 challenge and the BAL collected at 12, 24, or 48 hrs post infection. (A) Total concentration of RANTES in the BAL 12h post infection. (B) Total concentration of RANTES in the BAL 48h post infection. (C) Total concentration of MIP-1α in the BAL 48h post infection. (D) Total concentration of MCP-1 in the BAL 48h post infection. Results are representative of 3 independent experiments. One way ANOVA performed in GraphPad Prism version 5; (***$p<0.005$, **$p<0.01$, *$p<0.05$).

A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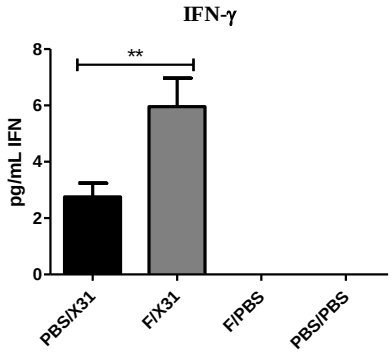
100
50
0
*
PBS/X31
F/X31
F/PBS
PBS/PBS

B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pg/mL IFN
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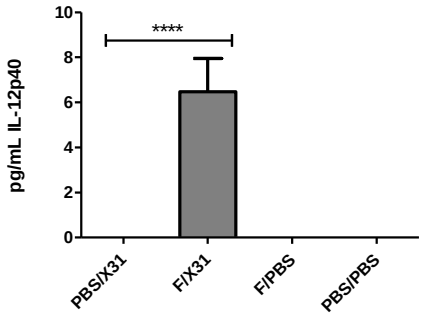
6
4
2
0
**
PBS/X31
F/X31
F/PBS
PBS/PBS

C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pg/mL IL-12p40
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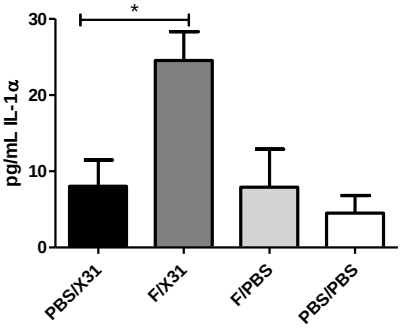
6
4
2
0
****
PBS/X31
F/X31
F/PBS
PBS/PBS

D


IL-1α
pg/mL IL-1α
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10
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*
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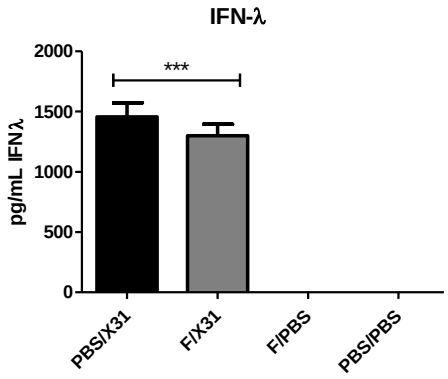
F/X31
F/PBS
PBS/PBS

E


IFN-λ
pg/mL IFNλ
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1500
1000
500
0
***
PBS/X31
F/X31
F/PBS
PBS/PBS

Figure 4.5: Cytokine response 48h post infection. Mice were immunized with the RSV F protein 7 days prior to influenza X31 challenge and the BAL collected 48h post infection. (A) Total concentration of TNFα in the BAL 48h post infection. (B) Total concentration of IFNγ in the BAL 48h post infection. (C) Total concentration of IL-12p40 in the BAL 48h post infection. (D) Total concentration of IL-1α in the BAL 48h post infection (E) Total concentration of IFNλ in the BAL 48h post infection. One way ANOVA performed in GraphPad Prism version 5; (****$p<0.0005$ ***$p<0.005$, **$p<0.01$, *$p<0.05$).

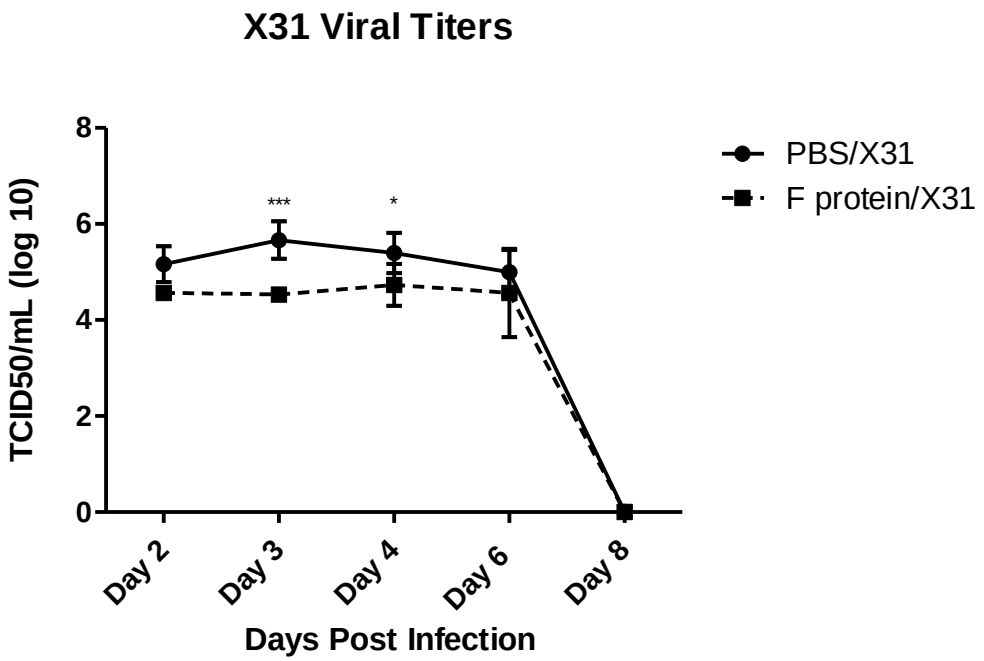

Figure 4.6: Influenza X31 viral titers. Mice were immunized with the RSV F protein and 7 days later challenged with 2.5 x 10^3 PFU/0.05mL influenza X31 influenza virus. At 2, 3, 4, 6, and 8 days post infection mice were sacrificed and the lung viral titer determined. Dashed line represent limit of detection. One way ANOVA performed in GraphPad Prism version 5.

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CHAPTER 5

CONCLUSION

Influenza and respiratory syncytial virus (RSV) are two of the leading causes of viral respiratory tract infections. Despite the availability of several annual influenza vaccines, influenza virus infections cause substantial morbidity and mortality worldwide every year. There is currently no vaccine for RSV. RSV causes significant morbidity and mortality in infants, children, and elderly individuals. There have been several attempts to develop a safe and effective RSV vaccine; however, there remain significant hurdles in regards to RSV vaccine production. Development of an attenuated RSV vaccine causes concern because natural infection does not confer long lasting immunity. Therefore it seems unlikely that an attenuated vaccine will induce long lasting immunity. It is more probable that annual vaccine boosters will be required due to waning immunity. For this reason, we proposed the use of a dual vaccine to combat influenza and RSV in an annual vaccine regimen. Our vaccine is composed of the HA protein of influenza, which was purified from 293 Freestyle cells, and F protein from RSV, which was purified from virally infected cells, either in equal molar concentrations or directly conjugated to one another. We compared our dual vaccines to HA only vaccinated mice to emulate the current vaccine strategy for influenza. Since there is no RSV vaccine, we could not compare our data to any existing vaccine. We also evaluated the ability of the F protein to adjuvant the immune response to the HA protein through TLR4 activation. The hypothesis addressed was that a vaccine composed of the HA protein of influenza and the F protein of RSV

would provide protection from both viruses. The working hypothesis was that the addition of the F protein, which is a known TLR4 agonist, enhance the immune response the influenza HA protein. The specific aims addressed here were:

Specific Aim 1. Generate mammalian cell-expressed HA protein using the 293 Freestyle system and evaluate the antigenicity of the protein. The *working hypothesis* is based on the ability of eukaryotic cells to produce recombinant HA protein that is antigenic, safe and protective. The data presented in chapter 3 demonstrates that the mammalian based cell line produce influenza HA protein from X31 virus. The HA protein was probed with hyperimmune serum from mice infected with X31 virus to determine if antibodies generated against the virus could recognize and bind to our HA protein. Native F protein extracted from virally infected Vero E6 cells. The native form of the F protein extracted from the viral envelope was used for all experiments to ensure proper folding and triggering of TLR4. The two glycoproteins were then conjugated to one another using chemical cross linkers EDC and NHS, which created amide bonds between the two proteins. Another western blot was then done to ensure that conjugation took place. The conjugated protein (HA-F) was detectable by RSV F specific antibodies.

Specific Aim 2. Evaluate the capacity of HA+F, HA-F, HA only, and F only vaccines to protect against homologous (A/HK/X31) and heterologous (A/PR/8/34) influenza virus challenge. The ability of the F protein to adjuvant the immune response to HA protein was evaluated to determine if antibody production was increased, neutralizing antibody titer was increased, and viral titer reduced to a greater degree when the F protein was directly conjugated to the HA protein (HA-F) or admixed with the HA protein (HA+F). The data represented in chapter 3 demonstrated that the inclusion of the F protein, either when conjugated to HA protein or given in equal molar concentration with HA protein, enhanced the overall antibody response as well as

the neutralizing antibody titer to the influenza HA protein relative to mice receiving the HA protein alone. Influenza X31 viral titers were also decreased in mice receiving dual vaccination at day 4 and 6 post infection when compared to HA only vaccinated mice. This data indicated that the addition of the RSV F protein adjuvants the immune response to the influenza HA protein during vaccination. To confirm that the adjuvant properties of the F protein were attributed to the F protein triggering through TLR4, TLR4 KO mice were utilized. Mice vaccinated with HA, HA+F, or HA-F had similar viral titers at day 4 and 6 post infection, indicating that TLR4 must be present to gain the adjuvant properties of the RSV F protein.

During these vaccination experiments, it was discovered that antibodies generated against the F protein were able to not only bind to X31, but to also neutralize virus replication *in vivo* and *in vitro*. In an attempt to deconvolute this phenomenon, peptides generated against the stock region of the F protein were generated. These peptides were used in a competition ELISA to determine if the peptides could compete with the binding of anti-F antibodies to influenza X31 virus. The 20mer peptides consisted of residues 1-160 of the F protein. Interesting, we found that resides 120-140 reduced anti-F antibodies binding to influenza X31 virus by 50%. This data confirms that anti-F antibodies are indeed able to bind to influenza X31 virus and that binding can be competed by the addition of peptides from the stalk region of F protein. To further elaborate on the interaction between influenza and the RSV F protein, a passive antibody transfer was done. Mice injected with 500μg of anti-F antibodies significantly reduced influenza X31 viral titers relative to PBS vaccinated mice. This phenomenon was only observed in X31 (H3N2) but not PR8 (H1N1), thus, other H3N2 influenza viruses were tested. Mice vaccinated with F protein and then challenged with A/Udorn/307/72 (H3N2) had significantly lower influenza viral

titer relative to PBS vaccinated mice, indicating that the F protein could reduce the viral titers of other H3N2 influenza viruses.

Specific Aim 3. Evaluate novel HA and HA- F vaccines ability to protect against homologous (RSV A2) and heterologous RSV (RSV B1) viral challenge. The hypothesis was that the F protein, which contain neutralizing epitopes would reduce RSV disease burden. Antibodies directed against the RSV F protein have been shown to reduce the lung viral burden and decrease morbidity associated with RSV infection in animal models. Thus, this aim encumbers examining the neutralizing antibody response, associated lung viral burden, and lung histopathology following RSV challenge as measurement of protection and disease. Data presented in chapter 3 demonstrates that F protein vaccination elicited a robust anti-F antibody response, which resulted in decreased viral replication relative to PBS vaccinated mice. F protein vaccination did not cause significant lung pathology nor did inclusion of influenza HA protein in conjunction with the F protein. This was important to evaluate in light of the failure of FI-RSV vaccine of the 1960s. Overall, data represented in chapter 3 indicates that dual HA and F vaccination results in a safe immunogenic vaccine capable of reducing both influenza and RSV disease burden while inducing minimal lung pathology.

Specific Aim 4. Evaluate the influenza-specific T cell response in RSV F immunized mice challenged with A/HK/x31 (X31) influenza virus. RSV surface glycoprotein, both F and G, have been implicated in host immune modulation following infection. Here, we determine if prior exposer to the RSV F protein can modulate the host immune response to a subsequent influenza infection. At the peak of the T cell response to influenza infection, there was not difference in the influenza specific T cell response between mice immunized with the F protein and challenged with X31 relative to those treated PBS and then infected with X31 influenza virus. T cells from F

immunized mice expressed higher levels of IL-4 after X31 influenza virus infection compared to mice treated PBS and challenged with X31. Earlier in the infection, day 4, mice immunized with the F protein exhibited higher numbers of cells in the BAL, of which included higher recruitment of CD8+ T cells and NK cells compared to PBS treated mice. There was no difference in the immunodominant influenza specific CD8+ T cells. Mice immunized with the RSV F protein expressed higher levels of specific chemokines and cytokines, including, MIP1-α, MCP-1, TNF-α, IFN-γ, IL-12p40, and IL-1α while expressing lower levels of RANTES, eotaxin, and IFN-λ. This data represented in chapter 4 indicates that prior exposer to the RSV F protein can modulate the immune response to influenza virus infection, but this did not correlate to a decrease in influenza virus titers.

Taken together, these data demonstrate that dual vaccination with the HA protein of influenza and the F protein of RSV is a safe, immunogenic, and effective vaccine. The RSV F protein can be used as an immunological adjuvant for the influenza HA protein thereby enhancing the immune response to HA. Dual vaccination resulted in decreased influenza and RSV viral titers and minimal lung pathology. The F protein enhanced the antibody titer to the influenza HA protein, while also reducing and neutralizing X31 virus when given alone. We identified a novel connection between RSV and influenza. Antibodies generated against the RSV F protein stalk region were able to bind to influenza X31 virus. Additionally, anti-F antibodies could reduce influenza H3N2 influenza viruses. Lastly, prior exposure to the F protein alone can modulate the immune response to influenza X31 infection, resulting in an altered cytokine/chemokine profile and increased CD8+ T cell and NK cellular trafficking to the lung after infection. The data presented here highlight the many properties of the RSV F protein. It is

feasible that the F protein can serve as an adjuvant for other non-immunogenic proteins through TLR4 activation.