THE FRACTALKINE RECEPTOR, CX3CR1, CONTRIBUTES TO INFLUENZA VIRUS REPLICATION

by

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(Under the Direction of Ralph A. Tripp)

ABSTRACT

Influenza A virus (IAV) places a high disease burden on the human population worldwide, especially in infants and the elderly. Due to the ability of IAV to mutate from year to year, it continues to present difficulties in generating effective methods of control, both with vaccination or antiviral therapies. Investigation of host cell interactions through genome-wide screens has provided new targets for therapeutic approaches in disease intervention. The chemokine receptor, CX3CR1, has become increasingly identified as a common host protein involved in viral infections. To begin to address the impact of CX3CR1 on IAV infection, RNA interference (RNAi) studies were performed to investigate the mechanism of CX3CR1 interaction with IAV. RNAi was used for efficient CX3CR1 gene knockdown *in vitro* followed by IAV infection. Knockdown of CX3CR1 resulted in significantly lower viral titers across multiple IAV strains. Using gene knockout mice, we determined that CX3CR1 was important for IAV replication *in* vivo at early stages of infection. Immunohistochemistry studies showed production of

IAV proteins in CX3CR1 silenced cells but a decrease in total infected cells. These findings suggest that CX3CR1 is involved in the late stages of IAV replication and could provide a new therapeutic target for IAV disease intervention.

INDEX WORDS: influenza, RNA interference, CX3CR1, therapeutic

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DEDICATION

This work is dedicated to my family to whom I will always love for helping me become everything that I am.

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CHAPTER 1

INTRODUCTION

Viruses are obligate intracellular pathogens that cause some of the highest disease burdens in humans. Being dependent on hosts for their life cycle, they must be able to utilize host cell machinery to replicate their relatively simple genomes. A viral life cycle, or replication cycle, involves several steps that are universal to all viruses. These key steps are attachment/entry, replication, and egress [1]. For each step, viruses must use many of the proteins present in the host cell. Identifying and understanding these host proteins as well as their interactions with viral proteins is of great interest in the field of virology. By learning the mechanisms by which a virus is able to utilize host proteins, novel therapies can be discovered to advance the methods and aspects of disease intervention.

Influenza viruses continue to be a major cause of disease in human populations with seasonal influenza causing around 36,000 deaths in the U.S. annually [1]. Vaccination remains the most effective treatment for preventing influenza virus infection, but current vaccines induce limited immunity due to antigenic drift, and must be updated in an expensive and prolonged process [2, 3]. Another concern is the emergence of novel avian and swine influenza viruses with pandemic potential caused by antigenic shift [4-6]. In addition to circumventing current vaccines, both antigenic drift and shift have

caused increasing resistance to antiviral drugs that were once or currently effective to treating influenza infection [7-13].

Influenza viruses belong to the family Orthomyxoviridae, possessing a genome consisting of eight segments of negative-sense, single-stranded RNA [14]. These segments encode for specific viral proteins, but most of the replication cycle is dependent on host cell components [15, 16]. Gaining knowledge of critical host cell determinants will help elucidate mechanisms of viral replication and biology, provide targets for novel drugs, and expand potential avenues of therapeutic intervention. Targeting host cell factors that are utilized by the virus will help prevent the emergence of viral mutants. Determining host factors important for viral replication has become the first step in the development of antiviral therapeutics.

RNA interference (RNAi) is a relatively new approach for performing gene silencing to identify host genes involved in influenza infection and replication and investigating virus-host interactions [17-22]. , RNAi is a natural pathway that inhibits gene expression transcriptionally or translationally in a homology-dependent manner [23], and is mediated by small non-coding RNAs [24, 25]. Generally, siRNA is transfected into host cells and is processed by the nuclease Dicer into single-stranded molecules that become incorporated into the RNA-induced silencing complex (RISC). Once incorporated, the antisense or guiding strand of the siRNA can bind to mRNA that contains homologous sequence to initiate degradation or inhibition of that mRNA [26-29]. Delivery of synthetic siRNA targeting host and viral genes has been successfully used against several respiratory viral infections such as influenza [30, 31], severe acute

respiratory syndrome coronavirus (SARS-CoV) [32, 33] and respiratory syncytial virus (RSV) [34].

Genome-wide RNAi screens have identified a multitude of human genes that are important for influenza replication. Among these, several chemokine receptors have demonstrated a possible role in infection. Chemokine receptors are known to have be involved in viral replication cycles [35, 36]. These receptors are primarily responsible for immune cell trafficking, however they have also been shown to be involved in autoimmunity, carcinogenesis, angiogenesis, and immunity to pathogens [37-40]. CX3CR1 is a chemokine receptor that has been shown to affect the replication of two viruses, including HIV-1 [41] and the respiratory pathogen RSV [42]. It also has been implicated in osteoarthritis, rheumatoid arthritis, and allergic rhinitis [43-45]. With a role in several viral diseases, CX3CR1 may provide an avenue for a novel disease intervention strategy against influenza.

The central hypothesis of this study is that CX3CR1 is involved in influenza replication and could provide a new target for novel therapeutics to reduce influenza infection without inducing viral mutation and resistance. The study includes the following specific aims:

Specific Aim 1. To determine the importance of CX3CR1 in influenza replication *in vitro* and *in vivo*. The *working hypothesis* is that RNAi silencing of CX3CR1 will reduce influenza replication *in vitro* and *in vivo*. After a strict validation process by a deconvoluted pool of siRNA that target a different seed site on CX3CR1 to confirm observations seen on a genome-wide screen, the replication phenotype of influenza will be measured to determine the impact of an absence of CX3CR1 in infection. It is often

beneficial to either inhibit immune effector proteins or directly use those proteins to decrease their effectiveness in an antiviral response. By utilizing a chemokine receptor, influenza may delay the function of the chemokine and in the case of CX3CR1 it would affect the infiltration of immune cells to the areas of infection. Validating CX3CR1 as a pro-viral gene will provide a new target for therapeutic intervention of influenza infection.

Specific Aim 2. To determine the role of CX3CR1 in influenza replication, spread, and cytopathogenesis. *The working hypothesis is that one or more influenza proteins interact with CX3CR1 for assembly of virions, trafficking of proteins to the cell surface, or egress of virions.* Understanding how CX3CR1 is utilized during IAV replication will enhance our knowledge of influenza biology and the immune response, as well as open possibilities for new avenues of influenza disease intervention.

Specific Aim 3. To determine the effect that prior infection or co-infection with respiratory syncytial virus has on influenza virus replication. *The working hypothesis is that pre-infection or co-infection with RSV negatively alters the innate and adaptive immune responses, resulting in an altered immune response to influenza virus infection causing enhanced disease.*

The evaluation of these specific aims will create a better understanding of the influenza viral replication cycle in the context of CX3CR1. The understanding of the importance of CX3CR1 in influenza replication can deepen our knowledge of virus biology and immune evasion. Determining the role of CX3CR1 in influenza infection may lead to further studies using drugs that inhibit viral-host interaction as a therapeutic in disease intervention.

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CHAPTER 2

LITERATURE REVIEW

Overview of influenza A virus (IAV)

Emerging infectious diseases (EIDs) remain a growing public health concern and those with pandemic potential are a risk for global health. A disease is recognized as an EID by meeting any of the following criteria: an old infection that has reemerged due to environmental changes, existing infections that have spread to new geographic areas and/or populations, previously unknown agents appearing in a changing environment, a newly discovered agent, and a new infection caused by changes in a known agent [1]. Respiratory pathogens such as influenza A viruses (IAVs) are well described EIDs that have continued to challenge methods for surveillance, prevention, and treatment. Newly emerging influenza viruses such as the 2009 pandemic H1N1 virus arose from the swine population and quickly became the dominant circulating strain in the human population [2]. Other outbreaks, such as the highly pathogenic H5N1 virus or the avian H7N9 virus continue to demonstrate their pandemic potential due to their zoonotic nature and ability to cause a high mortality in human and bird populations [3, 4].

IAVs continue to cause significant global disease burden in respect to economic and social cost due to morbidity and mortality. Seasonal influenza can cause an estimated 3-5 million cases of severe disease and up to 500,000 deaths globally [5]. In

the United States alone, the CDC reports that influenza can cause close to 50,000 deaths annually, possibly due to secondary bacterial infections that cause pneumonia [6]. The economic impact caused by this high morbidity and mortality; which consists of direct medical costs, projected lost earnings, and projected life values, is estimated to be around 87 billion dollars in the United States [7].

Vaccination remains the most effective treatment for preventing influenza virus infection, but the ability of the viruses to evolve and change their antigenic structure continues to force new vaccines to be produced. Antiviral drugs have been effective but due to the viruses' high mutation rate, resistance of one strain of influenza is often generated very quickly. Like all viruses, IAV must use the resources the host cells offer to complete replication for progeny virus. Investigating the viral-host interaction can provide new understanding of viral life cycles and mechanism of immune evasion. Genome-wide screening technology allows opportunity to study the host genes required for viral infection and replication. Discovering new host interactions could provide novel avenues of therapeutic disease interventions to circumvent both the difficulty in designing safe and effective vaccines, as well as the innate ability of the virus to evolve in defense of an antiviral environment.

IAV biology

IAV belong to the family *Orthomyxoviridae*, which are characterized enveloped viruses with a segmented genome of negative sense, single-stranded RNA. The virus is pleomorphic, having the ability to take a spherical or filamentous structure [8]. In the *Orthomyxoviridae* family, there consist three genera of influenza viruses, named IAV)

Influenza B, and Influenza C. IAV genome contains 8 negative sense RNA segments encoding up to 13 proteins depending on the viral isolate, 3 of which are more recent discoveries [9]. IAV encode 4 structural proteins: matrix protein 1 (M1), matrix protein 2 (M2), hemagglutinin (HA), and neuraminidase (NA). IAV encodes 3 polymerase proteins, polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), as well as a nucleoprotein (NP) that along with the polymerase proteins make up a viral ribonucleoprotein complex (vRNP) [10]. Additionally, IAV translates non-structural proteins for assistance in replication and immune evasion, these being the non-structural protein 1 (NS1) and the non-structural protein 2 or nuclear export protein (NEP). Finally, the three proteins named polymerase basic 1-frame 2 (PB1-F2), polymerase acidic-ORF-X (PA-X), and polymerase basic-N40 (PB1-N40) are results from splicing and open reading frame shifts in the PA and PB1 gene segments [11-13].

The IAV particle varies in size, ranging from 80-120 nm in diameter in the spherical conformation. However, due to the pleomorphic nature, more filamentous forms have occurred ranging from 40-100 nm in diameter and from 300 nm to 20 µm in length[14, 15]. While the transition to filamentous forms in not fully understood, it is known that M1 is the main determinant of viral morphology [16, 17].

Seasonal IAV generally infects the upper respiratory tract (URT) of humans while some strains of IAV can have a preferential tissue tropism in the lower respiratory tract (LRT). One of the main determinants of the tissue tropism is the receptor binding domain (RBD) on the HA globular head [18]. This RBD specifically binds sialic acids with terminal linkages with either alpha 2, 3 or alpha 2, 6 specific linkages. The binding

specificity between IAV strains are important in determining host range and transmission, as some viruses may target different sialic acid receptors which are distributed differently in the respiratory tract of different animals [19]. Cells that are known to be infected by IAV are: alveolar and bronchoepithelial cells, bronchoendothelial cells, alveolar macrophages, dendritic cells, monocytes, neuronal tissue, and NK cells [20, 21].

IAV replication

The life cycle of IAV can be separated into five phases: 1) attachment and entry into the host cell; 2) import of the vRNPs into the nucleus; 3) transcription and replication of the viral genome; 4) export of vRNPs and proteins from the nucleus; and 5) assembly, trafficking, and egress from the host cell membrane. The HA protein is responsible for binding and is a homotrimer that protrudes from the viral lipid membrane. The nascent form of HA, HA0, is made up of two subunits: HA1, which contains the RBD, and HA2, which contains the fusion peptide [22]. There are at least eighteen HA subtypes, being named H1-H18. The majority of these were found originating from humans and fowl, but H17 and H18 were recently discovered in bats [23]. It has also been shown that NA may play a role in the initial stage of IAV infection [24].

Once HA binds to sialic acid linked receptors on the cell surface, the virus in internalized by several mechanisms: clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin and caveolin independent pathways, and micropinocytosis [25, 26]. Once inside the endosome, following a decrease in pH, the HA is cleaved and undergoes a conformational change into the two disulfide-linked segments HA1 and HA2, which leads to fusion of the viral and endosomal membranes by way of the fusion peptide of the

HA2 [27]. Concurrently, in the acidifying endosome, the viral M2 protein forms a proton-selective ion channel to cause the M1 protein to dissociate from the RNPs [28]. Following uncoating, the RNPs must be transported to the nucleus for viral replication. Each component of the vRNPs, PA, PB1, and PB2 contain at least one nuclear localization signal (NLS)[9]. Also containing an NLS is NP, which is important for the import of the large RNP complexes [29, 30]. Importin 5, importin α1/α2, and importin α1/α5 have been shown to be involved in nuclear import with PB1, PB2, PA, and NP [30-33].

Once inside the nucleus, the viral RNPs undergo the two processes of replication and transcription. Replication involves the creation of a positive-sense full length complement of the negative-sense genomic viral RNA. This complement strand is known as cRNA, which is then used as a template for creation of more genomic negative-sense viral RNA. Transcription uses the genomic viral RNA as a template for the production of positive-sense viral mRNAs to be used in translation into proteins. Transcription must be performed first to acquire the correct amount of proteins to form viral particles[9]. Both processes require special promoters to verify that only viral templates are used. The 5' and 3' ends of vRNA and cRNA contain the promoters that the viral polymerase complex is able to recognize. The ends of these molecules have partial inverse complementarity, which allows them to form "panhandle" configurations [34]. These configurations provide the special promoters that allow the viral polymerase to recognize only viral genome.

During IAV transcription to mRNA, viral mRNA is capped through a PB2 mechanism and polyadenylated, to which the mRNA can now be used as a template [35].

The template mRNA can now be used for either translation into viral proteins for packaging or replication of the mRNA where a full-length positive-sense copy is made (cRNA), which is used as a template to make full-length copies of vRNA [36]. Transcription starts with the 5' end of vRNA binding to PB1. This activates two things, the PB2 subunit to bind at the 5'-capped ends of cellular RNAs, and the 3' end of the vRNA binds to PB1. Once the 3' end binds to PB1, the endonuclease site on PB1 is now open and will then allow PB1 to use the endonuclease activity to cleave the cap of the cellular RNA and bind it to the vRNA to now act as a primer for vRNA transcription [37]. During elongation of the mRNA, the polymerase continues until it reaches a stretch of 5-7 U residues where it stutters and results in the addition of a polyA tail [9].

Once the required amount of translated proteins has accumulated in the nucleus after the transcription phase, replication commences and is controlled by the viral polymerase. The viral polymerase complex itself consists of the PA, PB1, and PB2 proteins. PB1 is the catalytic subunit of the complex and also is the central protein as it binds to PA via the amino-terminus and the PB2 via the carboxy-terminus [38, 39]. PB2 is responsible for cap-snatching activity which is used to prime viral mRNA synthesis[9]. Other than allowing for viral mRNA synthesis, cap snatching results in preferential translation of viral mRNAs over host mRNAs as well as host mRNA degradation [40-42]. PB1 contains the endonuclease activity which cleaves the cap away from host mRNA and allows PB2 to transfer it to viral RNA[43].

Viral mRNAs require a polyA tail for translation, which is acquired through the use of cellular polymerase II [44]. Due to the nature of replicating in the nucleus, Influenza has access to splicing machinery that can be used to create two or more

proteins from the same gene segment. Both the M and NS segments of IAV require the use of cellular splicing to produce two different viral proteins from each segment. Unlike viral mRNA, cRNA does not require capping or polyadenylation, but it must be encapsidated by NP for stabilization, otherwise it will be degraded [45].

Once the viral mRNAs are exported out into the cytoplasm and translated into proteins, the proteins that are then re-imported into the nucleus via the same NLSs that were used before, thus the surface glycoproteins that do not associate with the RNPs do not re-import into the nucleus but are rather transported to ER and Golgi for folding and post-translational modification [46-49]. After replication is completed, NEP exports the vRNPs from the nucleus with the help of M1. The vRNPs associate with M1 to form an RNP complex and NEP connects the RNP complex to the cellular export machinery [50, 51]. Once into the cytoplasm, M1 association with the RNPs prevents their reimport into the nucleus [52].

Packaging and trafficking of RNPs into new virions is not very well understood but it has been observed that packaging signals exist in the 3' and 5' NCR of vRNAs. It is known that if the PB2 packaging signal is mutated, there are significant effects on virion formation[53]. Since the HA, NA, and M2 proteins are not imported back into the nucleus, they are exported to the plasma membrane. It is known that the HA, NA, and M2 undergo post-translational modifications, such as N-linked glycosylation and palmitoylation during their export through the endoplasmic reticulum and golgi [48, 49, 54, 55]. All three of the viral envelope proteins associate with lipid rafts which causes these areas to push out until the inner capsid containing the vRNPs become enveloped [56, 57]. Once at the surface, the NA is responsible for cleaving sialic acid residues that

would bind to HA and prevent proper virion release. NA is a homotetramer in its quaternary form and is responsible for cleavage of glycosidic linkages or neuraminic acids [58].

Immune response to IAV

The host immune response to IAV involves each arm of the immune system, beginning with the innate response. Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) that are often used during pathogen infections. Many of the anti-viral TLRs are located on the inside of endosomes for recognition of these internal pathogens. TLR3 was found to recognize double-stranded RNA in an IAV infection [59] and TLR7 recognized single-stranded RNA [60]. Activation of TLR3 induces transcription factors IRF3, AP1, and NFκB which ultimately lead to induction of type I interferons [61]. NOD-like receptors (NODs) also seem to have a role in response to IAV. Mice exhibiting deficiencies in NLRP3 have shown an increased mortality and reduced immune response when challenged with IAV, correlating to a reduction in cytokine and chemokine production, as well as reduced monocyte and neutrophil lung recruitment [62, 63].

Rig-I like receptors (RLRs) are cytosolic sensors of RNA that have been found to be the main sensor of IAV infection in host cells [64]. RIG-I, MDA5, and LGP2 are three members of the RLR family and have been found to be important for an antiviral response in IAV infection [65]. Each of these three RLRs have been shown to activate mitochondrial activated-signaling protein (MAVS) which initiates the antiviral signaling cascade through activation of type I interferon stimulating genes [66]. It was recently

shown that an IAV-induced activation of the interferon cascade requires RNA synthesis and nuclear export to occur, thus providing evidence that incoming vRNPs are not the predominant activator of RLRs [67].

The humoral arm of the immune system is a vital part in protection against IAV infection. During infection, neutralizing antibodies are primarily elicited against viral surface proteins HA, and to a lesser extent the NA and M2 [68]. These antibodies are critical to the clearance of virus during infection as well as protection from future infections, as demonstrated by passively transferring antibodies specific for IAV HA to severe combined immunodeficiency (SCID) mice, which provided protection against lethal challenge [69]. While antibodies against HA are neutralizing, NA antibodies can help elicit protection by preventing the NA from cleaving sialic acid on the host cell, thereby inhibiting the release of the virus and decreasing the spread of the infection [9]. Humoral mediated immunity can provide protection from several months up to a lifetime, depending on the strain of IAV.

Lastly, cell-mediated immunity is essential in eliminating virus-infected cells which eventually helps clear the infection. The cells responsible for this protection are $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs). These CTLs recognize epitopes from HA, M, NP, and PB2 on MHC I molecules [70], and upon recognition are able to lyse the cells which further assists in viral clearance [71]. This ability to recognize epitopes of internal viral proteins, which are more conserved than surface proteins, gives the CTLs a cross-reactive specificity aiding in protection from multiple strains of IAV.

IAV mechanisms of immune evasion and pathogenicity

Despite the multifaceted correlates of immune protection against IAV, the virus still is problematic throughout the lifetime of humans. Besides their roles in replication, several IAV proteins provide mechanisms of immune evasion. The IAV HA is most well described IAV protein as well as the best defined virulence factor. Due to the lack of proofreading of the viral polymerase, point mutations often occur in the surface protein genes leading to a high mutational rate of the virus. These mutations in amino acids of the antigenic regions of the HA (and NA) allow selective advantages for IAV, providing a mechanism of evasion of pre-existing immunity[72]. This selective mutation caused by the errors in polymerase activity is known as antigenic drift.

Due to the segmented nature of IAV, co-infection of a single cell can result in progeny viruses that contain genome segments from each parental virus. This process of genetic reassortment, termed antigenic shift, occurs when the new progeny virus contains a mixture of surface antigens from the parental strains. This phenomenon occurs in IAV due to the virus ability to infect more than one species. There are 18 known subtypes of HA, which are classified into two groups. Some subtypes are generally found in certain species due to their co-evolution with that species. The majority of HA subtypes are found in birds, with only a few being isolated from bats [23, 73]. Only three subtypes have been associated with human illness for long periods of time, H1, H2, and H3 [74]. If a reassorted virus with an avian HA infects humans, that virus has pandemic potential due to the lack of immunity in the human population to the avian HA.

Another pathogenicity factor related to HA is the proteolytic cleavage of the HA0 while in the endosome. The residues at the C terminus of the HA1, termed the cleavage

sequence, are important in determining the pathogenicity of IAV. The existence of several basic amino acids at the cleavage sequence allows for the HA to be cleaved by a wide range of host cellular proteases such as furin [75]. The distribution of furin in birds allows cleavage of the HA in most cell types, providing multiple sites of possible replication. If a virus possesses these multibasic cleavage sites, it is said to be highly pathogenic, while low pathogenic IAV is restricted to tissue types that possess the required protease for HA cleavage.

Similarly to HA, NA can also be involved in both antigenic drift and shift. There are presently 11 known subtypes of NA, with NA1 and NA2 being the only subtypes currently associated with human illness over an extended period of time. The NA protein of the IAV strain A/WSN/33 has been shown to be required for the neurovirulence phenotype that is associated with that virus, as well as lacking the requirement of any addition of exogenous serine protease in tissue culture. It is able to bind plasminogen in serum, which allows for cleavage of the HA and release of the virus [76]. This ability is linked to the loss of a carbohydrate chain at amino acid position 146 [49], which is also the cause of specific avian strains being able to survive a lower pH than human or swine strains [77].

IAV NS1 has been shown to be a potent inhibitor of the antiviral interferon response. One mechanism that NS1 uses is to block the interaction between RIG-I and double-stranded RNA [78]. NS1 has also been shown to directly interfere with interferon stimulating genes (ISGs) such as PKR by interacting with the N-terminal RNA binding-domain which blocks signaling [79]. Additionally, NS1 was shown to inhibit TNFα expression through direct interaction [80], as well as decrease IFN-β expression by

preventing activation of the transcription factor IRF-3 and NF-κB [81, 82]. This significant inhibition of the antiviral interferon response may also assist in a delayed adaptive immune response through infection of antigen-presenting cells (APCs). It has been shown that induction of antibodies against a non-related antigen is delayed when that antigen is given between 0 and 48 hours post-infection with IAV [83]. Finally, NS1 has also been shown to both prevent and induce apoptosis [84]. Preventing apoptosis may allow viral genome replication to finish and inducing apoptosis assists in the release of progeny virus. The NS1-apoptosis mechanism is unclear but it has been shown that apoptosis is mediated though NS1 induction of p53 in A549 cells [85].

Disease Intervention

Vaccination against IAV remains a mostly successful method for disease intervention; however the vaccine is directed against the highly variable surface proteins, which requires new formulations of the vaccine to be produced each year. This method is expensive, time consuming, and relies on accurately predicting which strains will dominate circulation for that year. There are two vaccine formulations that are currently in use: a trivalent or quadrivalent inactivated vaccine consisting of the HA and NA proteins from the predicted IAV or influenza B strains, and the live-attenuated vaccine known as FluMist though this is being retracted by the CDC for the2017 influenza season [86]. Due to IAV ability to undergo antigenic drift and shift, the efficacy of these vaccines is reduced and a constant surveillance must be maintained to predict which strains to design into that years vaccine. Also, the vaccine may not provide adequate efficacy in the populations that are in the most danger of severe disease caused by IAV,

the young, elderly, and immunocompromised [87]. Additionally, the vaccine is produced in eggs, which may exclude those in the population with an egg allergy [88].

Another disease intervention strategy against IAV is the therapeutic delivery of antiviral drugs. Currently, there are two types of drugs that target separate stages of the IAV replication cycle. The first are M2 inhibitors (Adamantanes), Amantadine and Rimantadine, which specifically inhibit the ion channel by entering into the channel and blocking the proton pore function [89]. Despite the early success of these drugs, there has been a decrease in usage due to the emergence of drug-resistant IAV strains [90]. The second class of antivirals against IAV is the neuraminidase inhibitors, Oseltamivir and Zanamivir. These drugs act as sialic acid mimics, competitively binding to NA [91]. This reversible reaction inhibits NA from cleaving the host sialic acids on the membrane to which HA is still bound to, hindering the release of the virion from the cell membrane [92-94]. Despite the effectiveness of both classes of drugs, resistance among the viral population is becoming increasingly common, as nearly all circulating IAV strains were resistant to amantadine since 2009 [95]. Adamantane resistance is characterized by mutations in the IAV M2 gene which causes either reduced binding of the drug to M2 or that M2 still retains efficient function [96]. Like the adamantane resistance, NA inhibitor resistance occurs through mutations in the NA that cause a reduced affinity of the drug for the ligand [97].

Due to the increasing occurrence of drug resistant strains as well as the difficult and cost of making effective vaccines against IAV, there is a need to develop novel therapeutics. RNA interference is an encouraging new method for creating safe and

effective therapeutics with the potential for less resistance among circulating strains. It also aids in the discovery of new targets for drug discovery.

RNA Interference (RNAi)

Prior to the 1990s, the only known mechanisms of transcriptional repression were modifications to DNA and proteins. It was then first discovered in plants that several gene silencing phenomena occur post-transcriptionally, which has since become to be known as post-transcriptional gene silencing (PTSG) or RNA silencing. It was originally called cosuppression due to the degradation of both an endogenous and exogenous gene in petunias [98]. It was later discovered in the nematode *Caenorhabditis elegans* that this suppression was caused by small RNAs [99]. The mechanism, termed RNA interference (RNAi) was studied more in this animal model and was found that injection of dsRNA resulted in efficient and specific gene silencing [100]. RNAi has since been studied in a wide range of organisms including plants, *Drosophila*, and mammals [101-103], and has become a crucial element to studying post-transcriptional gene control as well as providing new avenues for disease intervention.

RNAi is mediated short, non-coding RNA molecules that can control gene expression at both the transcriptional and translational levels [104]. These molecules interact with several proteins to allow targeting of specific sequences for direct degradation of mRNA or to suppress protein expression [105, 106]. There are several classes of RNAi molecules in eukaryotes: microRNA (miRNA), small interfering RNA (siRNA), piwiRNA (piRNA), and small nuclear RNA (snRNA) or small nucleolar RNA (snoRNA) [107].

It was found that up to 5% of the human genome encodes and produces over 1,000 miRNAs [108], which are responsible for regulating close to one-third of human genes [109]. These miRNAs are produced endogenously in the nucleus as opposed to the synthetic or virally induced siRNA, which are usually exogenously added to cells. Despite the difference in origin, both miRNAs and siRNAs function in the cytoplasm of the cell for gene regulation. The snRNAs and snoRNAs are produced and function entirely in the nucleus [110]. They are found to have a role in the regulation of transcription factors through the release of P-TEFb [110], as well as in splicing [111]. The piRNAs interact with the Piwi clade of Argonaute proteins, which is where they acquired their name[112]. While piRNA function in mammals has yet to be extensively studied, they have been shown to be required for germline cell development and maintenance [113, 114].

Biogenesis and post-transcriptional regulation of miRNA and siRNA

While each class of RNAi molecules may be mechanistically different in origin and function, miRNA and siRNA share a similar mode of action. This is to use a ribonucleoprotein complex that comprises an Argonaute family protein that is bound to a single strand of RNA about 20-30 nucleotides in length, which allows for sequence specific targeting. This complex is known as the RNA-induced silencing complex (RISC), which induces silencing of RNA through either direct degradation or transcriptional repression. The cellular origins of miRNA and siRNA are distinct, as miRNA is generated from endogenous transcript of the genome in the nucleus and siRNA

arises from exogenous sources such as plasmids or viral infection [104], but both have similar function in the cytoplasm.

In the nucleus, miRNAs begin their generation by being transcribed from an miRNA gene or intronic miRNA [115]. Generated by RNA polymerase II, the transcripts at least 1,000 nucleotides in length and now known as pri-miRNA, are capped and poly-adenylated [116]. They also contain double-stranded hairpins with both 5'- and 3'-terminal overhangs [117]. In the nucleus, a RNase III-type enzyme called Drosha cleaves the stem of the pri-miRNA resulting in a hairpin precursor called pre-miRNA. This new intermediate is about 65-70 nucleotides in length and is exported out of the nucleus into the cytoplasm by Exportin-5 and RanGTP [118].

Once in the cytoplasm, the processing and catalytic pathways for the endogenous miRNA and exogenous siRNA converge. Both start this pathway by interacting with the endoribonuclease Dicer, which makes two alterations in the pre-miRNA. Dicer first cleaves the loop from the top of the hairpin, creating a mature RNA duplex of around 22 nucleotides in length, followed by a cut at the 3' overhang on the other end of the duplex, producing a double-stranded miRNA. This duplex quickly associates with Argonaute, the catalytic enzyme of the RISC. Dicer is aided by a double-stranded RNA-binding protein (dsRBP). Dicer, Argonaute, and the dsRBP now make up the RISC-loading complex, which is required for creating diced dsRNA and associating it with the Argonaute protein [119]. Once loaded onto Argonaute, the miRNA unwinds into two strands [104]. The guide strand, or miRNA, is the strand most commonly loaded onto Argonaute while the passenger strand, or miRNA*, is usually discarded.

The RISC is now able to bind to single stranded RNA that is found in the cell cytoplasm such as mRNA with varying degrees of complementarity to the guide strand bound to Argonaute. Nucleotides 2-8 on the guide strand are referred to as the seed sequence, which is the initial binding site to the mRNA. This binding does not require perfect complementarity, as the position, number, and type of mismatches between the target gene and the miRNA can determine how gene silencing and regulation occurs [120]. The miRNA targets the 3'-UTR of the mRNA and if there is perfect complementarity, the mRNA is degraded by the Argonaute protein of the RISC, which is also identical to the mechanism of siRNA [116]. This is one way that miRNA regulates gene expression through transcriptional inhibition. If binding is not perfectly complementary, which occurs more with miRNA than siRNA, translation is repressed though multiple mechanisms. The miRNA can prevent ribosomal binding to the mRNA by competitively binding to the ribosome itself as seen in let-7 targeting by miRNA [121]. It was also shown that miRNA associate with eIF6 to inhibit the 60S subunit from binding to the 40S subunit for translation to occur [122]. Additionally, the miRNA can stimulate deadenylation of the poly-A tail of the mRNA as well as interfering with recruitment of eIF4E to the cap structure [121], both processes of which would cause a decrease in the stability of the mRNA leading to degradation. Finally, miRNA can promote degradation of the translational complex or cause the ribosome to fall off the mRNA [104, 123].

RNAi in disease intervention

RNAi-based intervention has become an attractive therapeutic method for controlling genes associated with a wide variety of diseases. This has an impact for cancer, viral disease, autoimmune disease, and genetic disorders. In cancer, RNAi can be used to target oncogenes and mutated tumor suppressor genes with high specificity, as well as multiple cellular pathways involved tumor progression. There is also a lack of side effects in RNAi that provides a significant benefit over other treatments such as chemotherapy [124, 125]. RNAi has been studied both *in vitro* and *in vivo* looking at the effective delivery of siRNA to treat rheumatoid arthritis, an autoimmune disease affecting joints. Several studies have showed a potential therapeutic effect by RNAi to decrease arthritis by targeting TNF-α [126, 127]. Delivery of siRNA has also been shown to decrease factors associated in causing genetic disorders such as Huntington's disease, Alzheimer's disease, and Parkinson's disease [128-130].

Due to viruses requiring host genes for their replication cycle, RNAi is seen as a therapeutic with great potential. It has been shown to be successful in multiple platforms, but the method for delivery remains a challenge. Synthetic siRNAs can be delivered in vivo for acute viral infections such as influenza [131, 132], severe acute respiratory syndrome coronavirus (SARS-CoV) [133, 134], and respiratory syncytial virus (RSV)[135, 136]. With siRNAs being short lived, viruses that have chronic infections such as HIV-1 or hepatitis, delivery of a plasmid encoding siRNA can provide a long term source for RNAi [137-140]. When designing an RNAi-based therapy to viral diseases, the life cycle of each virus must be considered as each virus utilize different host genes as well as cell locations for replication.

While it is both optimistic and plausible that more than one virus family utilizes the same genes for replication, the reality may be different. Determining which host genes are required for viral replication is an important step in designing potential RNAi therapeutics against viral infection. For IAV, several recent studies have performed genome-wide RNAi screens that allow for identification of host genes that IAV requires during the viral replication cycle [141-146]. Using these studies, it is now possible to identify host genes and cellular networks that are involved in IAV replication. These pro- or anti-viral gene targets can then lead to the design of RNAi-based therapeutic intervention or allow for a better understanding of the IAV-host interaction.

RNAi screening for host factors involved in viral replication

Understanding viral mechanisms of host interaction has greatly helped to expand the knowledge in cell biology as well as virology. The primary method for investigating virus-host interaction has been through the use of RNAi in genome-scale screens encompassing many gene families. There have been a number of studies in recent years that have utilized these genome-wide screens to identify hundreds of gene targets that are involved in viral replication amongst a wide variety of virus families [141-143, 147-154]. The goal of these studies is not only to determine mechanisms of virus-host interaction, but to identify potential targets for disease intervention.

Several genome-wide RNAi screens have been performed to identify host factors involved in IAV replication. The first was in a drosophila cell line using a dsRNA library targeting 13,7011 *Drosophila* genes, which was due to RNAi screening being poorly established in mammalian cell lines at the time as well as the unavailability of siRNA

libraries [141]. Using a recombinant influenza A/WSN/33 virus, the HA and NA segments were replaced with vesicular stomatitis virus glycoprotein (VSV-G) and a luciferase reporter, which allowed for infected cells to be measured by luciferase expression. However, the inability of the recombinant virus to replicate limited the screen to identify genes affecting viral entry only. Despite using *Drosophila* cells, which does not directly translate to genes important in IAV-human interaction, this study set the groundwork for the use of RNAi in genome-wide IAV screens.

The availability of siRNA libraries targeting each gene of the human genome was an important advancement for continuing genome-wide screens research. With siRNAs being very effective at gene silencing, as well as being cost-effective due to being easy to synthesize, they became ideal tools for RNAi [155]. To ensure efficient gene silencing, libraries usually consist of pools of siRNA, where each individual siRNA may target a separate region of the gene [142]. The next screen using human U2OS cells identified 133 host factors that were required for IAV replication [142]. Using A/Puerto Rico/8/34 H1N1 (PR8), HA was identified on the cell surface as an indicator of infection. This allowed for a more comprehensive analysis of the viral replication cycle, but still was only limited to a single round of replication. HBEC cells were used for a screen, which more closely mimics a natural infection [145] and targeted genes based off data obtained from a yeast two-hybrid system, but the system was hampered by a virus that lacked the NS1 protein, which may have skewed screen hits.

Two other groups used a human lung cell line (A549 cells) and an arrayed siRNA library [143, 144]. One used a recombinant IAV A/WSN/33 H1N1 (WSN) possessing a renilla luciferase gene that replaced the HA gene. This study therefore could not identify

host genes involved in viral assembly, trafficking, or release. It was however able to identify 219 host factors that were required for replication of IAV WSN. similar approach used a fully functional WSN virus to detect NP expression. First, the A549 cells were transfected with siRNA and then infected with wild-type WSN. Second, the viral supernatants were removed from the A549 cells and transferred onto HEK293 T cells that carried an influenza virus-driven luciferase reporter [144].

More recent screens have used lentiviral shRNA expression systems to identify host genes involved in IAV replication. A549 cells are infected with pools of lentivirus that are generated from an RNAi library. The cells are then challenged with IAV WSN at a dose to cause cytotoxicity. Any cells that survive infection undergo deep sequencing to identify the shRNA that has been integrated into the genome that is responsible for silencing the genes required for the decreased IAV infection and/or replication [156, 157]. This method of shRNA expression constructs allows for assays to be performed for longer durations than the transient siRNA method. Regardless of which type of silencing is chosen for the assays, a method for determining and identifying which genes have an effect on the result of the assay when they are silenced must be used. A common method for quality metrics is to calculate a Z'-factor, which determines the level of distinction between the positive and negative controls. This is followed by a Z-score to normalize the data to the mean value, which allows a comparison to the effectiveness of each siRNA relative to the assay distribution [158, 159]. The Z-score method is used to rank genes in effectiveness relative to the negative or positive control to allow for the identification of pro- or anti-viral genes.

Recently, an RNAi genome-wide screen was conducted to identify host kinase genes and protease genes involved in IAV replication, respectively [154, 160]. Along with these gene families, the G-protein coupled receptors (GPCRs) were included in this screen, with work currently unpublished. In this screen, a unique receptor called CX3CR1 was identified as a hit as being pro-viral.

Fractalkine receptor, CX3CR1

The family of chemotactic cytokines, appropriately named chemokines, are generally low molecular weight proteins (ranging from 8-17 kDA) [161]. Their primary function is for leukocyte trafficking and activation during disease. Chemokines contain conserved cysteine residues within their amino-terminal polypeptide sequence, and it is the number and structural arrangement of these cysteine residues that classifies each chemokine into one of four sub-families (C, CC, CXC, and CX3C) [162]. Chemokines signal through G protein-coupled, 7-transmembrane receptors to promote trafficking of cells along a chemoattractant gradient [163]. Upon interaction between a chemokine and chemokine receptor, the receptor triggers a flux in intracellular calcium ions leading to actin cytoskeleton assembly and cellular movement [163]. Chemokine receptors can be utilized by more than one specific chemokine ligand, which is hypothesized as a mechanism to protect against non-functional ligands or receptors [164]. While the primary function of chemokines is trafficking of immune cells, they have also been identified as important factors in immunity to pathogens, carcinogenesis, autoimmunity, and angiogenesis [165-168].

CX3CL1 (or fractalkine) is the only member of the CX3C chemokine sub-family and was first characterized in 1997, and is unique in that it exists in both a soluble and membrane-bound form [169]. The human CX3CL1 molecule that is about 95 kDA consists of 373 amino acids and it is functionally divided into 4 domains: an extracellular domain, a mucin-like stalk domain, a transmembrane domain, and an intracellular domain [169]. The soluble form, about 70 kDA and containing the N-terminal chemokine domain, has been shown to be a chemotactic factor for T cells [170], NK cells [171], monocytes [172], and mast cells [173]. Recently, CX3CL1 has been shown to play a role in angiogenesis and endothelial cell chemotaxis [174]. It has been shown that several proinflammatory cytokines such as IFN-γ and TNF-α can induce the expression of CX3CL1 [175, 176], as well as the presence of LPS [177].

CX3CR1 is a seven-transmembrane GPCR that is coupled to Gi and Gz subtypes of G proteins [178]. CX3CR1 has been shown to be expressed on T cells [179], dendritic cells and monocytes [179, 180], microglia [181], neurons [182], and lung epithelial cells [183]. It was previously thought that fractalkine, or CX3CL1, served as the only ligand for CX3CR1, however it was recently determined that the external loops of the polypeptide chain of CX3CR1 form a binding site for CCL26 [184]. Despite this, CX3CR1 remains the only known receptor for fractalkine, which aids in the identification and analysis of biological effects of fractalkine. $CX3CR1^{+}$ T cells have been shown to be important in viral infections such as vaccinia virus, as shown by CX3CR1-/- mice being more susceptible to vaccinia infection [185].

Other roles of CX3CL1 and CX3CR1 have been shown to have both positive and negative effects. CX3CR1 knockout mice have been shown to have reduced

neurogenesis and impaired performance in memory tasks [186, 187]. Neurotoxicity due to microglia can be controlled by the CX3CL1/CX3CR1 axis, as shown by using CX3CL1 to block LPS induced cell death [176, 188, 189]. Increased levels of CX3CL1 in serum and bronchoalveolar lavage fluids from lung and airway tissue have been found in patients with allergic asthma and rhinitis, which could be related to the general function of trafficking mast cells. $CX3CR1^{+}$ T cells also have been shown to have a role in inflammatory cell recruitment after stimulation with an allergen [190, 191], which is supported by the finding that adoptive transfer of wild type $CD4^{+}$ T into CX3CR1 deficient mice restores the features of asthma in those mice [170]. CX3CL1 contributes to the trafficking and localization of $CX3CR1^{+}$ T cells in the synovial joints, causing inflammation that is a characterization of rheumatoid arthritis (RA) [192]. This observation has been replicated in studies revolving osteoarthritis (OA), where increased levels of CX3CL1 have been found in OA patients [193-196].

Two isoforms of CX3CR1 have been identified that are created by alternative splicing [197]. In the open reading frame of CX3CR1, there are two single-nucleotide polymorphisms that each cause an amino acid change in the CX3CR1 protein, V249I and T280M, which are positioned in the sixth and seventh transmembrane domains, respectively [198]. Significant phenotypic differences relating to diseases have been observed in individuals with these allelic changes, such as a reduced risk for coronary artery disease [199], as well as an accelerated progression to AIDS in human immunodeficiency virus 1 (HIV-1) infected patients [200]. It has been reported that the CX3CR1 variants can affect the progression and survival time of amyotrophic lateral sclerosis (ALS), otherwise known as Lou Gehrig's disease [201]. This has currently been

the most significant gene factor associated with ALS, and further illustrates the importance of immune cell trafficking in human disease. The CX3CR1 variants have also been shown to play a role in multiple sclerosis [202], macular degeneration related to age [203], and Crohn's disease [204].

Viral interaction with chemokine receptors

Since the discovery surrounding HIV-1's ability to co-opt chemokine receptors for entry and replication, there has been a growing interest in the role of chemokine receptors during viral infections. New studies in immunology and virology have been opened, as well as the creation of novel avenues of disease intervention. Viruses are known to hijack chemokine receptors as a strategy for evasion, but little is known if they utilize these receptors during their replication cycles. Studying the way viruses utilize host chemokine receptors can provide new targets for drug design, as well as help elucidate mechanisms of immune evasion and replication cycle.

One mechanism of immune evasion through chemokine receptor utilization is to infect the immune cells themselves, such as in the case of HIV-1. This virus binds to several chemokine receptors as co-receptors: CXCR4 (fusin) [205], CCR5 [206], CCR3 [207], and CX3CR1 [197]. By binding to these receptors, the virus is not only able to utilize the receptor for cellular entry, but it also reduces interaction to any present chemokine ligand. The majority of these findings were discovered by identifying genetic variations in these receptors due to viral resistance being found in populations with these genetic changes. As discussed earlier, CX3CR1 has two isoforms which have been found as potent HIV-1 co-receptors and individuals infected with HIV-1 that are homozygous

for I249M280 progressed to AIDS more quickly than those of other allelic haplotypes [200].

Retroviruses are not the only virus family that has been shown to utilize chemokine receptors during infection. Herpesvirus, poxvirus, and papillomavirus families have been shown to exploit CXCR4 during replication. It is shown that in herpes simplex virus 1 (HSV-1) dendritic cell infection, CXCR4 is dramatically downregulated, although the precise mechanism is unknown [208]. Also, B lymphocyte infection by Epstein Barr virus (EBV) was shown to decrease surface expression of CXCR4 at early points of infection [209], as well as encode a GPCR called BILF1, which was found to form heterodimers with CXCR4 [210]. One herpes virus is particular adept at using GPCRs, and that is human cytomegalovirus (HCMV), which is able to encode four GPCRs (US27, US28, UL33, and UL78), all that have been shown to have similarity to chemokine receptors, with US28 able to signal in response to chemokines [211-213]. The remaining 3 GPCRs have been shown to have direct interaction with both CXCR4 and CCR5 [214].

Poxviruses, such as Myxoma virus, have been shown to utilize chemokine receptors for entry into the host cell. When CCR1, CCR5, or CXCR4 were transfected into 3T3 cells, Myxoma virus infection became possible [215]. Pappilomaviruses express have also demonstrated to utilize the chemokine-chemokine receptor axis during replication. Expression the oncogenes E6 and E7 by human pappilomavirus 16 and 18 (HPV16 and HPV18) results in increased expression of CXCR4 and CXCL12 [216, 217]. Chikungunya virus (CHIKV) is an alphavirus, which has a positive sense RNA genome.

Although the mechanism is unclear, it has been reported that CHIKV causes an upregulation of CXCR4 in 293T cells and human dermal fibroblasts [218].

Narrowing chemokine usage to CX3CR1, the first pathogen described utilizing this receptor was HIV-1. However, this is not the only virus to be shown to use CX3CR1 during the replication cycle. Recent studies have shown that CX3CR1 plays several roles in respiratory syncytial virus (RSV) infection. In addition to having the ability to modulate interferon and cytokine signaling [219-221], the RSV G protein contains a CX3C chemokine-like motif which allows the secreted form of the G protein to act as a CX3CL1 mimic by binding to CX3CR1 [222]. This causes an impaired ability for T cells to traffic to the lungs as well as causing cytotoxicity to the $CX3CR1^{+}$ cells. An increase in inflammation and eosinophilia in the formalin-inactivated RSV vaccine is also linked to the G protein CX3C-CX3CR1 interaction [223]. As mentioned earlier, CX3CR1 has been shown to be expressed on human airway epithelial cells. In the same study, it was determined that RSV co-localizes with CX3CR1 on the cilia in a G-protein dependent manner [183]. This is indicative of a possible co-receptor for RSV, and begins to illustrate the importance of CX3CR1 in lung viral infections.

Conclusions

Influenza virus is an infectious pathogen that can cause severe illness in humans on a global scale. While vaccination remains mostly effective, novel strains of influenza continue to arise creating the potential for a pandemic. Development and production of efficacious vaccines remains to be expensive, time-consuming, and inadequate to meet the public health demand. With incidence of resistance to current antiviral drugs

increasing, novel therapeutic approaches must be produced. By targeting host genes for drug design, resistance may be substantially delayed in the viral population. RNAi is an attractive technology to screen for host genes involved in virus replication cycle, as well as create avenues in disease intervention. CX3CR1 is an important gene in influenza replication and could be an excellent target for disease prevention and intervention.

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CHAPTER 3

THE FRACTALKINE RECEPTOR, CX3CR1, IS INFLUENTIAL IN INFLUENZA INFECTION

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Abstract

Influenza A virus (IAV) affects the human population worldwide, especially in infants and the elderly, and because IAV mutates from year–to- year it presents difficulties in generating effective methods of control. Investigation of host cell interactions has provided new targets for therapeutic approaches in disease intervention. The chemokine receptor CX3CR1 has become increasingly identified as a common host protein target involved in viral infections. To begin to address the impact of CX3CR1 on IAV infection, RNA interference (RNAi) studies were performed to determine the importance of CX3CR1 on IAV replication. RNAi was used for CX3CR1 gene knockdown *in vitro* followed by IAV infection. Knockdown of CX3CR1 resulted in appreciably lower viral titers across multiple IAV strains. Mice lacking CX3CR1 also showed decreased lung viral titers at early time points of infection, suggesting a role for CX3CR1 in IAV infection *in vivo*. These findings suggest that CX3CR1 is involved in the late stages of IAV replication and could provide a new therapeutic target for IAV disease intervention.

Introduction

IAV continue to be a major cause of disease in human populations with seasonal IAV causing around 36,000 deaths in the U.S. annually [1]. Vaccination remains the most effective treatment for preventing influenza virus infection, but the ability of the viruses to evolve and change their antigenic structure continues to force new vaccines to be produced. Due to IAV and the high mutation rate which often occurs in the virion surface glycoproteins, a consequence is evasion of the host immune system [1]. These mutations provide a selective advantage for specific viral strains, and such changes in viral structure is called antigenic drift. Due to the segmented nature of the virus, coinfection of a single host with two different influenza viruses can create progeny viruses that contain gene segments from both parents. If this process of reassortment involves the hemagglutinin (HA) and/or neurominidase (NA), the term antigenic shift is used [1]. Antigenic shift occurs less frequently but often results in a pandemic virus strain that increased mortality in the human population. Both types of influenza evolution have continued to present problems in human health throughout the world.

During IAV replication, the virus must utilize many host cell factors for every stage of replication. While some cellular components can be inhibitory of viral replication, some are essential. Knowledge of these host-virus interactions can provide further understand of IAV biology, as well as identify targets for the potential development of therapeutic disease interventions. RNAi interference (RNAi) is a new approach for investigating virus-host interactions. In eukaryotic cells, RNAi is a natural pathway that silences gene expression in a mRNA homology-dependent manner [2]. In this pathway, a special class of RNAs called microRNAs (miRNAs; refs.[2, 3]) regulate

host gene expression through sequence specific interaction with transcribed gene products [4].

With the knowledge of this natural process of the host cell, researchers have designed reagents that are able to capitalize on this pathway in order to regulate desired gene expression. One reagent in particular, synthetic dsRNA, also called short-interfering RNA (siRNA), is able to perform similar functions as the endogenous miRNA. siRNA is introduced to the cell exogenously but associates into the RNAi pathway in similar style. Once in the host cell, the siRNA associates with the RNA-induced silencing complex (RISC). When complexed with RISC, the siRNA guide strand can anneal to its complementary target mRNA, upon which the target mRNA is cleaved by Argonaute 2, which is an endonuclease of RISC [5, 6]. Once the gene is silenced, the effect on the viral replication cycle can be studied. Recently, several different laboratories have applied the use of RNAi in investigating the role of host proteins in multiple viral infections, including IAV [4].

The first RNAi screens for IAV were performed. in 2008 [7]. In these studies, IAV was modified genetically (HA was replaced with a glycoprotein from vesicular stomatitis virus and NA with luciferase) to be able to infect *Drosophila* cells and detect viral replication. The authors were able to identify 110 genes upon when depleted via RNAi in *Drosophila* cells showed a significant effect on IAV gene expression [7]. This primary study showed the benefit and applicability of genome-wide RNAi screens to identify host proteins involved in viral replication cycles.

Further development for genome-wide screening has led researchers into mammalian host platforms to increase the relevance of the findings. Using osteosarcoma

cells (U2OS) over 17,000 human genes were examined, while two different groups, Konig et al. and Karlas et al. used a human lung cell line (A549 cells) [8-10]. Brass et al. and Konig et al. used a recombinant IAV/A/WSN/1933, replacing the H1N1 HA with *Renilla* luciferase and were able to measure the activity of the luciferase as their detection method for IAV replication. This method allows for identification of host genes that are crucial in the early to middle stages of IAV life cycle, however with a non-existent HA gene, the virus cannot complete a full replication cycle. Karlas et al. used the wildtype strain A/WSN/33 (WSN), as well as a pandemic H1N1 strain A/Hamburg/04/2009, and quantified viral replication by first staining for NP in the siRNA treated and WSN infected cells [10]. Prior to staining, the supernatant was removed and transferred onto reporter 293T cells that had an influenza-specific luciferase reporter that allowed for quantification of viral particles released from the siRNA transfected cells [11]. This allowed Karlas et al. to identify host genes that were involved in any stage of IAV replication. Studies by Bakre *et al.* have performed genome-wide screens by looking at several specific classes of proteins including protein kinases and G-protein coupled receptors [12]. One particular gene that was found to be required for IAV replication in A549 cells was the fractalkine receptor (CX3CR1), a host factor also important for respiratory syncytial virus (RSV) infection [13].

CX3CR1, also known as V28 or GPR13, is a member of the chemokine superfamily, which consists of 4 families[14]. Each member of these families share the same ability to chemoattract cells to their G-protein coupled receptor. The CX3CR1 ligand fractalkine (CX3CL1) is the only member of the CX3C chemokine family. CX3CL1 consists of a chemokine domain that is linked to a transmembrane domain via a

mucin-rich stalk of an extracellular domain. The chemokine is synthesized as membrane-anchored form and may be cleaved in the soluble form by different metalloproteases [15]. Soluble CX3CL1 resembles a conventional chemokine exhibiting efficient chemotactic activity for human monocytes, NK cells, T cells, dendritic cells, and a subset of germinal center B cells [15, 16]. CX3CL1 expression has been reported in epithelial cells in the lungs, intestines, and kidneys [17, 18] and can also be expressed in endothelial and smooth muscle cells during inflammation [19, 20]. CX3CR1 is also expressed in a wide variety of hematopoietic cells, including most leukocytes such as monocytes[21], dendritic cells[22], NK cells and T cells[21, 23]. It is also found in cells of the central nervous system such as neurons and microglia [24]. Fractalkine and its receptor have been found to mediate migration, adhesion, and proliferation of the immune cells previously mentioned [25, 26], as well as promoting survival of CD4+ T cell subsets that causes an increase in airway inflammation, promoting asthma [27].

While CX3CR1 has not been described as being involved in IAV infection, its role in RSV infection has been studied. In addition to being able to modulate interferon and cytokine signaling[28-30], the RSV G protein contains a CX3C chemokine-like motif which allows the secreted form of the G protein to act as a CX3CL1 mimic by binding to CX3CR1[31]. This causes an impaired ability for T cells to traffic to the lungs as well as causing cytotoxicity to the CX3CR1+ cells[31]. An increase in inflammation and eosinophilia in the formalin-inactivated RSV vaccine is also linked to the G protein CX3C-CX3CR1 interaction [32]. In a murine model, mice that lacked CX3CR1 and infected with RSV showed a significant decrease in NK1.1 (+) and CD11b (+) cells trafficking to the lung with reduced IFNγ production when compared to WT mice [30].

Recently, it has been shown that CX3CR1 co-localizes with RSV particles in a human airway epithelial cell model, suggesting that it is a co-receptor for RSV [13].

Chemokine receptors have been shown to be important factors in viral life cycles. HIV-1 utilizes CCR5 and CXCR4 as co-receptors for cell entry [33, 34]. Along with RSV as mentioned before, human cytomegalovirus genome encodes four seven transmembrane chemokine-like receptors, which can bind to chemokines such as CX3CR1 with high affinity [35, 36]. In this study, we investigate the role of CX3CR1 in IAV replication *in vitro* and *in vivo*. The results suggest that CX3CR1 is involved in late stages of the IAV life cycle and may be being utilized by viral proteins for assembly and trafficking. This study provides insights into IAV replication and life cycle and could help elucidate new methods of disease intervention by utilizing drugs for targeting host genes.

Materials and Methods

*Cell cultures, virus stocks, and mice used***.** Adenocarcinomic human alveolar basal epithelial cells (A549) and Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 5% fetal bovine serum (FBS) and 5% L-glutamine. Homo sapiens lung adenocarcinoma (Calu-3) cells were cultured in DMEM with 20% FBS. All cells were incubated at 37°C in 5% CO_2. The influenza viruses used were A/WSN/33 (H1N1), A/Philippines/2/82, and A/Vietnam/1203/04;H5N1 and was propagated in the allantoic cavity of embryonated hen eggs at 37°C for 48-72 hours. The virus was aliquoted and stored at -80°C.

Female and male 4-8 week old CX3CR1 -/- mice were used for all studies. Female 4-8 week old C57BL/6 mice were used for all studies. All experiments were performed with 5 mice per group.

siRNA reverse transfection. 4 pooled siRNAs were used to target CX3CR1 (SMARTpool; Dharmacon ThermoFisher, Lafayette, CO). siRNAs were resuspended in Dharmacon siRNA buffer to a concentration of 1 μM and stored at -20°C until use. In all siRNA studies, an siRNA targeting the MAP2K gene (siMEK), a well characterized human kinase gene important for IAV replication [37], was used to control for the transfection efficiency, host gene silencing, and viral replication level. A non-targeting scrambled siRNA control (siNEG) was also used in all siRNA assays. Transfection of siRNAs was performed as previously shown [12]. Briefly, siRNAs were diluted 1:1 with HBSS and allowed to incubate for 5 minutes. Dharmafect-1 transfection reagent (Lafayette, CO) and HBSS were added such that each well received 0.004 mL of transfection reagent and 0.096 mL HBSS. The siRNA/transfection mix was allowed to incubate for 20 minutes at room temperature after which 0.08 mL of 1.5×10^4 A549 cells suspended in DMEM/5% FBS was added to each well, and the plate was incubated for 48 hours at 37°C in 5% CO_2. The final concentration of siRNA for all siRNA studies was 50 nM.

Quantitative RT-PCR. Total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA) and stored at -80°C until used. The quantity of total RNA was determined using a NanoDrop ND-1000 Fluorospectrometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription of pooled RNA was performed using random hexamers and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). cDNA diluted 1:10

was used as template using CX3CR1 gene expression assays (Applied Biosystems, Foster City, CA) and analyzed using MX300P software by Stratagene (La Jolla, CA). CX3CR1 expression is normalized to 18S ribosomal RNA (rRNA) gene expression. Data is presented as percent decrease in gene expression relative to 18S rRNA expression.

Cytotoxicity and virus infection. To determine if siRNA gene silencing was cytotoxic, the cell supernatants from siRNA transfected A549 cells were analyzed for adenylate kinase (AK) using a Toxilight kit (Lonza, Rockland, ME) as previously described [12]. A459 cells were subsequently infected with A/WSN/33 at an MOI of 0.001, 0.01, and 0.1 pfu/cell. Cells were incubated for 48 hours at 37°C in 5% CO_2. All assays were run in quadruplicate.

Viral titers. Virus titers in siRNA-treated A549 cells infected with A/WSN/33 were determined by plaque assay or by modified $TCID_{50}$ followed by a hemagglutination assay (HA). Briefly, virus infected A549 cell culture supernatants were serially diluted ten-fold and added to MDCK cells. The MDCK cell plates were incubated for 72 hours followed by an HA using 0.5% chicken red blood cells as previously described [38]. All viral titer assays were run in quadruplicate.

Immunofluorescence staining. A549 cells were fixed with cold methanol: acetone (80:20) for 15 min and incubated with primary antibodies (mouse anti-NP monoclonal antibody [ATCC; H16-L10-4R5] [5 µg/mL] followed by incubation with appropriate secondary antibodies (Alexa 488-conjugated goat anti-mouse [Invitrogen, Carlsbad, CA] and 4', 6-diamidino-2phenylindole (DAPI) counterstain (Invitrogen, Carlsbad, CA) (2µg/mL), as previously described [39]. Cells were visualized and counted using a

Cellomics ArrayScan system (Thermo Fisher Scientific), an automated fluorescence microscope coupled with image and analytical software.

Virus challenge experiments. C57BL/6 mice were anesthetized and intranasally (i.n.)-inoculated with 1 50% lethal doses (LD_{50}) A/WSN/33 (500 PFU diluted in 50 μL PBS. Mice were monitored daily for morbidity and mortality with body weights measured every day. On days 3, 5, and 7 post infection (p.i.), groups of mice were humanely euthanized and lungs collected into 1.0 mL PBS, homogenized at 4°C by use of gentleMACS^{TM} Dissociator (Miltenyi Biotec), clarified by centrifugation at 4°C for 10 min at 200xg, and aliquoted for storage at -80°C or used directly. A $TCID_{50}$ assay was then used to determine virus titers from clarified homogenates as previously described [38].

Statistical analyses. Statistical analyses were done using Student's *t* test or one-way analysis of variance (ANOVA), as indicated. Results were calculated as means ± standard errors. Values of $P < 0.05$ were considered significant.

Results

CX3CR1 is expressed in A549 cells. To confirm that CX3CR1 was expressed on A549 cells, we isolated genomic DNA and total RNA from A549 cells and amplified a fragment of the CX3CR1 gene and mRNA by PCR and RT-PCR, respectively. As shown in supplemental Figure 3.1A, both CX3CR1 and mRNA were amplified by PCR displaying a band size of 122 bp. To determine if these cells were expressing CX3CR1 at protein level, A549 cells were fixed, permeabilized, and stained with an anti-human CX3CR1 antibody conjugated to Phycoerythrin (PE) and then analyzed via flow

cytometry. As shown in supplemental Figure 3.1B, there is CX3CR1 expression in A549 cells measured from total RNA by qPCR. In supplemental Figure 3.1C, A549 cells stained with antiCX3CR1-PE showed medium-low fluorescence intensity for PE when compared to isotype controls, indicating that CX3CR1 was expressed to a low level in these cells.

siRNA specific for CX3CR1 reduce mRNA and protein expression. To validate the specificity of silencing of mRNA expression by the siRNAs, a pool of siRNA was deconvoluted into individual siRNAs labeled as siCX3CR1(1), siCX3CR1(2), siCX3CR1(3), and siCX3CR1(4). This method of validation is used when pools are used for the primary screen. The deconvolution of the pool and identification of two or more individual siRNAs from the pool induce the same phenotype allow for the validation. A549 cells were then transfected with these individual siRNAs (Figure 3.2A). Forty-eight hours post-transfection, total RNA was harvested and RT-qPCR was performed to measure the levels of CX3CR1 transcript. Each of the four CX3CR1 siRNAs were able to reduce CX3CR1 transcript levels significantly compared to non-targeting siRNA control (siNEG) (Figure 3.2A), demonstrating that the siRNA efficiently inhibited CX3CR1 expression. As expected, the non-targeting siRNA control (siNEG) and the siRNA against MAP2K (siMEK, siRNA that inhibits influenza replication) did not reduce CX3CR1 expression demonstrating that these siRNAs do not target CX3CR1 mRNA. The siRNAs siCX3CR1(2) and siCX3CR1(3) caused cytotoxicity in the form of monolayer loss (Figure 3.2B), and these siRNAs were excluded from future experiments. Taken together, our results show that siCX3CR1(1) and siCX3CR1(4) significantly

reduce CX3CR1 gene expression in A549 cells without causing cytotoxicity, thus we used these siRNAs for studying the effect of CX3CR1 deficiency on IAV replication.

Inhibition of CX3CR1 reduces IAV replication. To determine the effect of CX3CR1 on IAV replication, A459 cells were transfected siCX3CR1(4). At 48 hours post-transfection, cells were infected with influenza WSN at a MOI of 0.001-0.1 and incubated for 24 or 48h. The cells were fixed, permeabilized, and stained for influenza NP (Figure 3.3A-D). A Cellomics Array Scanner was used to quantitate the number of infected cells compared to total number of cells for each time point and condition (Figure 3.3B and 3.3D). Immunoflurescence antibody assay showed NP expression did not increase from 24 to 48h post-infection in cells that were treated with siCX3CR1 as opposed to those treated with siNEG. This phenotype was independent of the MOI, showing that IAV has decreased replication in CX3CR1-silenced cells when compared to cells that were transfected with siNEG. At 48 h post infection, cells transfected with siCX3CR1 had significant reductions in infected cells compared to control-treated (siNEG) cells, demonstrating that knockdown of CX3CR1 expression reduces influenza replication and spreading to the neighboring cells (Figure 3.3B and 3.3D). Surprisingly, the decrease in influenza WSN virus titer was greater in siCX3CR1 treated cells than in siMEK treated cells (positive control) (Figure 3.3E and 3.3F).

Inhibition of CX3CR1 reduces IAV titer in vitro. Since a significant decrease in influenza WSN virus titer was shown compared to the non-targeting control, we sought to investigate the replication kinetics of influenza WSN in cells treated with siCX3CR1(4). Thus, A549 cells were reverse transfected with siCX3CR1(4) for 48 h, then infected with increasing amount of influenza WSN for 12 to 48h and infection was measured by plaque

assay or IFA. As shown in Figure 3.4, CX3CR1 mRNA knockdown significantly decreased influenza WSN replication when the multiplicity of infection was 0.001 (Figure 3.4C), but not when the MOI was equal to 0.1 (Figure 3.4A), and a similar effect was observed in siMEK treated cells. This suggests that when the initial inoculum is high enough, influenza is able to overcome the absence of CX3CR1 expression probably by infecting the small fraction of cells that did not receive siRNA during the transfection reaction or by infecting the surrounding cells through cell-to-cell transfer [40]. Furthermore, the inhibition of influenza WSN replication was only significant at later time points indicating that CX3CR1 is being utilized during later stages of the IAV life cycle (Figure 3.4C). To corroborate that CX3CR1 is important for the replication of other influenza viruses, A549 cells were reverse transfected with siCX3CR1(4) for 48 h and then infected with IAV A/Philippines/2/82 (X79, H3N2) and a highly pathogenic avian influenza H5N1 virus (HPAIV). As shown in Figure 4D-F, siCX3CR1 treatment reduced both X79 and H5N1 replication in A549 cells, however the inhibitory effect on H5N1 replication was evident much earlier than in X79 infected cells. This difference is probably due to the fact influenza H5N1 replicates much faster than X79 and WSN in A549 cells. Taken together, these results suggest that CX3CR1 is required for influenza virus infection at stages later than virus attachment and entry.

IAV WSN replication is decreased in vivo in CX3CR1 null mice. As mice were the preferred model to show the importance of CX3CR1 in an influenza virus infection, we first wanted to demonstrate that mouse cells showed a similar reduction in influenza virus titer cells were treated with siCX3CR1. Thus, MLE-15 cells were transfected with each individual deconvoluted siCX3CR1 from the original pool for 48h, then infected with

influenza WSN for 48h and infection was measured by TCID50. As shown in figure 3.5A, siCX3CR1 treated cells had a significant reduction in influenza WSN replication, although to a lesser degree than the A549 cells, possibly due to the siRNA having a decreased knockdown efficiency in the MLE-15 cells. To assess the importance of CX3CR1 on influenza replication *in vivo*, C57BL/6 and CX3CR1^{tm1Litt} mice (CX3CR1 null) were infected with WSN at a non-lethal dose and lung viral burden was measured at days 3, 5 and 7 post infection by TCID50 (Figure 3.5B). At 3 days post-infection, WSN lung viral titers were decreased in CX3CR1 null mice when compared to wild-type C57BL/6 mice, but no difference was evident at days 5 and 7 pi. This data shows that influenza requires CX3CR1 expression during the first 3 days of infection, and absence of this chemokine receptor induces a delay in virus replication.

In summary, our *in vitro* studies suggest that influenza utilizes CX3CR1 at stages later than attachment and entry, possibly during viral protein trafficking and/or viral assembly, and our *in vivo* studies in CX3CR1 null mice, show a delay in the replication kinetic suggesting that CX3CR1 has a role in virus particle trafficking and release. More studies would need to be performed to define the mechanism by which CX3CR1 aids influenza virus release from infected cells.

Discussion

A previous study in our laboratory showed that CX3CR1 was a pro-viral gene during IAV infection (unpublished data). The role of CX3CR1 during respiratory syncytial virus infection and pathogenesis is better known, leading us to investigate whether

influenza utilizes CX3CR1 in a similar fashion. CX3CR1 is a G-protein coupled receptor expressed on T cells [21], dendritic cells and monocytes [22], microglia [41], neurons [42], and lung epithelial cells [43]. Here, we show that A549 cells express intermediate levels of CX3CR1 (Sup Fig 1) and siRNA knockdown of CX3CR1 completely abolishes its expression (Fig 1). Furthermore, absence of CX3CR1expression results in a reduction of IAV replication (Fig 2), confirming the genome-wide screen previously done in our laboratory (unpublished data). Interestingly, IAV infection of siNEG control-treated A549 cells with a MOI of 0.001 resulted in 40% infection (% NP positive cells) by 24 h and nearly 100% by 48 h, on the other hand, IAV infection of siCX3CR1-treated A549 cells resulted in 20% infection by 24h with no increase by 48h. This result was independent of multiple MOI used, suggesting that IAV was able to infect these cells, but unable to spread to neighboring cells (Fig 2).

This reduction in % infection of siCX3CR1-treated cells correlated with a reduction in virus production and release to the cell supernatant (Fig 3). While a high MOI did not result in decreased viral titer, lower MOIs demonstrated the importance of CX3CR1 in influenza infection, as these lower doses are more representative of a natural infection. Moreover, the same results were observed with other IAV strains including and H3N2 (A/Philippines/2/82) and a highly pathogenic H5N1 virus (A/Vietnam/1203/04), suggesting that the use of CX3CR1 during IAV replication is conserved across strains.

To speak to a role for CX3CR1 for influenza replication *in vivo*, we first demonstrated that treating MLE-15 cells, a mouse lung epithelial cell line, resulted in reduced influenza viral titers in the supernatant(Fig 4). This result led us to pursue an *in*

vivo model, as CX3CR1 null mice were i.n. infected with influenza and the lung virus titers determined at days 3,5,and 7 (Fig 4). It was observed that absence of the CX3CR1 resulted in a delay in viral replication, with lower titers at day 2-3 and higher viral titers at day 8 pi compared to wild type mice. The reduction in virus titer at early time points correlates with the *in vitro* results, suggesting that lack of CX3CR1 expression could result in impaired virus trafficking or egressing from epithelial cells.

As IAV readily change/mutate, and often become resistant to current antiviral drugs, a different approach must be considered when designing antiviral therapeutics. Targeting host genes to decrease viral replication is becoming an attractive approach for drug design. CX3CR1 expression is associated with inflammatory diseases such as osteoarthritis and rheumatoid arthritis [44]. It may be possible for therapeutics for these diseases to provide an intervention in IAV infection.

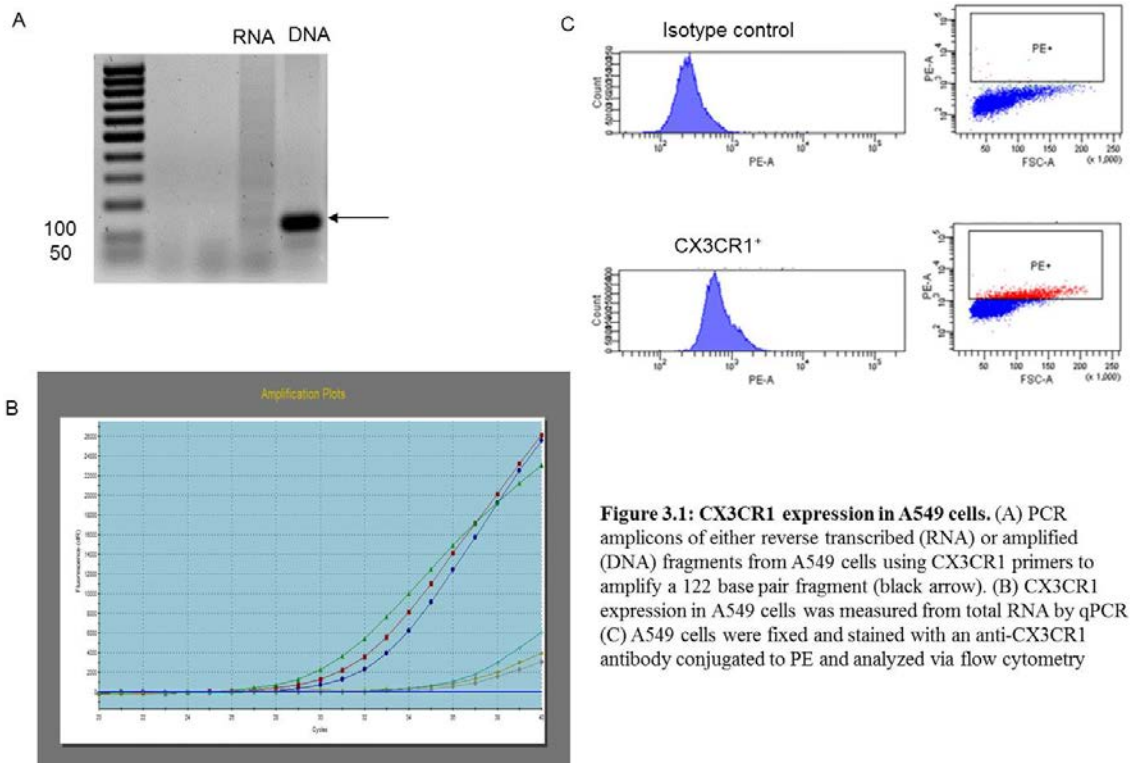

Figure 3.1: CX3CR1 expression in A549 cells. (A) PCR amplicons of either reverse transcribed (RNA) or amplified (DNA) fragments from A549 cells using CX3CR1 primers to amplify a 122 base pair fragment (black arrow). (B) CX3CR1 expression in A549 cells was measured from total RNA by qPCR (C) A549 cells were fixed and stained with an anti-CX3CR1 antibody conjugated to PE and analyzed via flow cytometry

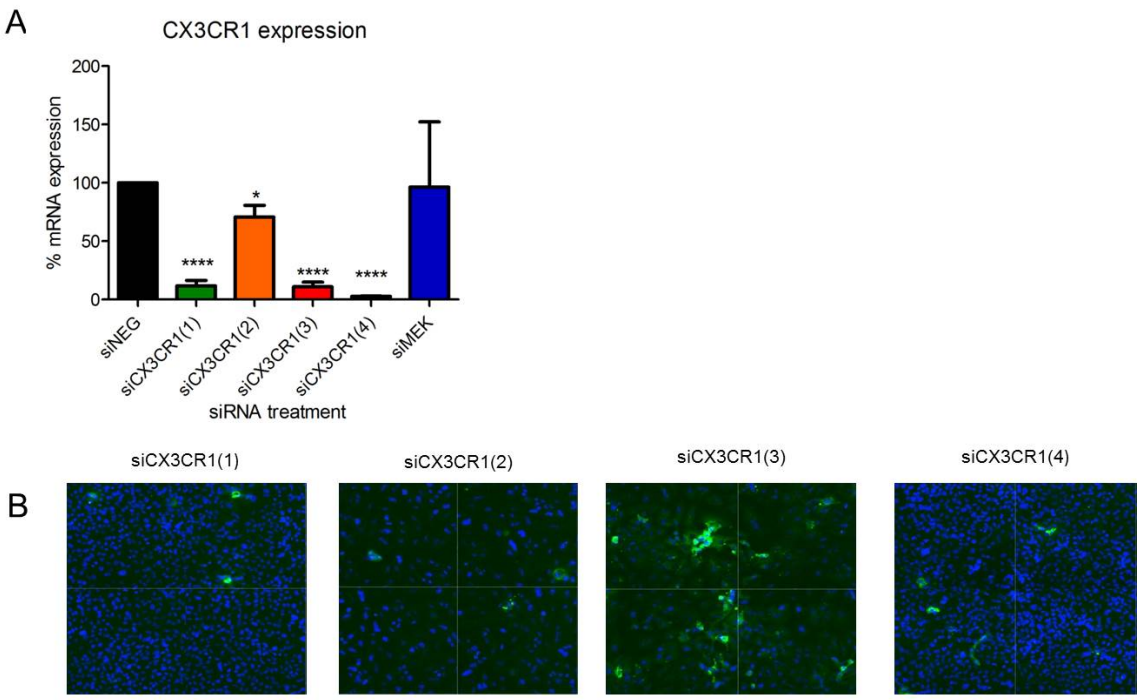

Figure 3.2: siRNAs decrease CX3CR1 expression resulting in decreased infection and viral replication *in vitro*. (A) A549 cells were transfected with 50 μM of designated siRNA for 48 hours followed by RNA isolation and RT-qPCR . (B) 48 hours post-transfection, A549 cells were infected with WSN at 0.001 MOI. Cells were stained 48 hours p.i. for IAV NP and DAPI (B). *p<0.05

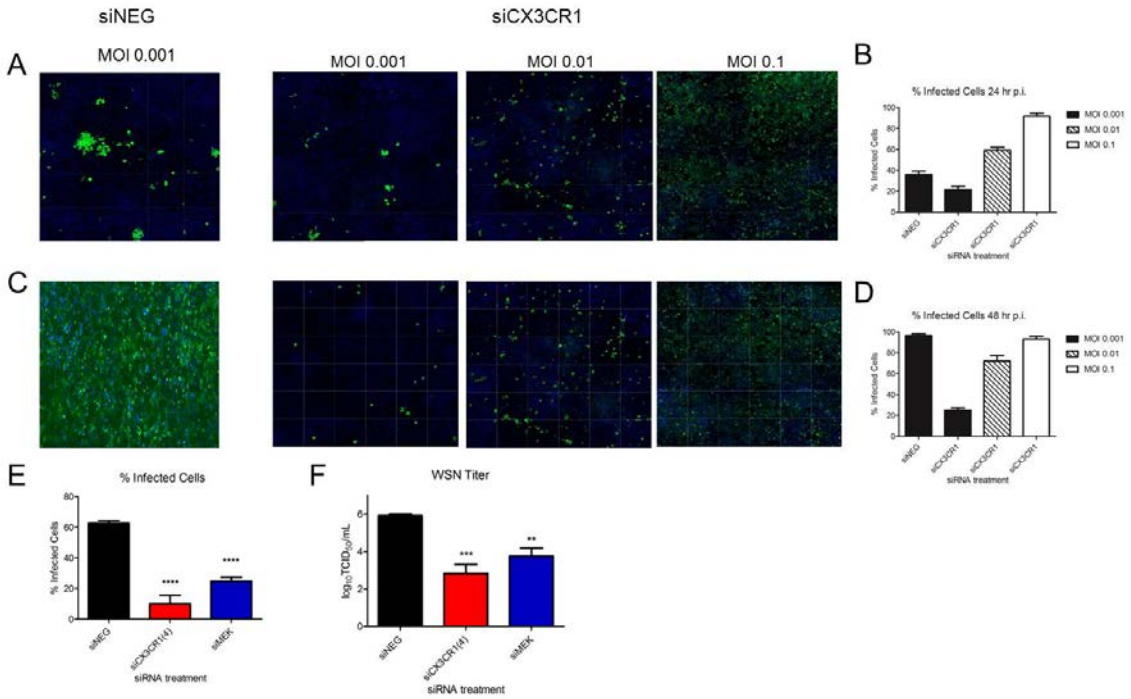

Figure 3.3: Inhibition of CX3CR1 reduces IAV replication. A549 cells transfected with siNEG and siCX3CR1(4) for 48 hours, infected with WSN at indicated MOI for 24 hours (A,B) and 48 hours(C,D), and stained for IAV NP. Cells were stained 48 hours p.i. for IAV NP and infected cells were measured via cellomics array scanner. (E) A549 cells transfected with siNEG, siCX3CR1(4), and siMEK for 48 hours and infected with WSN at an MOI of 0.001 for 48 hours and stained for NP. (F) Supernatant from infected cells was removed for use in a $TCID_{50}$ for determination of viral titer of WSN.

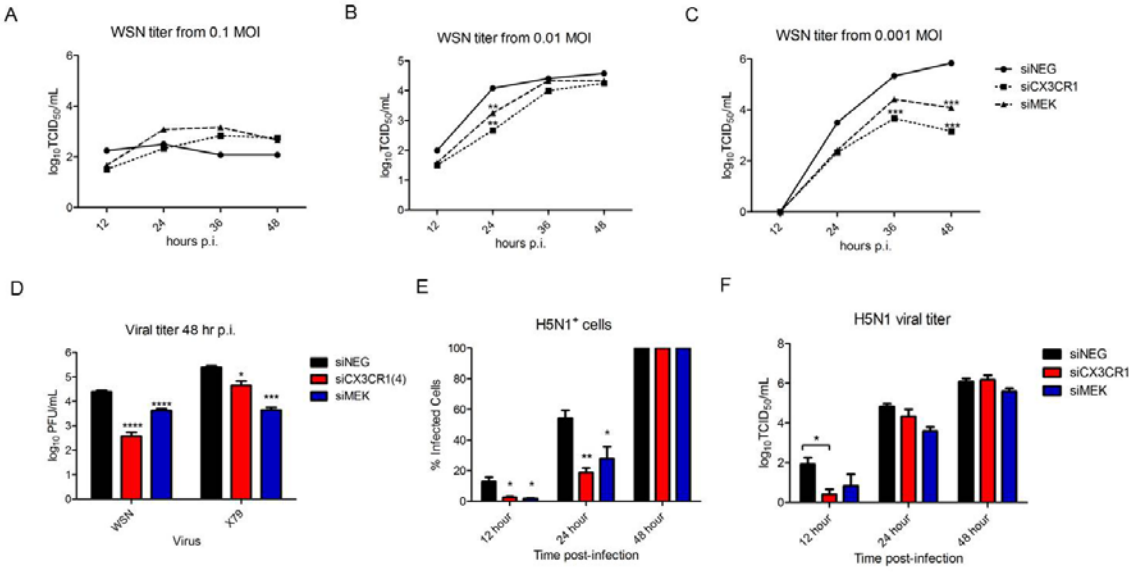

Figure 3.4: Inhibition of CX3CR1 reduces IAV titer *in vitro*. (A-C)48 hours post-transfection of siNEG, siCX3CR1, and siMEK, A549 cells were infected with WSN at indicated MOI and supernatant was removed at indicated time points p.i. for use in a $TCID_{50}$ for determination of viral titer of WSN. (D-F) A549 cells were infected with WSN at 0.001 MOI, 0.01 MOI with X79, and 0.001 HPAIV H5N1. (E) Cells were stained 48 hours p.i. for IAV NP and infected cells were measured via cellomics array scanner. (D,F) Supernatant was removed at 48 hours for X79 and at indicated time points for H5N1 for use in plaque assay or $TCID_{50}$ for determination of viral titer of WSN *$p<0.05$

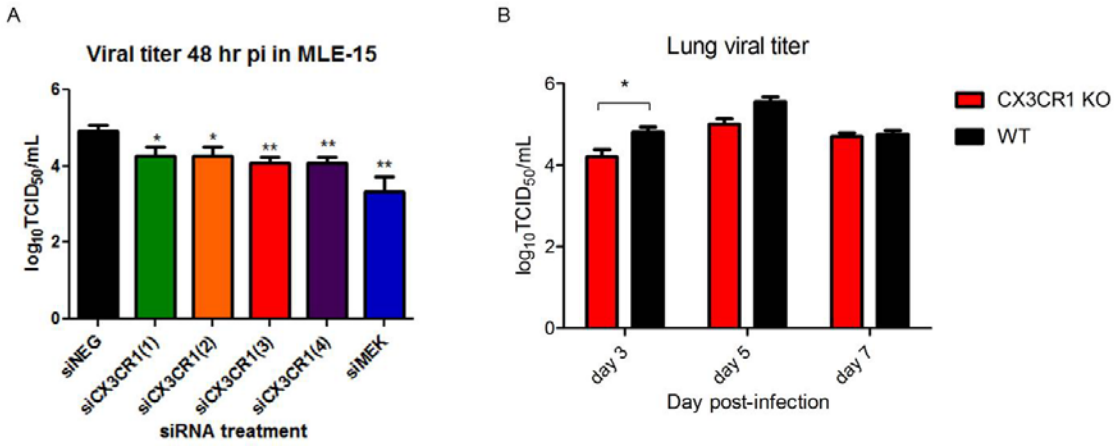

Figure 3.5: IAV WSN replication is decreased in CX3CR1 null mice. (A) MLE-15 cells were transfected with indicated siRNAs for 48 h, then infected with IAV WSN at 0.001 MOI. Supernatant was removed at 48h p.i. for use in a $TCID_{50}$ for determination of viral titer of WSN. (B) Both C57BL/6 and $CX3CR1^{tm1Litt}$ mice were infected with 0.5 LD50 WSN. Virus titers in the lungs 3, 5, and 7 days post-infection as measured by $TCID_{50}$.

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CHAPTER 4

CX3CR1 CONTRIBUTES TO INFLUENZA REPLICATION AT LATER STAGES OF INFECTION

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Abstract

Influenza A virus (IAV) has the ability to circumvent current vaccination methods. While antiviral drugs can be effective in inhibiting viral replication, resistance amongst the influenza viral populations is becoming increasingly common. As influenza must utilize host factors for replication, targeting host interacting partners has become an attractive method for inhibiting influenza replication, which reduces the chance for viral mutant populations arising. CX3CR1 was identified as a pro-viral gene for in a genome-wide screen using RNA interference (RNAi). To begin to address how influenza utilizes CX3CR1, we used RNAi to efficiently knockdown CX3CR1 and followed with an IAV infection. Blocking surface expression of CX3CR1 did not result in a decrease in influenza infection. Knockdown of CX3CR1 resulted in an accumulation of influenza nucleoprotein in the cytoplasm of cells silenced for CX3CR1, suggesting CX3CR1 affects influenza during late stages of infection. Co-transfection with viral protein constructs with CX3CR1 constructs showed a co-localization of viral and host proteins. These findings suggest that CX3CR1 is involved in the late stages of IAV replication and could provide a new therapeutic target for IAV disease intervention.

Introduction

IAV is a major cause of disease in human populations with seasonal influenza causing substantial deaths in the U.S. annually [1]. Vaccination remains the most effective treatment for preventing influenza virus infection, but the ability of the viruses to evolve and change their antigenic features continues to force new vaccines to be produced. IAV continues to threaten millions of people annually[2]. While vaccines are generally available, the ability of the virus to antigenially drift and cause may lead to vaccine failure [3]. Along with influenza vaccines, the human population is dependent on two licensed antiviral drugs that inhibit the viral neuraminidase: oseltamivir and zanamivir [4-7]. However, considerable levels of oseltamivir and zanamivir-resistance seasonal influenza viruses have arisen, which has been associated with the single amino acid change in the positions H274 and Q136, respectively [8, 9]. Due to the ability of IAV to mutate and circumvent our current vaccines and antiviral drugs, new approaches are being used by researchers to identify novel disease intervention strategies. Aiming at the host factors rather than the virus directly is a concept to combat evolution of viral resistance and develop broad-spectrum antiviral therapeutics.

Influenza virus is part of the family *Orthomyxoviridae*, and thus is an enveloped virus with a segmented genome of negative sense, single-stranded RNA. The virus has 8 segments of RNA that encode for up to 13 proteins, 3 of which are recent discoveries [10]. Three viral proteins are present in the host-derived lipid envelope, which are the hemagglutinin (HA), the neuraminidase (NA), and the matrix protein 2 (M2) ion channel. The matrix protein 1 (M1) is the most abundant component of the virion [11]. Each gene segment is encapsidated in the nucleoprotein (NP) with the ribonucleoprotein (RNP)

complex containing the three subunits of the viral polymerase (PB1, PB2, and PA) [1]. The last two proteins are the non-structural proteins NS1 and NS2. NS1 is involved in immune modulation while NS2 has been found to be a nuclear export protein (NEP) [12, 13]. The 3 new proteins (PB1-F2, PB1-N40, PA-X) are results from splicing and ORF shifts in the PA and PB1 gene segments [14-16] .

Packaging of RNPs into new virions is not very well understood but it has been observed that packaging signals exist in the 3' and 5' non-coding regions (NCR) of vRNAs [17] .It is known that if PB2's packaging signal is mutated, there are significant effects on virion formation [18]. Since the HA, NA, and M2 proteins are not imported into the nucleus, they are transported to the plasma membrane. It is known that the HA and NA undergo post-translational modifications such as N-linked glycosylation during their export through the endoplasmic reticulum and Golgi [19, 20]. Once the surface glycoproteins reach the plasma membrane, the HA and NA associate with lipid rafts while the M2 does not [21]. The cytoplasmic tails of the HA and NA contain palmitoylation sites that are believed to recruit M1 with the RNPs to bud properly [22].

Due to the ability of IAV to mutate and circumvent our current vaccines and antiviral drugs, new approaches are being used by researchers to identify novel disease intervention strategies. One approach is to target the host factors required for viral replication, with the idea that targeting host genes and not viral genes will decrease the chance for viral mutation [23]. Before designing drugs to target these host factors, the targets must first be identified to have an interaction with the virus in question, as well as identify where in the viral replication cycle the host gene is impacting the virus [23].

Genome-wide screens are utilized to identify potential targets for antiviral therapy, and the use of RNA interference (RNAi) has greatly enhanced the abilities of these screens.

RNAi interference (RNAi) is an effective approach for investigating virus-host interactions and detection of novel host factors required for viral replication [24-28]. RNAi is a natural pathway that silences gene expression in an mRNA homology-dependent manner [29]. In this pathway, a special class of RNAs called microRNAs (miRNAs;.[29, 30]) can regulate host gene expression through sequence specific interaction with transcribed gene products [31]. Several recent RNAi-based studies have identified host genes important for IAV infection and replication [32-36] to which further studies have identified pathways these genes are associated with virus life cycle [33-37]. Studies performed by our group using genome-wide screens aimed at several kinase genes and G-protein coupled receptors [38], have revealed that the CX3CR1 gene is required for efficient IAV replication in A549 cells. The fractalkine receptor, CX3CR1, is a host factor also important for respiratory syncytial virus (RSV) replication [39].

Viruses have been shown to utilize chemokine receptors during their replication cycle. Retroviruses such as human immunodeficiency virus 1 (HIV-1), use a number of chemokine receptors as co-receptors for entry into the host cell [40-42]. A number of herpes viruses, including human cytomegalovirus, have been shown to interact with multiple chemokine receptors, but their mechanisms of interaction are not well understood [43-46]. Poxviruses have also been shown to utilize chemokine receptors as co-receptors for entry, as in the case of Myxoma virus [47]. Looking closer a viral interaction with CX3CR1, it has been shown that HIV-1 uses CX3CR1 as a co-receptor [48]. As mentioned before, CX3CR1 is involved in RSV replication, as RSV can utilize

CX3CR1 as a co-receptor [39] as well impair trafficking of leukocytes by having a viral protein act as a CX3CL1 mimic to bind to CX3CR1 [49]. In this study, we investigated the mechanism by which IAV uses CX3CR1 *in vitro* and *in vivo*. The results suggest that CX3CR1 is involved in late stages of the IAV life cycle and may be being utilized by viral proteins for assembly and trafficking. This study provides insights into IAV replication and life cycle and could help elucidate new methods of disease intervention by utilizing drugs for targeting host genes.

Materials and Methods

Cell Cultures, Virus stocks, and Mice used. Adenocarcinomic human alveolar basal epithelial cells (A549) and Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 5% fetal bovine serum (FBS) and 5% L-glutamine. Homo sapiens lung adenocarcinoma (Calu-3) cells were cultured in DMEM with 20% FBS. All cells were incubated at 37°C in 5% CO_2.The influenza viruses used were A/WSN/33 (H1N1, kindly provided by Richard Webby, St. Jude Children's Research Hospital, Memphis TN and was propagated in the allantoic cavity of embryonated hen eggs at 37°C for 48-72 hours. The virus was aliquoted and stored at -80°C.

Female and male 4-8 week old CX3CR1 -/- mice were used for all studies. Female 4-8 week old C57BL/6 mice were used for all studies. All experiments were performed with 5 mice per group.

siRNA reverse transfection. 4 pooled siRNAs were used to target CX3CR1 (SMARTpool; Dharmacon ThermoFisher, Lafayette, CO). siRNAs were resuspended in

Dharmacon siRNA buffer to a concentration of 1 µM and stored at -20°C until use. In all siRNA studies, an siRNA targeting the MEK gene (siMEK), a well characterized human kinase gene important for IAV replication [50], was used to control for the transfection efficiency, host gene silencing, and viral replication level. A non-targeting siRNA control (siNEG) was also used in all siRNA assays. siRNAs were diluted 1:1 with HBSS and allowed to incubate for 5 minutes. As described previously [38], Dharmafect-1 transfection reagent (Lafayette, CO) and HBSS were added such that each well received 0.004 mL of transfection reagent and 0.096 mL HBSS. The siRNA/transfection mix was allowed to incubate for 20 minutes at room temperature after which 0.08 mL of 1.5×10^4 A549 cells suspended in DMEM/5% FBS was added to each well, and the plate was incubated for 48 hours at 37°C in 5% CO_2. The final concentration of siRNA for all siRNA studies was 50 nM.

Cytotoxicity and virus infection. To determine if siRNA gene silencing was cytotoxic, the cell supernatants from siRNA transfected A549 cells were analyzed for adenylate kinase (AK) using a Toxilight kit (Lonza, Rockland, ME). Results were normalized to a siTOX control, i.e. a siRNA control (Dharmacon) causing complete cell death by 48 hours. siRNA transfected cells with luminescence greater than or equal to 20% of the siTOX control were not considered for further evaluation. A459 cells were subsequently infected with A/WSN/33 at an MOI of 0.001, 0.01, and 0.1 pfu/cell. Cells were incubated for 48 hours at 37°C in 5% CO_2. All assays were run in quadruplicate.

Viral titers. Virus titers in siRNA-treated A549 cells infected with A/WSN/33 were determined by plaque assay or by modified $TCID_{50}$ followed by a hemagglutination assay (HA). Briefly, virus infected A549 cell culture supernatants were serially diluted

ten-fold and added to MDCK cells. The MDCK cell plates were incubated for 72 hours followed by an HA using 0.5% chicken red blood cells as previously described [51]. All viral titer assays were run in quadruplicate.

Immunofluorescence staining. A549 cells were fixed with cold methanol: acetone (80:20) for 15 min and incubated with primary antibodies (mouse anti-NP monoclonal antibody [ATCC; H16-L10-4R5] [5 µg/mL], rabbit-anti-M2 polyclonal antibody [GeneTex GTX125951], rabbit-anti-HA polyclonal antibody [GeneTex GTX127357]) followed by incubation with appropriate secondary antibodies (Alexa 488-conjugated goat anti-mouse [Invitrogen, Carlsbad, CA], Alexa 647-conjugated donkey anti-rabbit [Invitrogen, Carlsbad, CA], and 4', 6-diamidino-2phenylindole (DAPI) counterstain (Invitrogen, Carlsbad, CA) (2µg/mL) as previously described [52]. Cells were visualized and counted using a Cellomics ArrayScan system (Thermo Fisher Scientific), an automated fluorescence microscope coupled with image and analytical software.

Analysis of pFLAG IAV constructs. Following transfection of HEK293T cells, cells were lysed at 24 h post-transfection. Total protein content was determined for clarified cell lysates using the BCA protein assay kit (Pierce, Rockford, IL). Lysates were separated by SDS-PAGE, with the same amount of total protein being loaded into each lane, and then transferred to nitrocellulose paper. Immunoblots were blocked for 1 h in PBS contained -.5% Tween 20 and 5% nonfat dry milk, washed in PBS containing 0.5% Tween 20, and incubated overnight at 4°C with rabbit anti-FLAG antibody in PBS containing 0.5% Tween 20 and 5% nonfat dry milk. Membranes were washed three times with PBS containing 0.5% Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated donkey anti-rabbit.

Co-localization of IAV/CX3CR1 and Confocal microscopy. HEK293T cells were either co-transfected with IAV-FLAG constructs and plasmid expressing CX3CR1-HA for 24 hr, or transfected with plasmid expressing CX3CR1-HA for 24 hr followed by IAV infection for 12 hr. Cells were fixed with cold methanol: acetone (80:20) for 15 min and incubated with primary antibodies (mouse anti-HA tag monoclonal antibody

Virus challenge experiments. C57BL/6 mice were anesthetized and intranasally (i.n.)-inoculated with 1 50% lethal doses (LD_{50}) A/WSN/33 (500 PFU diluted in 50 μL PBS. Mice were monitored daily for morbidity and mortality with body weights measured every day. On days 3, 5, and 7 post infection (p.i.), groups of mice were humanely euthanized and lungs collected into 1.0 mL PBS, homogenized, and clarified by centrifugation. A $TCID_{50}$ assay was then used to determine virus titers from clarified homogenates as previously described [51].

CX3CR1 surface blocking. A549 cells were incubated with either purified rabbit polyclonal anti-CX3CR1 (Torrey Pines Biolabs Inc, East Orange, NJ) or isotype control antibody (rabbit IgG) for two hours. Cells were then either infected with WSN at an MOI of 0.01 or co-treated with WSN and mouse sera against WSN, twenty-four hours post-infection, the cells were fixed and stained

Statistical analyses. Statistical analyses were done using Student's *t* test or one-way analysis of variance (ANOVA), as indicated. Results were calculated as means ± standard errors. Values of $P < 0.05$ were considered significant.

Results

Blocking of surface CX3CR1 surface expression does not inhibit IAV replication. This data suggests influenza could be using CX3CR1 as an alternate receptor and that silencing CX3CR1 is decreasing entry into the cells. To address this possibility, A549 cells were incubated with either an anti-CX3CR1 monoclonal antibody to block CX3CR1 receptor availability or an isotype control antibody (rabbit IgG) for two hours. After incubation, cells were either infected with WSN at an MOI of 0.01 for co-treated with WSN and mouse sera against WSN (positive control for blocking WSN binding). Twenty-four hours post-infection, the cells were fixed and stained as previously described. As observed in Figure 4.1, blocking of CX3CR1 with a monoclonal antibody slightly decreased the percentage of cells infected with influenza, but this decrease was not statistically significant ($P>0.05$) when compared to the isotype control, while treatment of WSN with mouse antiserum completely prevented virus infection (Figure 3). These results demonstrate that CX3CR1 surface availability is not required for influenza infection in A549 cells, and suggest that CX3CR1 is not acting as a co-receptor.

NP localizes in the cytoplasm in CX3CR1-deficient cells. To determine where in the IAV replication cycle that CX3CR1 is being utilized, we silenced CX3CR1 expression for 48 h and infected cells with WSN at a MOI of 0.01 for 8 hr to 48 h (Figure 5). Influenza replication was measured by IFA and detection of NP expression using the cellomics array scanner. At 48 hours post-infection, NP intensity in cells previously transfected with siCX3CR1 was significantly increased when compared to cells transfected with the non-targeting control, siNEG (Figure 4.2A-B). At 8 hours post-infection, NP intensity and localization was similar between siNEG, siMEK, and

siCX3CR1 transfected cells. However, at 12 hours post-infection, NP intensity was higher in siCX3CR1(4) and siMEK-transfected cells while NP intensity decreased in siNEG transfected cells. This decrease in intensity also corresponds to an increase in infected cells in the siNEG group (Figure 4.2G). As shown in Figures 4.2E and 4.2H, NP can be visualized either accumulating in the cytoplasm of siCX3CR1(4) transfected cells or leaving and entering new cells as in the siNEG cells. Taken together, these results demonstrate that CX3CR1 expression is required for influenza replication, and CX3CR1 knockdown induces cytoplasmic accumulation of NP while decreasing viral particle release to the cell supernatant. This suggests a potential role for CX3CR1 in IAV replication possibly in assembly, trafficking, or viral egress.

Reduced CX3CR1 expression does not affect IAV WSN protein trafficking. To determine how a reduction in CX3CR1 affected different viral proteins, a time course was first performed to determine at which time point each IAV protein could be visualized for a difference in localization in siCX3CR1 treated cells compared to siNEG treated cells. A549 cells were infected at an MOI of 1 and fixed at 4, 8, 10, 12, and 14 hours post-infection, permeabilized, and stained for influenza nucleoprotein (NP), hemagglutinin (HA), neuraminidase (NA), non-structural protein 1 (NS1), and non-structural protein 2 (NS2) (Figure 4.3). At 12 hours post-infection, each IAV protein that was stained for demonstrated significant expression and localization to the cellular compartments that each protein is known to localize in [53-55]. Thus 12 hours post-infection was determined to be a time point that would allow a potential difference in viral protein accumulation to occur in cells with reduced CX3CR1 expression.

In Figure 4.4, A549 cells were transfected with siCX3CR1 or siNEG for 48 h and infected with WSN at an MOI of 1. At 12 hours post-infection, the cells were fixed, permeabilized, stained for influenza HA, NA, NS1, and NS2, and visualized on the cellomics array scanner. Cells treated with siCX3CR1 had no visible difference in accumulation of IAV proteins when compared to siNEG treated cells at this early stage in an *in vitro* infection, suggesting that a reduction in CX3CR1 may cause an inhibition at a later stage in replication.

IAV WSN proteins co-localize with CX3CR1. To determine if CX3CR1 and individual influenza proteins co-localize *in vitro*, pFLAG-IAV constructs were generated by subcloning individual IAV genes into a pFLAG-tag vector. These were verified by performing a Western blot staining on a nitrocellulose membrane with an anti-FLAG antibody after running harvested protein on SDS-PAGE (Figure 4.5). Following verification that these plasmids could express each IAV protein in an immunofluorescence assay, the plasmids were co-transfected into HEK293T cells with a plasmid expressing CX3CR1 tagged with HA (pHA-CX3CR1). As the classical HA tag is not cross-reactive with WSN HA, we could use it for co-transfection/infection experiments to stain both for CX3CR1 and IAV WSN HA. As shown in Figure 4.6, multiple influenza virus proteins co-localize with CX3CR1, including HA, M2, and PB1.

While these proteins co-localized during a co-transfection, an infection with live virus would be a more realistic representation to show co-localizing proteins. For this experiment, HEK293T cells were transfected with pHA-CX3CR1 for 24 h and infected with IAV WSN at an MOI of 1. The cells were fixed, permeabilized, and stained for IAV HA and M2. Upon visualization through confocal microscopy, both HA and M2 co-

localize with CX3CR1 (Fig 4.7). This co-localization appears to be occurring at the Golgi, as wheat germ agglutinin has been used as a Golgi marker, as well as the cell membrane.

A lack of CX3CR1 causes a decrease in replication and clearance of IAV in vivo. As previously shown, influenza virus replication was decreased at an early time point in CX3CR1 null mice. In order to confirm that lack of CX3CR1 expression induced delayed viral clearance in mice we repeated this study and measure lung viral burden at days 2 to 8 pi. As shown in Figure 4.8, influenza WSN titers were lower at days 2 and 3 pi in CX3CR1 null mice compared to C57BL/6, and higher at day 8 pi, however these differences were not statistically significant ($p>0.05$). Taken together, these results demonstrate that absence of CX3CR1 expression leads to a delay in virus replication and clearance.

Discussion

CX3CR1 is used by RSV and HIV-1 as a co-receptor during viral attachment and infection, and blocking of CX3CR1 with antibodies results in reduced RSV and HIV-1 infection [56, 57]. However, we showed that surface blocking of CX3CR1 had no significant effect on IAV infection suggesting that CX3CR1 is not utilized as a co-receptor by IAV (Fig 4.1). We also showed that siCX3CR1-treated cells infected with IAV produced NP in the cytoplasm therefore describing viral entry, nuclear trafficking, and genome transcription and translation are not affected by the lack of CX3CR1 expression. Nevertheless, siCXCR1-treated/IAV infected cells showed increased NP accumulation in the cytoplasm by 12 to 48 h pi (Fig 4.2) with no increase in the overall

amount of infected cells, suggesting that virions may be packaging, trafficking, and egressing insufficiently in these cells. IAV NP has not been implicated in viral progeny assembly or egress; other proteins could be interacting with CX3CR1 during late states of infection.

When CX3CR1 expression was reduced, a there was no visible change in localization of several IAV proteins (Fig 4.4), but more time points will be needed to further investigate this, as well as visualization through confocal microscopy. When transfecting a plasmid expressing CX3CR1 and infecting with IAV WSN, co-localization between proteins was observed between CX3CR1 and IAV M2 and HA around the cell membrane as well as the Golgi. Since CX3CR1 is a membrane protein, it is possible that it is interacting with the hemagglutinin (HA) or matrix protein (M2) of IAV. These two viral proteins are both palmitoylated which possible lends them to associating with lipid rafts and other membrane bound proteins such as CX3CR1[58]. Palmitoylation would occur at the Golgi, which is where CX3CR1 is glycosylated and possibly palmitoylated [59]. Any interaction between the HA and M2 with CX3CR1 could have an impact on viral trafficking and egress, which may provide more insight into the reasoning behind the decreased viral replication seen in cells knocked down for CX3CR1. Further studies will be needed to address a possible interaction between viral membrane proteins and CX3CR1.

To speak to a role for CX3CR1 for influenza replication in mice, CX3CR1 null mice were i.n. infected with influenza and the lung virus titers determined at days 2-8 (Figure 4.8). It was observed that absence of the CX3CR1 resulted in a delay in viral replication, with lower titers at day 2-3 and higher viral titers at day 8 pi compared to

wild type mice. The reduction in virus titer at early time points correlates with the *in vitro* results, suggesting that lack of CX3CR1 expression could result in impaired virus trafficking or egressing from epithelial cells. The delay in viral clearance at later time points may be due in part to the role of CX3CR1 in immune cell trafficking. It has been shown that *CX3CR1+ cells* constitute a major component of the *cytotoxic* response to viral infections [49, 60, 61], thus lack of CX3CR1 expression on immune cells may result in inefficient elimination of infected cells and allowing the virus to persist for a longer duration. Additional experiments will be needed to address this hypothesis.

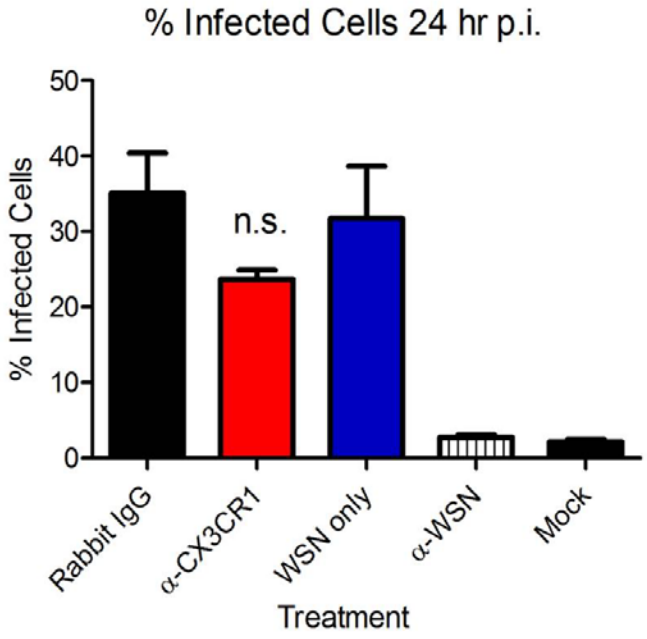

Figure 4.1: Blocking of surface CX3CR1 surface expression does not inhibit IAV replication. A549 cells were incubated with either anti-CX3CR1 polyclonal antibody or isotype control antibody (rabbit IgG) for two hours. Cells were then either infected with WSN at an MOI of 0.01 or co-treated with WSN and mouse sera against WSN, twenty-four hours post-infection, the cells were fixed and stained.

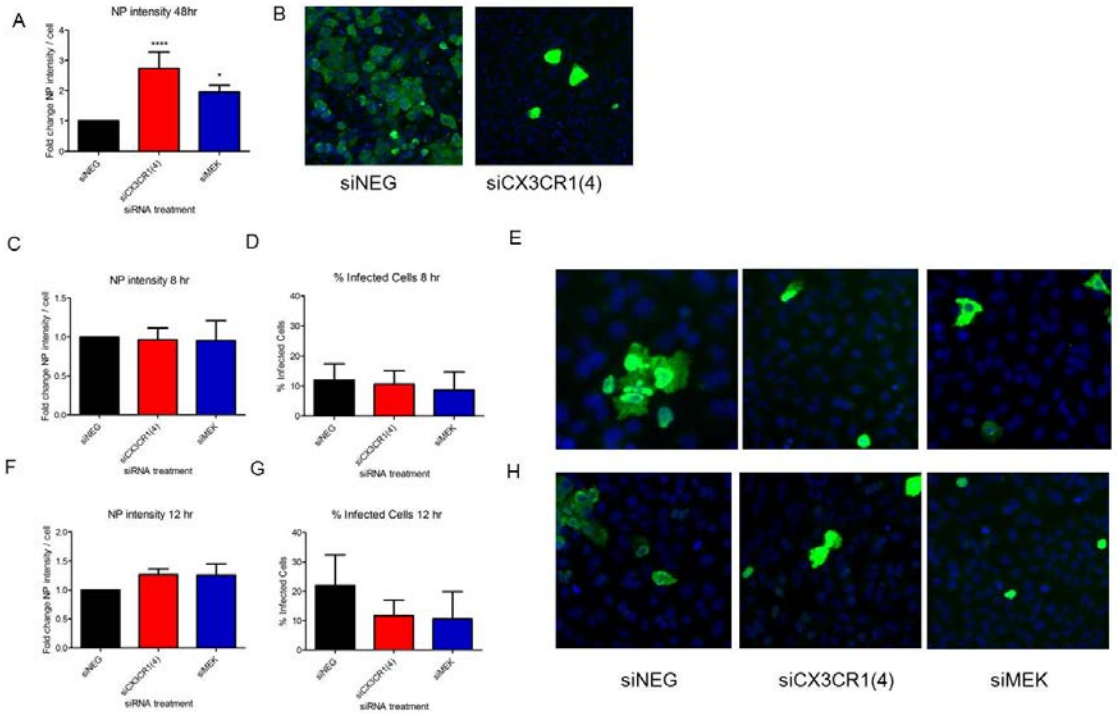

Figure 4.2: IAV NP localizes in the cytoplasm in cells that lack CX3CR1. (A-H) 48 hours post-transfection of siNEG, siCX3CR1, and siMEK, A549 cells were infected with WSN at MOI 0.001. (A,C,D,F,G) Cells were fixed and stained for NP after the indicated time post-infection(p.i) and analyzed for NP intensity via cellomics array scanner. Representative images of each siRNA treated infection are shown for 8 hours (E) and 12 hours (H)*p<0.05.

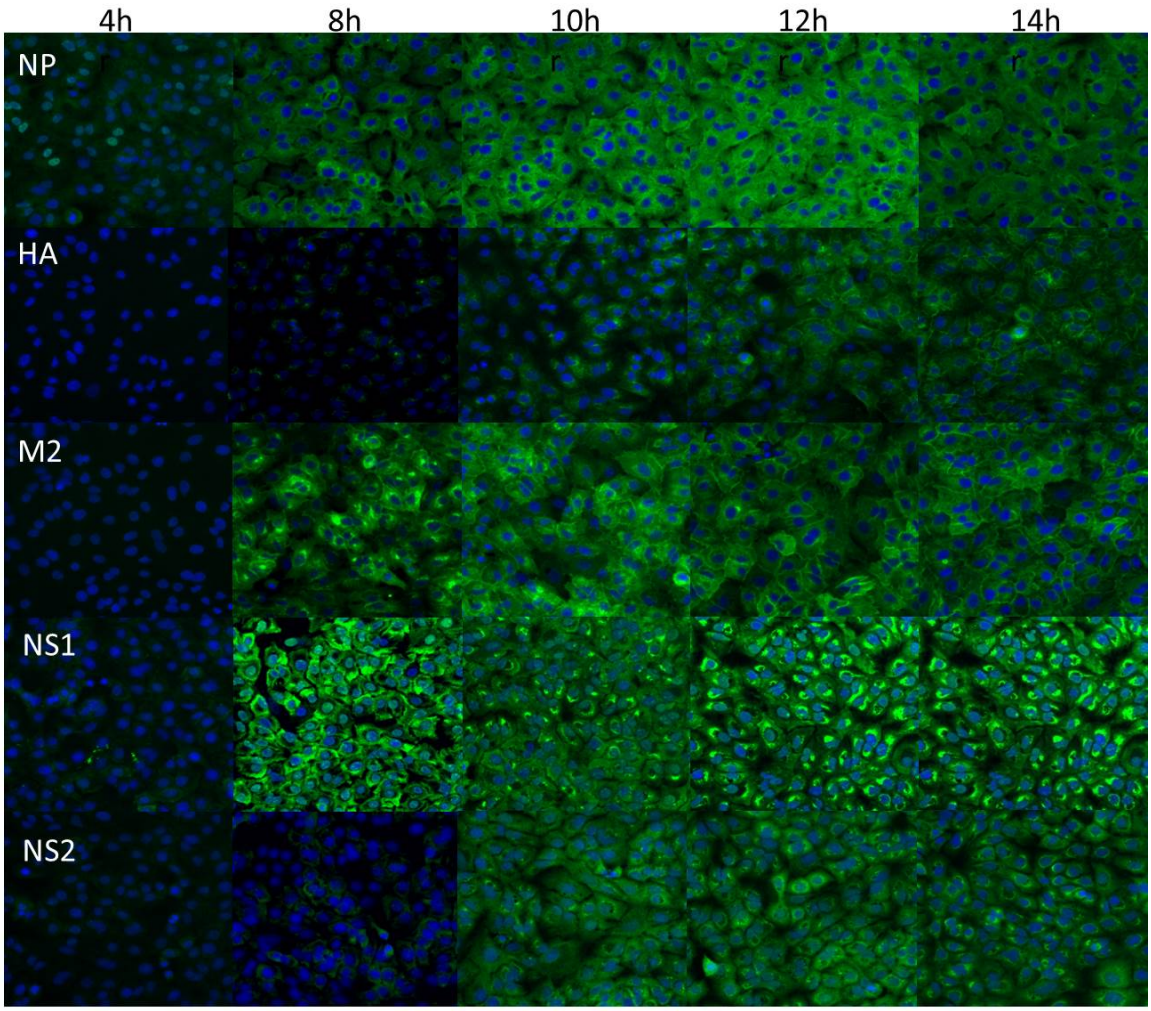

Figure 4.3: IAV proteins localize traditionally by 12h post-infection. A549 cells were infected with WSN at MOI of 1. Cells were fixed and stained for NP, HA, M2, NS1, and NS2 at the indicated time post-infection(p.i) and analyzed via cellomics array scanner. Representative images of each

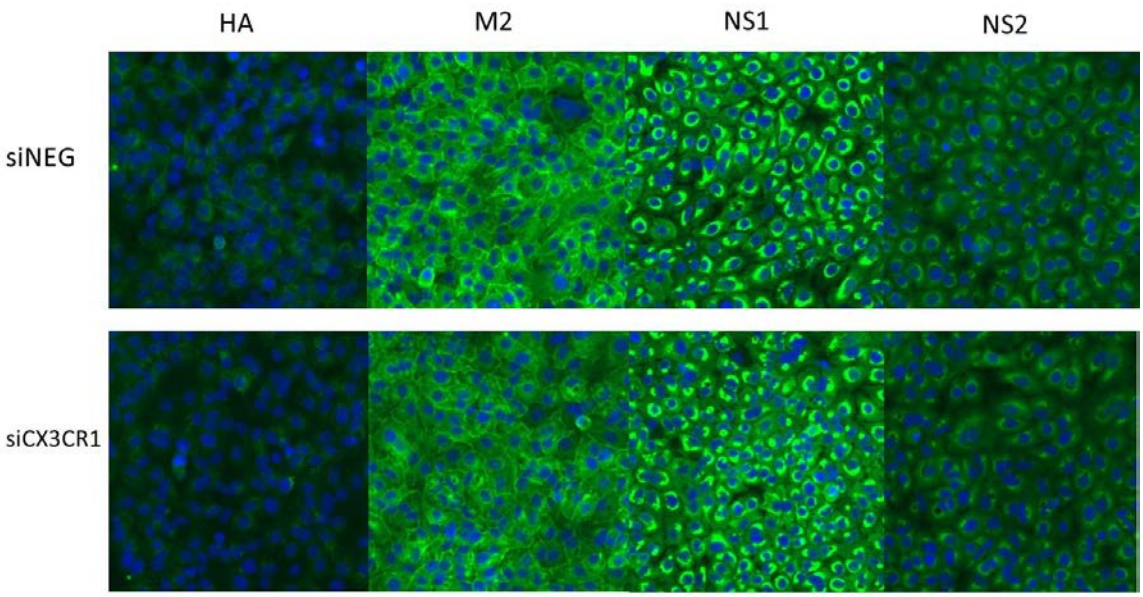

Figure 4.4: Reduced CX3CR1 expression does not affect IAV WSN protein trafficking. 48 hours post-transfection of siNEG and siCX3CR1(4) , A549 cells were infected with WSN at MOI 1. Cells were fixed and stained for HA, M2, NS1, and NS2 at 12h p.i and analyzed via cellomics array scanner. Representative images of each

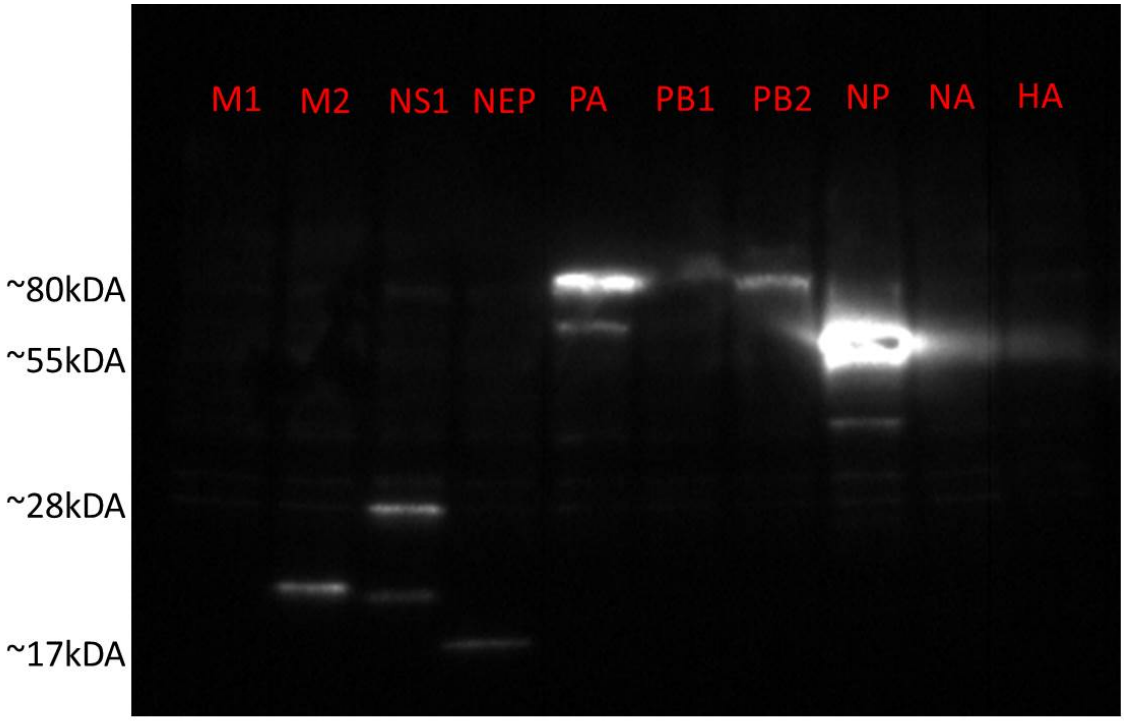

Figure 4.5: IAV proteins express FLAG on pFLAG vector. HEK293T cells were transfected with individual pFLAG constructs containing IAV WSN genes. Total protein was harvested 24 h post-transfection and separated by SDS-PAGE, then transferred to nitrocellulose membrane. Membrane was stained with anti-FLAG antibody

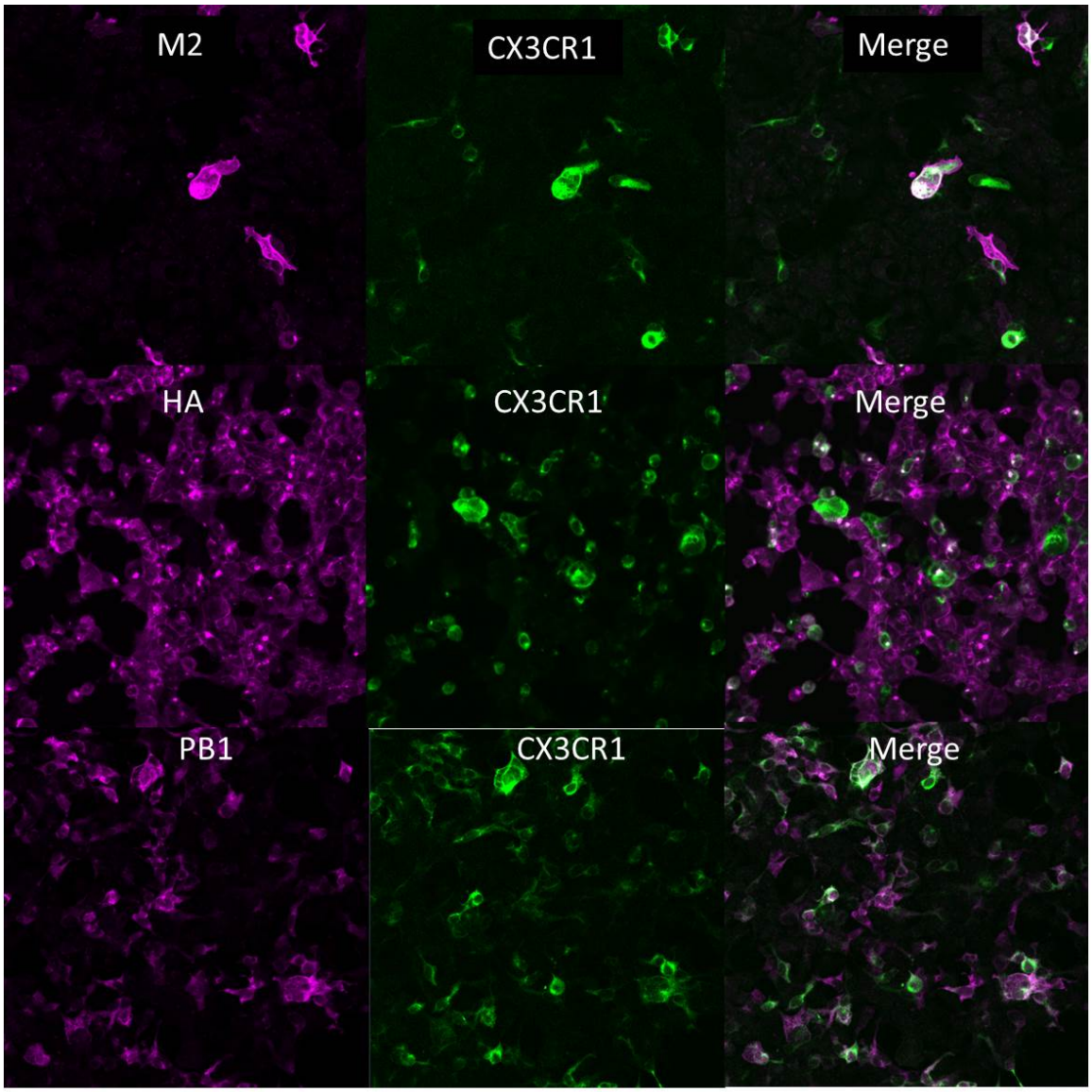

Figure 4.6: IAV proteins co-localize with CX3CR1 when co-transfected. HEK293T cells were co-transfected with pHA-CX3CR1 and pFLAG-M2, pFLAG-HA, pFLAG-PB1. Cells were fixed, permeabilized, and stained 24 h post-transfection and analyzed via confocal microscope Zeiss 710. Representative images of each

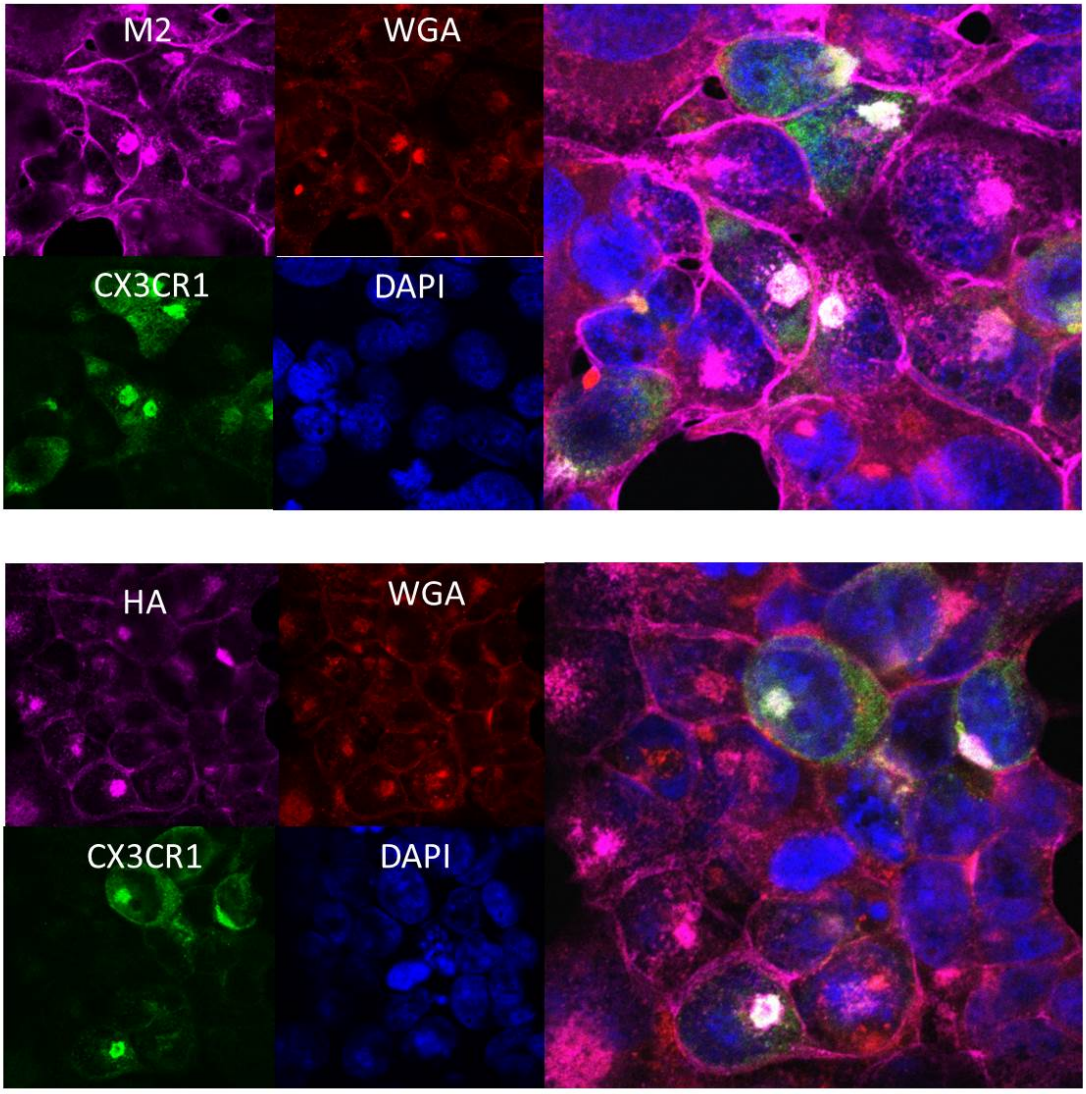

Figure 4.7: IAV proteins co-localize with CX3CR1 when during infection. HEK293T cells were co-transfected with pHA-CX3CR1 and pFLAG-M2, pFLAG-HA, pFLAG-PB1. Cells were fixed, permeabilized, and stained 24 h post-transfection and analyzed via confocal microscope Zeiss 710. Representative images of each

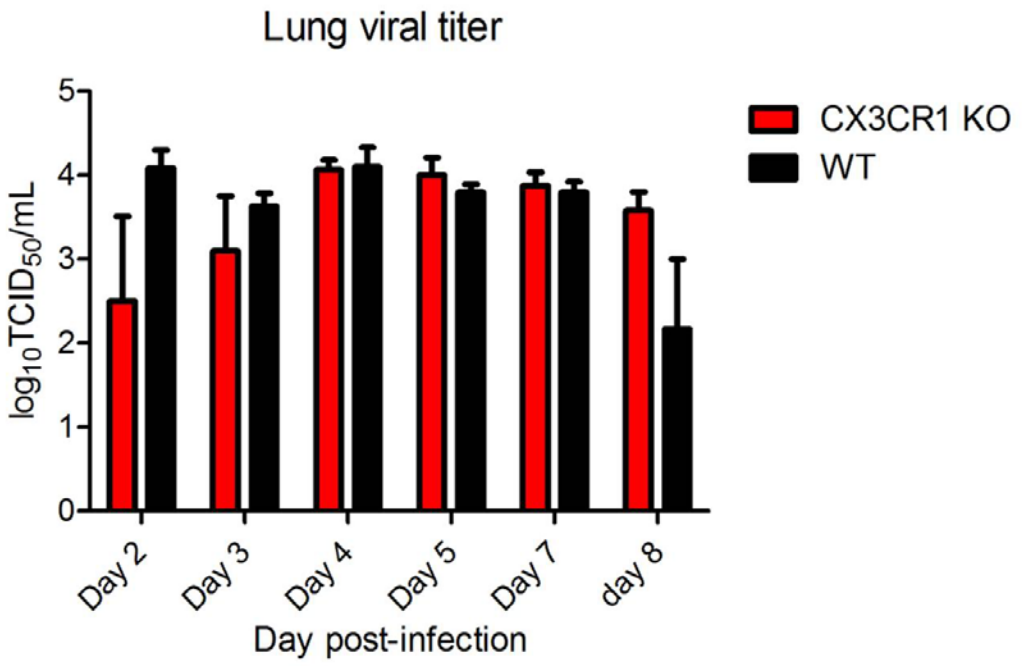

Figure 4.8: IAV WSN lung viral titer is decreased in CX3CR1 null mice. Both C57BL/6 and CX3CR1 null mice were infected with 0.5 LD50 WSN. Virus titers in the lungs 2,3,4,5,7, and 8 days post-infection as measured by $TCID_{50}$.

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CHAPTER 5

THE EFFECT OF RESPIRATORY SYNCYTIAL VIRUS (RSV) INFECTION ON (IAV) INFECTION AND PATHOGENESIS

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Abstract

Influenza virus (IAV) and respiratory syncytial virus (RSV) are seasonal viruses that co-circulate and cause substantial morbidity and some mortality among the extremes of age and immune compromised. RSV has been shown to modulate the immune response following infection, a feature that may affect the outcome of influenza virus infection and disease pathogenesis. To begin to address the impact of RSV infection on subsequent IAV infection, immune studies were performed to investigate changes in the immune response on lung viral titers. BALB/c mice were intranasally infected with RSV, allowed to recover, then intranasally challenged with RSV, influenza, or were co-infected with RSV and influenza. These groups were compared to mice without prior exposure to RSV. While the co-infection of naïve mice showed little difference in lung virus titers compared to mice infected with IAV or RSV alone, mice previously infected with RSV had increased IAV titers in the lung when co-infected with RSV, and generally had decreased cytokine responses linked to differences in immune cell populations. These findings suggest that RSV infection and modulation of the host immune response indirectly impacts the immune response to IAV, and provides new avenues for exploring the contribution of influenza and RSV-specific immune responses to disease pathogenesis.

Introduction

Human respiratory syncytial virus (RSV) belongs to the *Pneumovirus* genus, within the family *Paramyxoviridae*. RSV has enveloped, negative-sense single-stranded RNA genome of approximately 15 kb that encodes 11 viral proteins [1]. In human infections, the replication of the virus occurs in the superficial cells of the respiratory epithelium, as ciliated cells appear to be the primary targets [2].

RSV is the principal cause of infant hospitalizations from respiratory tract infections, causing an estimated 17 hospitalizations for every 1000 children under 6 months of age [3]. One study that followed a birth cohort of 125 infants reported that within the first year of life, 68% experienced an RSV infection [4]. The clinical outcomes in RSV infection vary from a mild upper respiratory tract infection to a life threatening lower respiratory tract disease. An estimate of 15-50% of infection among infants occurs in the lower respiratory tract, and the most common clinical manifestation in these incidents is bronchiolitis [4]. The RSV season occurs in the United States generally from late October to early May, with a peak incidence occurring in December [5, 6]. RSV often co-circulates with influenza viruses, which causes difficulty in determining the disease impact of each virus. In young children, the estimates of burden of the disease for either virus may be confounded by the other [7].

Influenza viruses cause disease among persons in all age groups, and influenza accounts for 3–5 million infections per year, representing the overall leading cause of human respiratory disease due to viral infection [8]. In the United States alone, seasonal influenza is responsible for 20,000–40,000 deaths per year and poses a considerable economic burden due to decreased productivity and healthcare costs associated with

hospitalization and/or treatment. Morbidity and mortality associated with pandemic and emerging, highly pathogenic influenza strains can be substantially higher [9, 10].

It is known that many cases of bronchiolitis in infants that require hospitalization involve co-infections with at least two different viruses [11, 12], and that infection with multiple respiratory viruses correlates to an increase in disease severity [13-17]. Furthermore, an analysis of co-infection clinical manifestations compared to those that arise from single infections showed that independent of the secondary viral pathogen, RSV controlled the severity of obstructive airway disease and duration of hospitalization [18]. In a study that determined the prevalence of multiple viral pathogens involved in lower respiratory tract infections of infants, RSV was found to be the most common cause, as well as being the pathogen that was involved in the most frequent co-infections [15]. The influenza A virus (IAV) season generally occurs just during or after the RSV season [19], and like RSV, causes significant disease in the young and elderly populations [20, 21]. Due to the overlap of seasons, similar tissue tropism, and the similar age groups that are most affected, it is likely that co-infections of RSV and IAV occur and cause a significantly increased disease outcome.

RSV is known to modulate the host immune system in a number of mechanisms of viral-host interaction, some of which may allow for an enhanced secondary viral infection. RSV contains two non-structural proteins, NS1 and NS2, each of which have been shown to antagonize the interferon response [22, 23] as well as repress dendritic cell (DC) maturation [24]. The RSV G protein has been shown to induce suppressor of cytokine signaling proteins (SOCS), specifically SOCS1 and SOCS3, inhibiting IFN-β production [25]. It has been shown that the G protein can be produced as a secreted form

(sG) that promotes a CD4 $^{+}$ Th2 bias phenotype causing several enhanced disease outcomes, such a severe pathology, decreased viral clearance, and increased pulmonary eosinophilia upon subsequent RSV challenge, a mechanism that depends on IL-5 and IL-13 secretion from CD4^{+} T cells [26]. The RSV G protein also contains a CX3C chemokine motif that allows the sG to act as a CX3CL1 mimic, bind to CX3CR1 expressed on leukocytes, modulate CX3CL1-mediated responses and disrupt CX3CR1^{+} T cell activity[27]. CX3CR1^{+} T cells constitute the major component of the cytotoxic response to RSV infection, therefore by inhibiting the recruitment of CX3CR1^{+} T cells to the airways, the G protein reduces the antiviral T cell response to RSV infection. Each mechanism of immune modulation provides evidence that a prior infection with RSV may provide an optimal environment for a secondary viral infection, which may lead to enhanced disease pathogenesis.

In this study, we sought to address the impact of RSV infection on subsequent IAV infection, co-infection studies were performed to investigate changes in immune response to infection and associated lung virus titers. Mice were infected with RSV, allowed to recover, then challenged with RSV, influenza, or were co-infected with RSV and influenza. These groups were compared to mice without prior exposure to RSV. While the co-infection of naïve mice showed little difference in lung virus titers compared to mice infected with influenza or RSV alone, mice previously infected with RSV had increased influenza titers in the lung when co-infected with RSV. These findings suggest that RSV can impact the immune response to influenza co-infection, and provide new avenues for exploring the contribution of influenza and RSV-specific immune responses to disease pathogenesis.

Materials and Methods

*Cell Cultures, Virus stocks, and Mice used***.** Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 5% fetal bovine serum (FBS) and 5% L-glutamine. Vero E6 cells (ATCC CRL-1586) cells were cultured in DMEM with 5% FBS and 5% L-glutamine. All cells were incubated at 37°C in 5% CO_2.The influenza virus used was mouse-adapted H3N2 reassortant A/Aichi/2/68 (x31) and was propagated in the allantoic cavity of embryonated hen eggs at 37°C for 48-72 hours. The virus was aliquoted and stored at -80°C.

Virus challenge experiments. BALB/c mice were anesthetized and were intranasally (i.n.) infected with 10^6 p.f.u. RSV, boosted 28 days later with 10^6 p.f.u. RSV, and challenged 2 days later with 10^3 p.f.u IAV(x31) diluted in 50ul PBS. Mice were monitored daily for morbidity and mortality with body weights measured every day. On days 3, 6, and 9 p.i., groups of mice were humanely euthanized and lungs collected into 1.0 mL PBS, homogenized at 4°C by use of gentleMACSTM Dissociator (Miltenyi Biotec), clarified by centrifugation at 4°C for 10 min at 200xg, and aliquoted for storage at -80°C or used directly. Plaque assays were performed to determine lung viral titer on Vero cells and MDCK cells for RSV and IAV respectively.

BAL collection and quantification of cytokines. 4-6 week old BALB/c mice were intranasally infected with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both. On days 7 and 14 p.i, mice were humanely sacrificed and tracheotomy was performed. The mouse lungs were washed three times with 1 mL of PBS and the BAL was centrifuged at 200 x g for 10 min at 4°C. The recovered

supernatants were collected and stored at -80°C until assessed for cytokine concentration, and the cell pellet were resuspended in 200 µL of FACS staining buffer (PBS containing 1% BSA). Total cell numbers were counted using a hemocytometer. Cytokines were measured as previously described [28]. Briefly, the assay was analyzed on a Luminex 200 instrument (Luminex Corporation, Austin, TX) using Luminex xPONENT 3.1 software. The presented cytokines were the only cytokines found to be statistically significant.

Quantification of virus-specific splenocytes. As previously described [28], the day prior to the assay, 96-well Multiscreen plates (Millipore) were coated with anti-mouse IFN-γ capture antibody (R&D Systems) and incubated overnight at 4°C. The plates were then blocked by the addition of 200 µL of RPMI-10 media (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol and 2 mM L-glutamine) and incubated for 2 h at 37°C. In parallel, spleens were harvested from mice at 7 and 14 days post infection with RSV A2, IAV x31, or both RSV and IAV, and prepared to a single cell suspension using a syringe plunger and a 70 µm mesh nylon strainer. The cell suspensions were collected by centrifugation for 10 min at 200 x g and suspended in RPMI-10 at a concentration of 10^7 cells/mL. Spleen cell suspensions were added to each well, and cells were stimulated with either 5µg/mL $M2_{82\text{-}90}$ (SYIGSINNI), 5µg/mL $G_{\mathbf{183\text{-}197}}$ (WAICKRIPNKKPGKK), 5µg/mL $NP_{147\text{-}155}$ (TYQRTRALV) or without peptide for 24h at 37°C and 5% CO_2. Plates were washed 4 times with wash buffer (0.05% Tween-20 in PBS), anti-mouse IFN-γ detection antibody (R&D Systems) was added and plates were incubated overnight at 4°C. Detection antibody was removed, plates were washed and cytokine spots were developed using ELISpot blue color module

(R&D Systems). Spots were counted using an ELISPOT reader (*AID EliSpot Reader* System). RSV-specific ELISPOT numbers were determined from triplicate wells/cell population by subtracting the mean number of ELISPOTs in the unstimulated wells.

Flow cytometry. For flow cytometry analysis, cell suspensions were incubated in FACS staining buffer and blocked with FcγIII/II receptor antibody (BD), and subsequently stained with antibodies from BD bioscience, i.e. anti-CD3e (clone 145-2C11), anti-CD8α (clone 53-6.7), anti-CD4 (clone RM4-5) and optimized concentration of MHC class I H-2K^d tetramer complexes bearing the peptide SYIGSINNI (Beckman Coulter) representing the immunodominant epitope of the RSV M2-1 protein [29]. To determine cell types in BAL, cell suspensions were stained for 60 min at 4°C with an optimized concentration of anti-CD3e (clone 145-2C11), anti-CD11c (clone HL3), anti-CD49b (clone DX5), anti-Gr-1 (clone RB6-8C5). Cells were acquired on a LSRII flow cytometer (BD bioscience) with data analyzed using FlowJo software (v 7.6.5).

Statistical analyses. Statistical analyses were done using Student's t test or one-way analysis of variance (ANOVA), as indicated. Results were calculated as means ± standard errors. Values of $P < 0.05$ were considered significant.

Results

Prior memory to RSV slows pulmonary IAV clearance.

To investigate the effect that a primary RSV infection has on a secondary IAV infection, we intranasally infected BALB/c mice with 10^6 p.f.u. RSV, boosted 28 days later with 10^6 p.f.u. RSV or PBS, and challenged 2 days later with 10^3 pfu IAV. Looking

at the percent of original body weight (Figure 5.1A), there is no difference in weight loss between the mice that were primed and boosted with PBS and challenged with IAV and the mice that were primed with RSV, boosted with PBS, and challenged with IAV. However when the mice are both primed and boosted with RSV and challenged with IAV, the mice are protected from the IAV induced weight loss. This suggests that priming alone with RSV does not affect IAV weight loss, however co-infection with RSV and IAV after an initial RSV prime does result in protection from weight loss, possibly due to a memory response to RSV in the boost which also helps protect against IAV disease.

Although the protection in IAV induced weight loss from mice that were both primed and boosted with RSV, we see that there is significantly higher lung viral titers in these mice at 6 days p.i. (Figure 5.1B). This suggests that despite a protection from IAV induced weight loss, there is a delay in IAV clearance, possibly due to the ability of RSV to influence the immune system towards a Th2 $CD4^+$ T cell profile, which is counteractive to the Th1 desired response for helping clear an IAV infection.

Pulmonary cell infiltration is differentially affected in single infections and co-infections. We next sought to determine what immunological factors were causing the delay in influenza viral clearance when co-infected with RSV. To accomplish this we intranasally infected BALB/c with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both (10^6 pfu RSV and 10^3 pfu IAV in the same inoculum). Performing BAL and counting cells, we first noticed that IAV induces close to 10-fold more cell recruitment than RSV at day 7 p.i. (Figure 5.2A), but this infiltration is more acute than RSV, as evidence as the severe reduction by day 14 as opposed to equal levels

of infiltration at days 7 and 14 p.i. for RSV. Also, IAV and RSV co-infection induces more cell recruitment than RSV at day 7, but not more than IAV alone. However, this infiltration due to co-infection is sustained for a longer time than IAV alone, possibly due to the RSV, as evidence that RSV alone maintains similar levels from day 7 to day 14 p.i. Furthermore, RSV primed mice followed by a co-infection of RSV and IAV have increased cell infiltration most likely due to the recruitment of RSV-specific memory cells.

Prior RSV infection is associated with decreased cytokine expression by BAL cells responding to IAV challenge. To determine if the cytokine mileiu in response to an IAV infection is affected by prior infection with RSV, we intranasally infected BALB/c with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both (10^6 pfu RSV and 10^3 pfu IAV in the same inoculum). Analysis of cytokines in BAL fluid revealed that prior infection with RSV results in the reduction of several T cell cytokines in response to a subsequent IAV infection, including the pro-inflammatory cytokines IFN-γ, IL-5, IL-6, and TNF-α (Figure 5.3). This is suggesting that RSV is affecting the $CD4^+$ T cell population as to more of a Th2 type response, where upon inflammatory cytokines are reduced.

Prior RSV infection alters immune cell infiltrate into the lung in mice challenged with IAV. To investigate the effect that RSV induces on immune cell types infiltrating into the lung, mice were primed with RSV or PBS then challenged with RSV, IAV, RSV and IAV, or PBS. As shown in Figure 5.4, prior RSV infection is linked to a decrease in pulmonary cell infiltration of granulocytes after challenge with IAV or co-infected with RSV and IAV. This may demonstrate the decrease in inflammatory cytokines such as IL-

5 that we saw in mice primed with RSV and challenged with IAV or both RSV and IAV. Another effect observed in RSV primed mice was the reduction in NK cells in mice infected with IAV (Figure 5.5A-B), which may explain the delay in clearance observed in Figure 5.1B. We also observed an increase in T cell recruitment in RSV primed mice at day 7, demonstrating a memory response was induced when challenged with RSV or RSV and IAV (Figure 5.5C).

RSV-specific CD8+ T cell levels are affected co-infection. We next investigated the effect of co-infection on RSV-specific $CD8^+$ T cell infiltrating into the lung. As shown in figure 5.6A, RSV priming induced a M2-specific CD8 T cell response on day 7 when primed with RSV and challenged with PBS. A similar effect was seen at day 14, when PBS primed mice were challenged with RSV (Figure 5.6B). Co-infection with RSV and IAV reduced the percentage of RSV-specific cells in PBS primed mice, suggesting that challenging with both viruses may induce immune exhaustion in the $CD8^+$ T cell population in the lungs.

Investigation of RSV-specific $CD8^+$ T cell in the spleen revealed similar effects, as M2-specific T cells were seen in the spleen in mice primed with PBS and challenged with RSV as well as mice primed with RSV and challenged with PBS (Figure 5.6C). Interesting, when mice were primed with PBS and co-infected, the number of M2-specific T cells were greater than in mice only infected with RSV at day 7, suggesting that IAV is helping to induce immunity to RSV. However at day 14, the levels of M2-specific T cells in the co-infected mice has reduced to levels of single-infected mice (Figure 5.6D)

RSV infection decreases levels of IAV-specific CD8+ T cell in the spleen. To determine the effect of RSV on levels of IAV-specific CD8+ T cells, we stimulated splenocytes harvested from all infection groups with IAV NP. There is a slight decrease in NP-specific T cells in mice primed with RSV and co-infection compared to mice primed with PBS and co-infected at day 7 (figure 5.7A), however the effect is exaggerated at day 14 in mice primed with RSV and infected with IAV alone compared to mice primed with PBS and infected with IAV alone (Figure 5.7B). We also see a significant reduction in NP-specific T cells when observing PBS primed mice with a co-infection compared to PBS primed mice with IAV infection alone. In both cases, an RSV infection reduces IAV-specific CD8+ T cells in the spleen, perhaps due to an immune exhaustion mechanism as seen in the reduction of RSV-specific CD8+ T cells in co-infected mice.

Discussion

With similar host tropism, age distribution of infection, and seasonal overlap, it is not surprising that co-infections with RSV and IAV occur and could be the cause of increase in disease severity in some children that develop bronchiolitis [13, 30]. We showed that when mice primed and boosted with RSV were then challenged with IAV had less disease severity as measured by weight loss, but also displayed a delayed lung viral clearance of IAV (Figure 5.1). An increase in disease severity in a co-infection or a prior RSV infection followed by an IAV infection could be related to pulmonary lung infiltrate, and we demonstrated that mice co-infected or having prior RSV infection and

IAV challenge resulted in increased levels of immune cell infiltrate compared to PBS controls (Figure 5.2).

Despite the increase in pulmonary cell infiltrate in mice that received a prior RSV infection, the cytokine profile displayed decreased pro-inflammatory cytokines that would result in the delayed clearance of a subsequent viral infection (Figure 5.3). It is possible that RSV is influencing the immune system towards a Th2 type response which would explain the decrease in pro-inflammatory cytokines. Also, when mice were primed with RSV and subsequently infected with IAV, there were decreases in granulocytes and NK cells (Figure 5.4 and 5.5). A reduction in these cell types would help explain the reduction in cytokine levels seen in RSV primed mice. The reduction in NK cells would also allow IAV to replicate for a longer duration, and possibly provides evidence in the delayed clearance that we observed.

RSV priming induced M2-specific $CD8^+$ T cell response, and same effect was seen in PBS primed mice infected with RSV, which is expected (Figure 5.5). However, co-infection with IAV reduced the percent of RSV-specific $CD8^+$ T cells in PBS primed mice. Having a co-infection with two viruses may induce an exhaustive phenotype in the T cell population, as T cells targeting the first virus may become limited as other naïve T cells may become devoted to targeting the second virus instead of the first. We saw a similar effect in IAV-specific $CD8^+$ T cells when mice were primed with RSV and co-infected with RSV and IAV (Figure 5.6). The observation of reduced IAV-specific $CD8^+$ T cells may also be explained by the ability of RSV to induce an immune response that is not conducive for maturation of antigen-specific T cells.

Further studies will be necessary to elucidate the mechanism of RSV impact on subsequent IAV infection. It would be beneficial to investigate pulmonary cell infiltrates at more time points, as different cell types have different peak levels post-infection. Exploring the cytokine profiles at a more time points could help link the differences in pulmonary cell recruitment with the observable delay in clearance of IAV phenotype observed in RSV primed and co-infected mice.

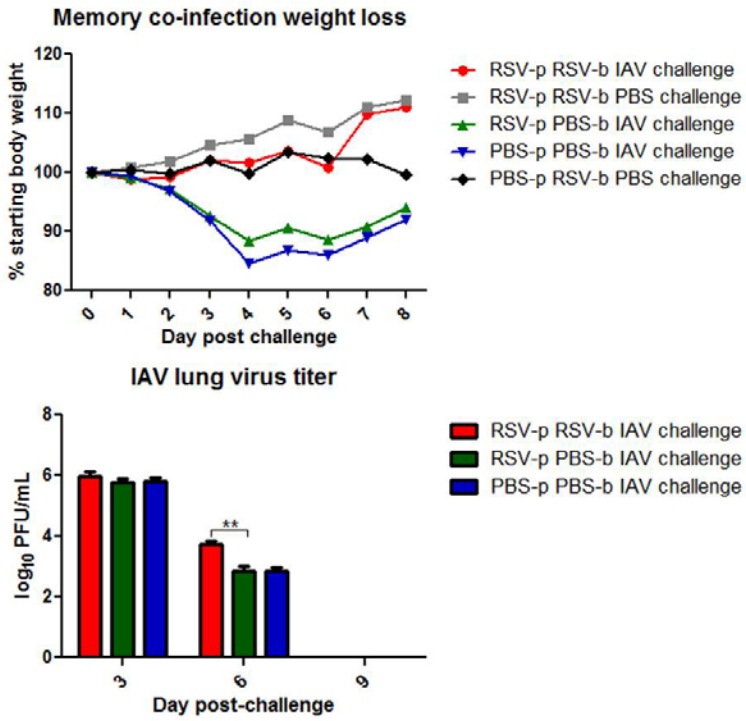

Figure 5.1: Prior memory to RSV does not affect weight loss linked to IAV challenge but does slow pulmonary IAV clearance. 4-6 week old BALB/c mice were intranasally infected with 10^6 p.f.u. RSV, boosted 28 days later with 10^6 p.f.u. RSV, and challenged 2 days later with 10^3 pfu IAV. Weights and clinical signs were evaluated on days 1,2,3,4,5,6,7, and 8 post-challenge. One cage from each group were sacrificed on days 3,6, and 9 to determine IAV lung virus titer via plaque assay.

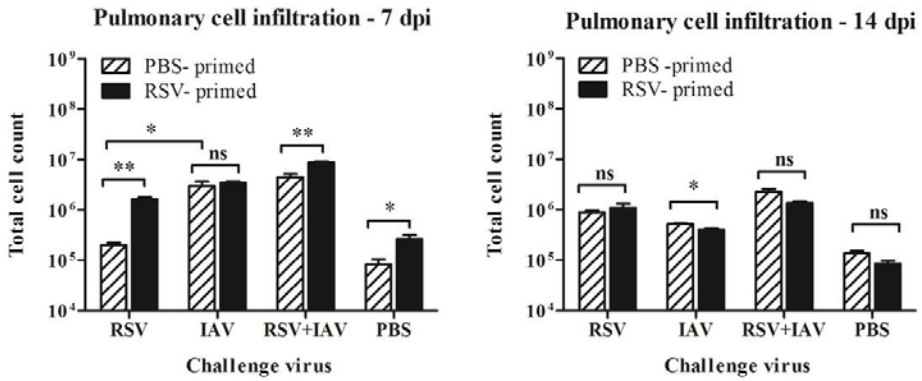

Figure 5.2: Pulmonary cell infiltration is differentially affected in single infections and co-infections 4-6 week old BALB/c mice were intranasally infected with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both. BAL was performed with 3 washes with 1 mL PBS

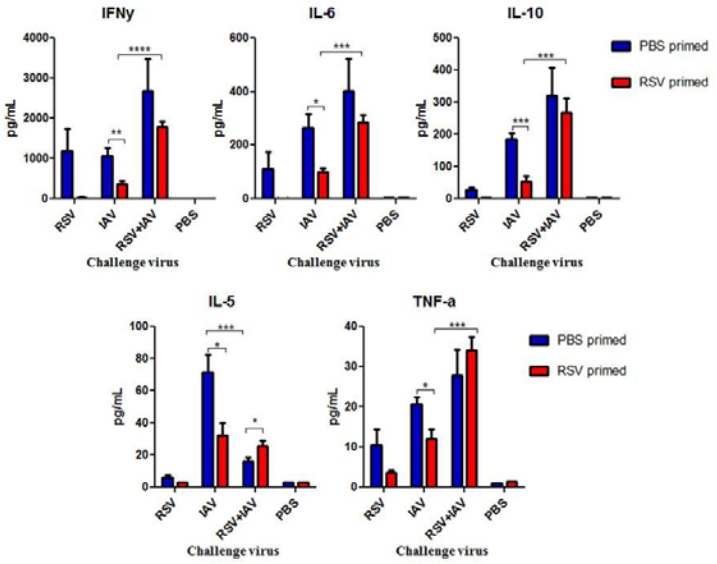

Figure 5.3: Prior infection with RSV affects T cell cytokine expression after IAV challenge. 4-6 week old BALB/c mice were intranasally infected with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both. BAL was performed on day 7 and cytokines were measured using the mouse cytokine/chemokine kit (Millipore).

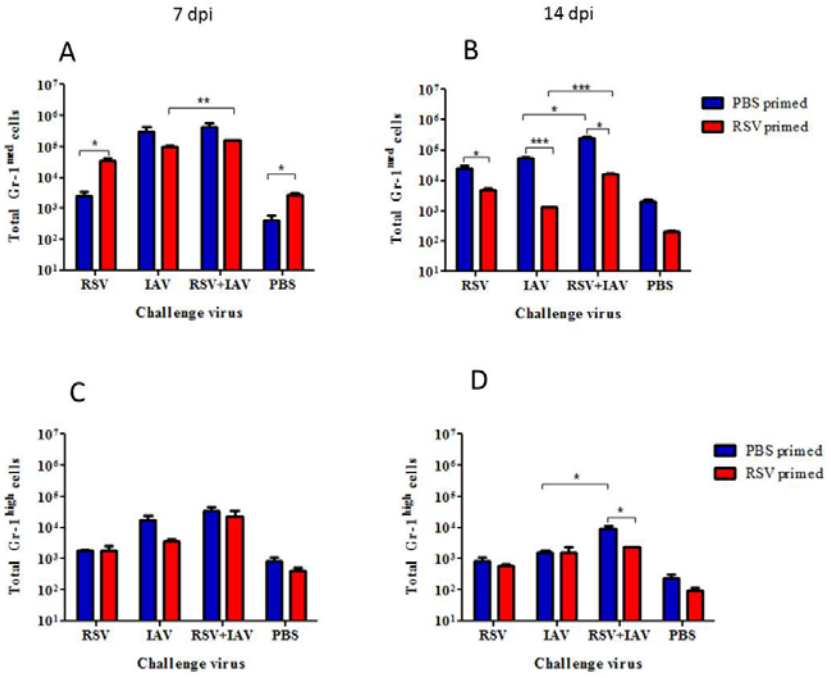

Figure 5.4: Prior infection with RSV affects granulocyte lung infiltration. 4-6 week old BALB/c mice were intranasally infected with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both. BAL was performed on day 7 and 14 and stained with anti-Gr1. Cells were quantified on LSRII flow cytometer.

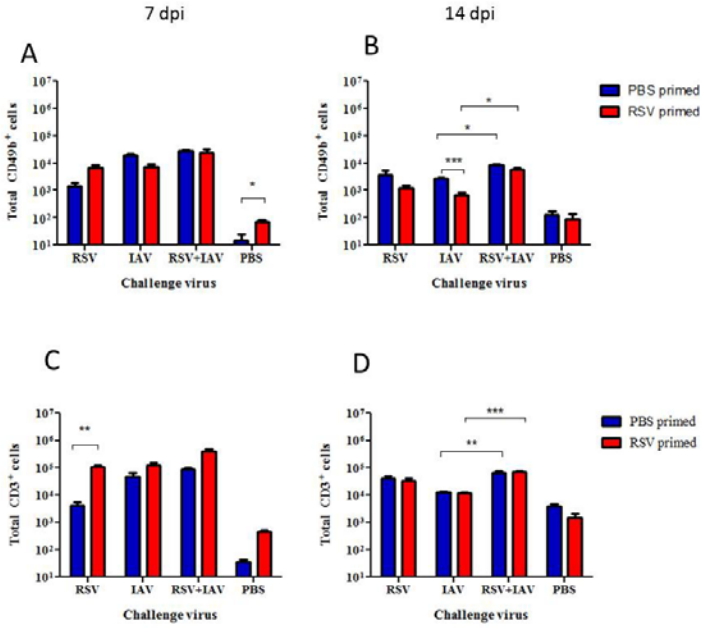

Figure 5.5: Prior infection with RSV affects T cell and NK lung infiltration. 4-6 week old BALB/c mice were intranasally infected with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both. BAL was performed on day 7 and 14 and stained with CD49b and CD3. Cells were analyzed on LSRII flow cytometer.

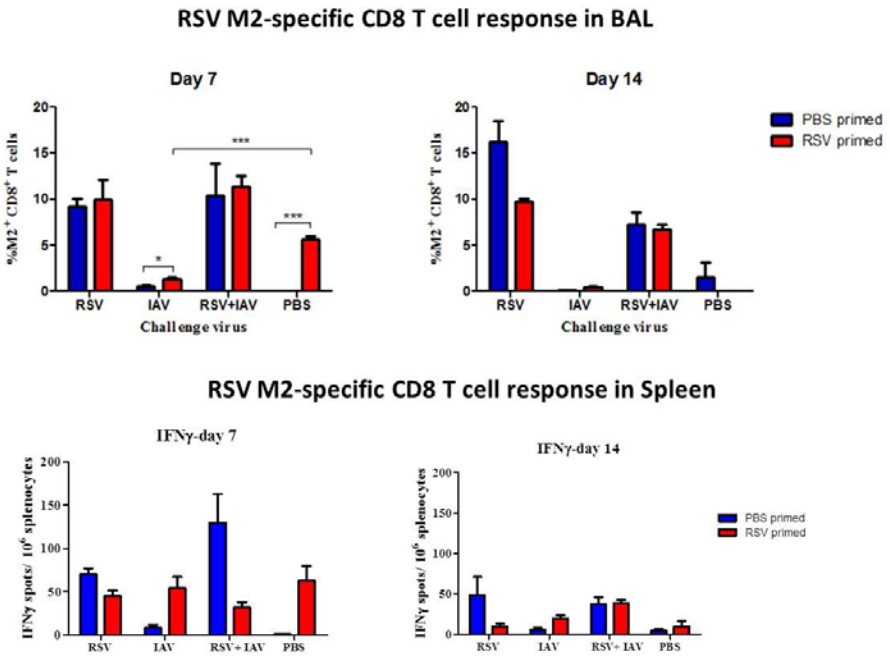

Figure 5.6: RSV-specific CD8+ T cell levels are affected co-infection. 4-6 week old BALB/c mice were intranasally infected with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both. BAL was performed and splenocytes were harvested on day 7 and 14. Cytokines were measured using the mouse cytokine/chemokine kit (Millipore) and splenocytes were prepared as single cell suspensions for use in ELISPOT.

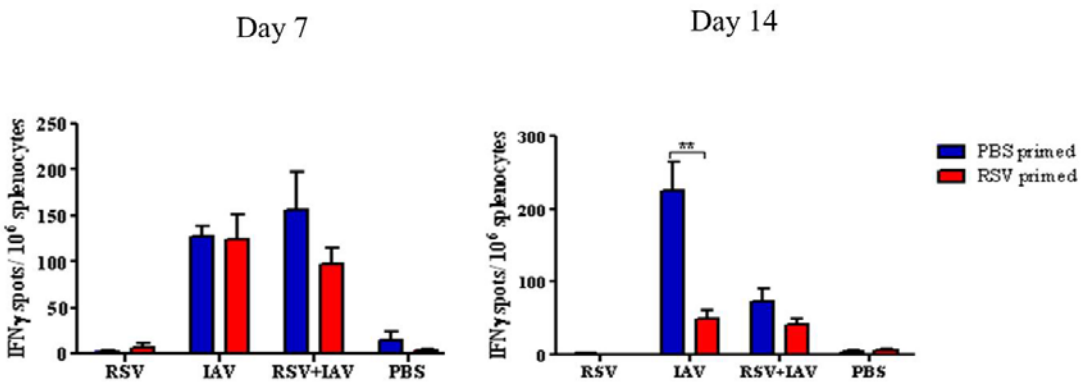

Figure 5.7: IAV-specific CD8+ T cell levels are affected co-infection. 4-6 week old BALB/c mice were intranasally infected with 10^6 p.f.u. RSV and challenged 30 days later with 10^6 pfu RSV, 10^3 pfu IAV, or both. Splenocytes were harvested on day 7 and 14 and splenocytes were prepared as single cell suspensions for use in ELISPOT.

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CHAPTER 6

CONCLUSIONS

Influenza A virus persists as a global heath disease threat due to the ability of the virus to continually mutate. This is evident as the constant demand for surveillance for novel circulating strains, as well as the requirement of new vaccine formulations in order to combat those novel strains. In addition to the difficulty and economic cost of vaccine design for influenza, antiviral drug-resistant populations in circulating strains are becoming increasingly common, describing the need for developing novel approaches for disease intervention. While the majority of previous methods involve targeting the virus, which places a selective pressure on the virus to allow mutations in the viral populations to occur, the more recent approach to target host genes that are utilized during viral replication has become a very attractive methodology for avenues of disease intervention. This has opened a new discipline of drug design, and has generated genome-wide screens that are able to identify potential genes involved in viral replication pathways. Investigation of these genes must be performed to validate their efficacy in affecting viral replication, as well as determine a mechanism by which they are utilized in the host-viral interaction. The *hypothesis* addressed was that CX3CR1 is a potential target for therapeutic influenza disease intervention. The *specific aims* addressed were:

Specific Aim 1: To determine the importance of CX3CR1 in influenza replication *in vitro* and *in vivo*. The *working hypothesis* is that by using RNAi to silence CX3CR1 will significantly reduce influenza replication *in vitro* and *in vivo*. The data presented in Chapter 3 show that siRNA against CX3CR1 reduces transcript expression close to 90%, which results in a reduction in influenza replication. Additionally, inhibiting CX3CR1 expression also correlated to a reduction in influenza titer in a lung epithelial cell line. Furthermore, utilizing multiple strains of influenza a virus, the importance of CX3CR1 in viral replication was present in all strains, suggesting that the viral-host interaction of influenza and CX3CR1 is conserved across multiple strains. Finally, a lack of CX3CR1 in a mouse model resulted in decreased lung viral titers at early stages of infection, suggesting that the absence of this chemokine receptor delays influenza viral replication.

Specific Aim 2: To determine the role of CX3CR1 in influenza replication, spread, and cytopathogenesis. The *working hypothesis* is that one or more influenza proteins interact with CX3CR1 for assembly of virions, trafficking of proteins to the cell surface, or egress of virions. Upon blocking surface expression of CX3CR1, there was no significant decrease in influenza replication, suggesting that the virus is not using CX3CR1 as a co-receptor. Influenza NP accumulated in the cytoplasm of cells that were treated with siRNA against CX3CR1 and infected with influenza, demonstrating that viral protein production was not being affected by a reduction in CX3CR1. When CX3CR1 was expressed in HEK293T cells and subsequently infected with influenza, localization of M2 and HA with CX3CR1 was observed at the golgi and cell membrane, suggesting that these two proteins may interact with CX3CR1 after post-translational modifications such a palmitoylation. Finally, it was observed that in mice lacking

CX3CR1, a delay in replication of the virus was noticed, suggesting a utilization of the chemokine receptor early in infection and replication, but also a delay in clearance possibly due to the lack of a chemoattractant molecule in the immune system preventing proper sufficient immune cell trafficking.

Specific Aim 3. To determine the effect that prior infection or co-infection with respiratory syncytial virus has on influenza virus replication. The *working hypothesis* is that pre-infection or co-infection with respiratory syncytial virus (RSV) negatively alters the innate and adaptive immune responses, resulting in an altered immune response to influenza virus infection causing enhanced disease. A delay in influenza viral clearance was observed in mice primed and co-infected with RSV. This may be due to the fact that a decrease in pulmonary immune cell recruitment as well as a decrease in pro-inflammatory cytokines was observed in these mice as well. Specifically, granulocytes and NK cells were decreased in mice primed for RSV and challenged with influenza, suggesting a prior infection with RSV negatively alters the immune profile that is required for effective influenza virus clearance.

Taken together, this research validated the importance of the fractalkine receptor CX3CR1 in influenza virus replication in both *in vitro* and *in vivo* models. It was determined that influenza virus utilizes CX3CR1 during late stages of replication, possibly viral assembly, trafficking, or egress. In addition, it was observed that specific influenza proteins co-localize with CX3CR1 during an infection, and may provide a potential mechanism of interaction. Finally, in vivo infections with RSV resulted in an altered influenza disease that is marked by modified immune responses caused by a prior infection with RSV.