EFFECTS OF A PRIMARY INFLUENZA INFECTION ON SUSCEPTIBILITY TO A SECONDARY *STREPTOCOCCUS PNEUMONIAE* INFECTION

by

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ABSTRACT

Influenza infections result in increased susceptibility to a secondary *Streptococcus pneumoniae* infection. The aim of the present studies was to determine the mechanism(s) responsible for this increase in susceptibility. Using an *in vivo* co-infection model, we found that susceptibility to *S. pneumoniae* was significantly increased at 6 days but not 3 days after an influenza infection. We depleted mice of neutrophils and found that neutrophils were important in the response to *S. pneumoniae* in mice infected with bacteria only or those infected with influenza for 3 days prior to a *S. pneumoniae* infection. However, at 6 days, neutrophil depletion did not alter the response to bacterial growth, indicating that neutrophil function was altered. We measured reactive oxygen species (ROS) generation and phagocytosis of *S. pneumoniae* by lung and bone marrow neutrophils isolated from mice infected with influenza for 3 or 6 days and compared these to neutrophils from either mice stimulated with LPS to induce neutrophil migration or from uninfected mice. We found that neutrophils from influenza-infected mice were not able to either phagocytose bacteria or produce ROS in response to incubation with *S. pneumoniae* as readily as those from uninfected mice. In addition, neutrophil-depleted mice infected with influenza for 6 days were more susceptible to *S. pneumoniae* infection than either uninfected depleted mice or depleted mice infected with influenza for 3 days. Lung cytokine levels, such as IL-10, were elevated in mice infected with influenza for 6 days followed by *S. pneumoniae*. These data indicate that influenza-induced changes in neutrophil-independent mechanisms increase susceptibility to a *S. pneumoniae* infection. We developed a novel *ex vivo* tracheal explant system to determine whether influenza-induced tissue damage increases adherence of *S. pneumoniae*. Using this system, we were able to model an influenza infection from the initial stages of infection to denudation and repair of the respiratory epithelium. We found that adherence was only increased at the initial stages of influenza infection. Increases in adherence may be due primarily to decreased mucociliary clearance. Together, these data indicate that an influenza infection increases susceptibility to a *S. pneumoniae* infection by altering both neutrophil-dependent and –independent mechanisms.

INTRODUCTION

Influenza Background

Influenza Virus

The influenza virus belongs to the virus family *Orthomyxoviridae*, which consists of influenza A, B, and C viruses, and thogotovirus (also called influenza D viruses), with influenza A viruses being the most pathogenic [1, 2]. Viruses belonging to this family have a segmented, negative single-stranded RNA genome and are enveloped with the plasma membrane of the host cell [1]. The genomes of influenza A and B viruses consist of 8 RNA segments, and the influenza C genome consists of only 7 RNA segments [1]. *Orthomyxoviridae* viruses are distinguished from other enveloped negative-stranded RNA viruses (*Paramyxoviridae*) based on their ability to bind mucus via their hemagglutinin protein [1]. The influenza viral messenger RNAs are transcribed from the viral RNA segments, making influenza viruses negative-sense viruses [1]. The genomic RNA of influenza viruses must serve as a template for both mRNA synthesis and the antigenome positive strand, which is used to produce additional copies of genomic RNA [1]. Even though these viruses encode and package their own RNA-dependent RNA transcriptase, they are dependent on infected cells for mRNA synthesis and can replicate only after synthesis of both viral mRNAs and proteins [1]. One of the properties of influenza viruses that make them unique is that all RNA synthesis occurs in the nucleus of an infected cell [1].

Nucleocapsid (NP) and matrix (M) proteins help to distinguish between the influenza A, B, and C viruses [1]. Within influenza A, further characterization into subtypes is based on the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins [1]. There are 16 known types of HA (H1-H16) and 9 known types of NA (N1-N9) which are found in wild birds and poultry worldwide [3, 4]. Of these, H1, H2, and H3 hemagglutinin subtypes, and N1 and N2 neuraminidase subtypes have been isolated in human influenza infections during influenza epidemics and pandemics since 1900 [2, 4, 5]. Both glycoproteins are found outside the lipid envelope, with HA appearing rod-shaped and NA appearing mushroom-shaped [1]. The 8 RNA segments of the influenza A virus encode for at least 10 viral proteins: three polymerase proteins (PB1, PB2, and PA), two envelope glycoproteins (HA and NA), NP, matrix protein (M_1), ion channel protein (M_2), and two nonstructural proteins (NS_1 and NS_2, although NS_2 is now known to associate with the M_1 protein and is no longer considered nonstructural) [1, 2, 6, 7].

While influenza A viruses are able to infect avian species, humans, and other mammalian species, including swine and horses, influenza B viruses have only been found to infect humans and influenza C viruses infect primarily humans but can also be found in swine in China [1]. The reservoir for influenza A viruses is wild birds such as ducks, geese, gulls, and shorebirds [3, 8]. The tissue tropism for avian influenza A viruses is the epithelial cells of the avian intestinal tract, resulting in excretion of virus in their feces [3]. Although they generally do not cause disease in wild birds, they can spread to domestic birds and mammals, with increased morbidity and mortality and the

possibility of an influenza pandemic [8-10]. Since they are able to infect a variety of species, influenza is not considered an eradicable disease [5].

The M_1 and M_2 genes are located on segment 7 of the viral genome [1]. The most abundant virion protein is M_1, which is located within the lipid envelope and provides rigidity to the membrane as well as structure to the virus particle [1]. In addition, M_1 may be associated with the cytoplasmic tails of the HA, NA, and M_2 proteins, as well as the RNP [1]. It is an influenza virus type-specific antigen which is highly conserved [11]. M_2 is encoded by a spliced mRNA [12] and is expressed abundantly on the plasma membrane of virus-infected cells but not in virions [13-15]. The primary function of the M_2 protein appears to be ion channel formation, which is necessary for viral uncoating within the host cell [1, 5].

The ribonucleoprotein (RNP) complex consists of four viral proteins, with NP the most abundant, which interact with the eight RNA segments [1]. The NP is unique for each influenza virus (A, B, or C) and is a major target of cytotoxic T lymphocytes for recognition of viral-infected cells [16]. The NP gene is located on segment 5 of the viral genome, and NP is synthesized in the cytoplasm and transported to the nucleus [1].

The RNA-dependent RNA polymerase complex is comprised of three polymerase proteins (PB1, PB2, and PA) which are encoded for on RNA segments 2, 1, and 3, respectively [1]. PA is acidic while PB1 and PB2 are basic [17]. The complex is synthesized in the cytoplasm and later transported to the nucleus [1].

The NS_1 and NS_2 genes are located on segment 8 of the influenza virus genome [1]. NS_1 is encoded by a colinear mRNA transcript and NS_2 is encoded by a spliced

mRNA [1]. Although NS_1 is readily found in the nucleus and associated with polysomes of influenza-infected cells, its presence has not been detected in virions, leading to its classification as nonstructural [18-22]. NS_2 was originally classified as nonstructural, but it has been found in virions associated with the M_1 protein [6, 7]. Within the host cell, it can be found in both the nucleus and the cytoplasm [23, 24].

Hemagglutinin

HA plays a major role in the infectivity of the influenza virus because it attaches the virus to the host cell by binding to sialic acid-containing receptors on the cell surface, thereby initiating viral penetration [1, 25]. In addition, HA mediates entry of the viral nucleocapsids into the cytoplasm of the host cell by mediating the fusion of the endosomal and viral membranes [1]. Immunity to and clearance of the influenza virus is mediated by neutralizing antibodies against HA, the major influenza antigen [1]. HA also plays a role in influenza epidemics due to changes in its antigenic structure which are not recognized by previously developed antibodies [1]. HA can be found in two forms: an uncleaved precursor form (HA_0) or a cleaved form which consists of two disulfide-linked chains (HA_1 and HA_2) [1]. Infectivity and spread of the virus is dependent upon the cleavage of HA, resulting in the initial fusion of the viral envelope and host cell membrane to allow for entry into the host cell [1, 4]. Linkages between sialic acid and galactose determine the species-specificity of different HA molecules to various hosts [1]. Human tracheas have α2,6 linkages, avian intestines have α2,3 linkages, and pig tracheas have both α2,3 and α2,6 linkages [26]. Since pigs contain receptors for both avian and human influenza viruses, a new influenza strain can emerge

if a pig is infected with both a human influenza virus and an avian influenza virus at the same time through reassortment of the viral genes between the two viruses.

Neuraminidase

The NA gene is located on segment 6 of the viral genome and is an important virulence factor which undergoes genetic mutation, similar to HA, leading to influenza epidemics [1]. Unlike HA, antibodies to NA are not neutralizing [1]. NA is used, along with HA, to subtype influenza A viruses and consists of an enzymatically active head domain with a membrane-embedded stalk region [1]. NA removes terminal sialic acid residues from glycoproteins by catalyzing the cleavage of the α-ketosidic linkage between terminal sialic acid residues and D-galactose or D-galactosamine, which allows the virus to move throughout the respiratory tract [25, 27]. In addition, NA may use the same mechanism to facilitate transport of the virus through the respiratory tract mucin layer in order to gain access to respiratory epithelial cells [1, 25]. NA activity is also necessary to disrupt self-aggregated influenza virions [25].

Antigenic Drift

The influenza virus maintains its antigenicity through two mechanisms of genetic variation: antigenic drift and antigenic shift. Antigenic drift occurs when point mutations in the HA and/or NA genes result in a new variant which previously circulating influenza antibodies do not recognize, resulting in yearly outbreaks and epidemics [28, 29]. The influenza virus RNA genome has a high mutation rate and does not have mechanisms to proofread or repair errors that occur during replication, so antigenic drift

is a common occurrence and is part of the evolution of influenza viruses [5, 8, 28]. Antigenic drift is the reason why new influenza vaccines have to be developed each year and why previous vaccines usually do not protect against future influenza epidemics [28].

Antigenic Shift

Antigenic shift is a more drastic way in which the virus alters itself and results in the emergence of a new influenza subtype with a novel HA (or novel HA and NA) to which the general population has no immunity, often resulting in a global pandemic [28, 29]. In order for antigenic shift and pandemics to occur, avian influenza virus genes must be introduced into humans [28]. One way in which this may occur is through genetic reassortment where avian and human influenza virus genomes mix in an animal (usually a pig or human) infected with both an avian influenza virus and a human influenza virus [28, 29]. Since the viral genome is segmented, reassortment of genes between two different viruses is possible if they infect the same host cell [4]. Another possible way for antigenic shift to occur is by direct transmission of an avian or pig virus to a human, with adaptation of the genome to the new host resulting in a new subtype capable of human-to-human transmission [4, 5, 28]. If this virus is able to spread readily between humans and there is little to no immunity to the virus in the general population, a pandemic is likely to occur [5].

Influenza Virus Infection

In the United States, the influenza virus infects up to 20% of the population and causes up to 40,000 deaths each year [1]. It is a highly contagious disease which affects

every age group, but the morbidity and mortality is greatest in persons over 65 years of age and in those with underlying pulmonary and cardiac diseases [30-32]. The influenza virus infects primarily the columnar epithelial cells of the upper respiratory tract and causes sporadic infections, large epidemics, or pandemics [5, 31]. The influenza virus can also replicate in monocytes/macrophages and other leukocytes [33]. The virus is transmitted between humans by the spread of infectious droplets through coughing, sneezing, and speaking [5, 34]. Once infected, there is a one to four day incubation period before symptoms of infection are evident, but viral shedding occurs as early as within 24 hours before the onset of symptoms [4, 5, 34]. Some common symptoms of an influenza infection are malaise, myalgia, anorexia, a dry cough, an abrupt onset of fever which lasts up to 5 days, and respiratory symptoms [5, 34]. Most influenza infections do not cause epidemics or pandemics. However, during an epidemic, the death toll is up to eight times higher than in years with no epidemics due to the increase in morbidity and complications, especially among the elderly [4, 32, 34]. Influenza infections normally result in 21,000 deaths per year [29]. The influenza viruses which are currently circulating include the H1N1 and H3N2 subtypes of influenza A viruses, as well as influenza B viruses [5]. Of the two influenza A viruses currently circulating, the H3N2 subtype has caused more severe disease and excess pneumonia and influenza mortality than the H1N1 subtype [35].

The influenza virus interferes with the functions of the host cell in a variety of ways, including the loss of protein synthesis due to the degradation of host mRNA by the viral cap endonuclease, leading to enhanced viral protein synthesis and eventual cytolysis

of the host cell [2, 4]. The influenza virus also downregulates RNA-activated protein kinase (PKR) activity, which normally responds to double-stranded RNA, by directly binding of PKR by the viral NS1 protein and by interfering with PKR dimerization and activation through the activation of a latent chaperone-associated protein (p581PK) [2, 36-39]. Cytolytic death usually occurs within 20-40 hours of influenza infection [2]. The double-stranded RNA produced during viral replication may also induce apoptosis due to the production of Fas antigen [4].

Virus-infected cells respond to the infection by activation of several transcription factors and production of chemotactic, proinflammatory, and antiviral cytokines, resulting in eventual clearance and resolution of the infection [2]. Monocytes/macrophages infected with influenza A virus secrete chemokines such as MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, MIP-3α, and IP-10 [40-42], whereas epithelial cells produce RANTES, MCP-1, and IL-8 [43, 44]. Type I interferons (IFN-α/β) are important anti-viral cytokines produced during an influenza infection by epithelial cells and monocytes/macrophages which play a role in both the innate and adaptive immune responses to the infection [33, 45-48]. In addition, IL-1β, IL-6, and TNF-α are produced by influenza-infected monocytes/macrophages [33, 46, 49-52]. Transcription factors which are activated due to an influenza infection and induce chemokine and cytokine production include nuclear factor kappa B (NF-κB), activating protein (AP)-1, interferon regulatory factors (IRFs), signal transducers and activators of transcription (STATs), and nuclear factor-IL-6 (NF-IL-6) [42, 45, 53-56].

Influenza Pandemics

Influenza pandemics occur when a new strain of influenza A virus emerges which is spread readily between humans, resulting in serious disease worldwide [8]. Since influenza pandemics have been noted, they have been found to occur almost every 30 years [4, 57]. The most deadly influenza pandemic of recent history was the 1918 "Spanish Flu" when up to 40-50 million people died worldwide and more than 500,000 people died in the United States [8, 34]. This pandemic was caused by the H1N1 influenza A virus and was unique in that it affected young adults 20-40 years old rather than the normally affected elderly, with mortality reaching 50% in some regions [34]. Death was rapid and usually occurred within a few days of infection [8]. The life expectancy in the United States decreased by 12 years due to the 1918 pandemic [29]. Recently, it has been shown that the HA of this virus was highly pathogenic [58]. Another pandemic, the "Asian flu" caused by the H2N2 influenza A virus, occurred in 1957 and is estimated to have caused over a million deaths worldwide with around 70,000 deaths in the United States [8, 34, 57]. In 1968, the "Hong Kong flu" pandemic occurred and was caused by the influenza A subtype H3N2, resulting in 34,000 deaths in the United States [8, 34].

Current Pandemic Threat: H5N1

A new subtype of influenza virus, H5N1, has emerged from an avian influenza virus during the past decade and has the potential to cause a global pandemic. H5N1 binds primarily to the α-2,3-linked sialic acid receptor found on the respiratory and alimentary epithelium of birds, but this receptor is also found on the ciliated portion of

the human pseudostratified columnar respiratory epithelium, resulting in the ability to infect humans [59]. The virus first appeared in humans in Hong Kong in 1997, where it was confirmed to have infected 18 people, resulting in 6 deaths [5]. In addition, asymptomatic or mildly asymptomatic infections were present during the 1997 outbreak in Hong Kong [60, 61]. Since 1997, it has continued to infect humans but has yet to develop the ability to spread efficiently between humans to cause widespread disease and a global pandemic. It initiated in poultry and has now spread to migrating birds, resulting in the spread of cases to countries other than China, including Cambodia, Thailand, and Vietnam [4, 8]. In addition, it has caused 108 confirmed human cases, resulting in 54 deaths, as of June 28, 2005 [8]. H5N1 remains a threat as it can both mutate rapidly and obtain genes from influenza viruses infecting other animal species [8]. If a human is infected with both H5N1 and a subtype which circulates readily among humans (such as H1N1 or H3N2), the H5N1 virus may reassort with these subtypes to produce a novel subtype that can be rapidly transmitted between humans [8]. Since no prior immunity exists to this novel subtype, a global influenza pandemic can result [8]. Since human ciliated respiratory epithelial cells can be infected by both avian and human influenza viruses during the late phase of infection, reassortment could occur in this cell type [4]. In addition, the respiratory epithelium of pigs contains receptors for both avian and human influenza viruses, allowing for another situation where reassortment could happen if a pig were infected with both types of influenza [4]. If such a situation were to occur, it is likely that the mortality rate would be drastic, since H5N1 already exhibits a high mortality rate in humans [4]. In addition, the H5N1 virus is stable for up to 6 days in the

environment and is resistant to both amantadine and rimantadine, making treatment and prevention of spread difficult [4].

Influenza Virus Vaccine

The most effective way of preventing and controlling influenza infections is by vaccination [29]. In the case of influenza, vaccines are formulated to stimulate an immune response against the HA and NA proteins, which results in neutralization of virus infectivity and reduction of the severity of disease, respectively [5]. There are currently two vaccines licensed for use in the United States: the inactivated viral vaccines and the intranasal live, attenuated vaccine [5]. The inactivated vaccine is composed of HA from H1N1 and H3N2 subtypes of the influenza A and B virus strains [5]. In children and adults, it is 60-90% effective at preventing influenza infections, but its immunogenicity is lower in the elderly [5]. While the inactivated vaccine does offer substantial protection, it is not able to induce sufficient mucosal or cell-mediated immune responses [5]. An alternative to the inactivated vaccine is the live, attenuated vaccine, which is administered intranasally and replicates in the upper respiratory tract [5]. Since the virus replicates, it is able to induce a specific protective immune response by the host to the influenza virus [5]. The advantages of the live, attenuated vaccine compared to the inactivated vaccine are it is able to induce mucosal and systemic immune responses [5]. In addition, this vaccine is more easily administered (needle-free) [5].

Antivirals

Recently, two new classes of influenza antivirals have been developed and licensed in the United States to help control and/or prevent influenza infections [29]. One class of influenza antivirals is the adamantine compounds which consist of amantadine and rimantadine hydrochloride. These compounds are 70-90% effective at preventing an influenza A virus infection if given prophylactially and can also reduce the severity of an infection if given within 48 hours of the onset of symptoms [5, 29, 62]. The adamantine compounds interfere with influenza A viral replication by blocking the function of the M_2 protein, resulting in prevention of virus uncoating after entry into the host cell [5]. Since influenza A viruses mutate rapidly, viruses resistant to amantadine and rimantadine have already been isolated [5].

Another class of influenza antivirals is the neuraminidase inhibitors. These compounds are sialic acid analogues which specifically inhibit neuraminidase activity of both influenza A and B viruses [5, 25]. They are able to inhibit all influenza A NA subtypes found in the avian reservoir [25]. The neuraminidase inhibitors are zanamavir (Relenza), which is administered by inhalation, and oseltamivir (Tamiflu), which is administered orally [5]. Both are given within 48 hours of the onset of symptoms and can reduce the length of disease by 1-2 days [5]. In addition, both are effective at preventing influenza infections [25]. Some advantages of these compounds over adamantine compounds are fewer cases of resistant viruses have been noted, they are effective at eliminating amantadine and remantadine resistant influenza viruses, and they

have a broader spectrum of antiviral activity since they can affect the NA activity of all 9 NA subtypes [5, 25].

Secondary Bacterial Infections

Influenza infections predispose individuals to the development of secondary bacterial pneumonia infections, which are characterized by a new fever and productive cough after recovery from the influenza infection [5]. The bacterial pathogens which most commonly cause such infections include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, and group A β-hemolytic streptococci [5]. While either infection alone is rarely fatal, a combined infection leads to a more drastic outcome, which is caused in part by failure of antibiotics to treat a *S. pneumoniae* infection after a prior influenza infection [63]. Influenza and pneumonia combined were the number one cause of infectious death in the United States in 2002, despite the widespread availability of antibiotics [64]. Since *S. pneumoniae* is the most common cause of community-acquired pneumonia [65, 66], it is important to better understand the mechanisms whereby a prior influenza infection increases susceptibility to a secondary *S. pneumoniae* infection.

Streptococcus pneumoniae Background

Classification

Streptococcus pneumoniae are gram-positive, α-hemolytic, lancet-shaped, facultative anaerobes with a polysaccharide capsule which normally occur in pairs (diplococci) or in long chains. *S. pneumoniae* was first isolated in 1881 [67]. At least 90

different serotypes of *S. pneumoniae* exist which are classified based on the composition of the oligosaccharides which make up their capsular polysaccharides [68, 69]. Each of these serotypes is able to colonize the nasopharynx and establish a carrier state, although some serotypes are able to colonize more readily [70]. The carriage rates of *S. pneumoniae* vary from 5-30% in healthy adults and 20-50% in healthy children [71-73]. Of the 90 serotypes of *S. pneumoniae*, approximately 20 serotypes are able to cause disease in other areas of the body, such as the lungs, blood, or brain [70]. Importantly, 10 of the 90 *S. pneumoniae* serotypes cause 62% of invasive pneumococcal disease cases worldwide [74]. Young children and the elderly are not able to mount an effective immune response against capsular polysaccharides and are therefore more susceptible to *S. pneumoniae* infection than healthy adults [70]. Pneumococcal pneumonia remains a common cause of death for young children [75]. Protection from *S. pneumoniae* disease relies on both the innate and the adaptive immune responses [70].

Innate and Adaptive Immune Responses to *S. pneumoniae*

S. pneumoniae triggers an immediate innate immune response [70]. When it first enters the lungs, a variety of opsonins, such as complement, C-reactive protein, lectins, and IgM anti-carbohydrate antibodies, bind to the *S. pneumoniae* [70]. Opsonization leads to phagocytosis by neutrophils and macrophages, which then produce cytokines and other inflammatory mediators to initiate the adaptive immune response [70]. *S. pneumoniae* inhibits the deposition of opsonins and resulting phagocytosis with its capsular polysaccharide [70]. In addition, pneumococcal surface protein A, which extends beyond the capsule, has an electronegative charge which reduces complement

deposition [76]. Neutrophils are recruited to the site of infection through the activation of complement by pneumolysin and C-polysaccharide [70]. In addition, *S. pneumoniae* infection leads to the production of a variety of pro-inflammatory cytokines (IL-1, IL-6, TNF-α, IL-12, and IFN-γ) as well as anti-inflammatory cytokines (IL-4 and IL-10) which help to stimulate the immune response [77].

S. pneumoniae induces an adaptive immune response with the production of both anti-capsular and anti-surface protein antibodies [70]. Protection from *S. pneumoniae* infection is due primarily to antibody-complement dependent phagocytosis [70]. Antibody-dependent clearance of *S. pneumoniae* that results from prior exposure to *S. pneumoniae* is the basis for the development of the current *S. pneumoniae* vaccines [78].

S. pneumoniae Infections

S. pneumoniae colonizes the upper respiratory tract and causes respiratory diseases such as pneumonia, sinusitis, and otitis media and invasive infections such as meningitis and bacteremia [65, 79, 80]. It is the most common cause of acute otitis media, sinusitis, and pneumonia [81, 82]. This pathogen is especially deadly in developing countries, where it is the most common pathogen isolated from the over 5 million deaths per year due to pneumonia in children under 5 years old [79]. Each year it causes approximately 1.1 million deaths worldwide [83]. In the United States alone, pneumococcal pneumonia results in death in 5-7% of the estimated 1 million yearly cases, making it one of the top 10 causes of death [82, 83]. *S. pneumoniae* is the most common cause of community-acquired pneumonia worldwide, and it is estimated that *S. pneumoniae* causes 25% of all cases of community-acquired pneumonia in the United

States [65, 66]. The reservoir for *S. pneumoniae* is in the human nasopharynx, where it normally exists asymptomatically in 40-60% of the population [65, 79, 84]. Children, the elderly, the immunocompromised, and individuals with chronic diseases are the most susceptible to *S. pneumoniae* infections [84]. African-Americans, American Indians, Native Alaskans, and Australian Aborigines are also more susceptible to *S. pneumoniae* infection [80]. *S. pneumoniae* is spread via droplet transmission from person to person [80]. Infection usually results when a new serotype is acquired and spreads from the nasopharynx to the eustachian tube, resulting in otitis media, or to the lungs via aerosolization, resulting in lobar pneumonia [66, 85, 86]. This spread from the nasopharynx to the lungs often occurs when pneumococci are aspirated during sleep [87, 88]. The pneumonia caused by *S. pneumoniae* proceeds through four stages of development: engorgement (0-4 hours), red hepatization (4-24 hours), gray hepatization (1-5 days), and resolution (5-10 days) [78, 89, 90]. This pneumonia results in irreparable damage during the initial stages of the disease and leads to death in 5-22% of cases [91]. Once in the alveoli, the *S. pneumoniae* can spread rapidly into the blood [92].

Pneumococcal pneumonia is characterized by a sudden onset of chills and chest pain followed by the development of a fever and rusty sputum [93]. However, other symptoms such as nausea, vomiting, or diarrhea may also be present [94]. Several techniques are employed to determine if *S. pneumoniae* is responsible for the clinical symptoms of pneumonia. Decreased white blood cell counts often occur in patients as well as elevated C-reactive protein and bilirubin levels [95-97]. Pneumococcal pneumonia is often diagnosed by x-ray and is characterized by pulmonary infiltrates in

one or more segments within a single lobe [98, 99]. Identification of *S. pneumoniae* as the causative agent of pneumonia using alveolar fluid can be challenging since *S. pneumoniae* is a common resident of the nasopharynx and may be present in patient samples even though another organism is causing the pneumonia. A blood culture is the most definitive diagnostic tool; however, not all cases result in bacteremia and therefore this detection method is not optimal for all cases [100].

Antibiotics: Treatment of *S. pneumoniae* and Resistance

Antibiotics such as penicillin are currently the primary method of control of pneumococcal infections [101]. One of the problems associated with *S. pneumoniae* infections is the increasing amount of resistance to antibiotics, particularly penicillin [102, 103]. Treatment of *S. pneumoniae* with penicillin began in the mid-1940s, and drastic increases in *S. pneumoniae* resistance to penicillin began to occur during the 1970s and 1980s [80]. As of 1999, approximately one-third of clinical isolates were resistant to penicillin with highly resistant strains comprising 14% of *S. pneumoniae* isolates [104-106]. The serotypes which are most frequently isolated from children (serotypes 6, 14, 19, and 23) are also the ones that have developed the most antibiotic resistance [100]. In addition, *S. pneumoniae* have been isolated which are multi-drug resistant, limiting the choices of antibiotics which can be used to treat infections [102]. More powerful and narrower spectrum antibiotics such as vancomycin are being used as a last resort for treatment of multi-drug resistant *S. pneumoniae*, but *S. pneumoniae* has the mechanisms needed to develop resistance to vancomycin as well [78]. In addition, *S.*

pneumoniae antibiotic tolerance, which allows bacteria to survive in the presence of antibiotics, has been found for both penicillin [107-109] and vancomycin [110, 111].

S. pneumoniae vaccines

Currently, there are two pneumococcal vaccines licensed in the United States: the 23-valent pneumococcal polysaccharide vaccine (covers 23 of the most common serotypes) and the 7-valent pneumococcal conjugate vaccine (covers 7 of the most common serotypes in children) [80]. The 23-valent vaccine is composed of the capsular polysaccharides from 23 different serotypes of *S. pneumoniae* (serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) which covers up to 93% of the serotypes found in the United States [79, 80, 112]. While this vaccine is effective at protecting adults from pneumococcal infection, it is not very effective in children under 2 years of age or in the elderly due to the poor antibody response elicited by polysaccharide-based vaccines [113, 114]. In addition, it generates a T-cell independent response that does not induce memory [76]. To overcome this problem, a 7-valent pneumococcal polysaccharide-conjugate vaccine was developed which generates antibody responses in children under 2 years of age to the 7 most common serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) that cause invasive disease in the United States [70]. It consists of polysaccharides from these 7 serotypes of *S. pneumoniae* individually conjugated to diphtheria toxoid CRM_{197} [80]. Interestingly, widespread use of this vaccine in infants has been associated with decreased rates of pneumococcal disease in the general population, including older children and adults [80].

One drawback of both of these vaccines is the failure to protect against disease from the other *S. pneumoniae* serotypes which are prevalent in other parts of the world [70, 112]. Through international travel, these serotypes could colonize the nasopharynx of an individual who then brings that serotype to another country. This newly introduced serotype could potentially cause infection due to the lack of immunity. Vaccines which contain pneumococcal surface proteins are currently being developed [80]. Some of the proteins being used in these vaccines include neuraminidase, autolysin, pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), pneumococcal surface adhesion A (PsaA), and proteinase maturation protein (PmpA) [115-119]. In order to be useful as a vaccine, the protein must not differ antigenically between *S. pneumoniae* serotypes and must elicit a strong protective antibody response [70].

Adhesins

The pneumococcus adheres to GlcNAcβ1-3Gal glycoconjugate receptors on nasopharyngeal cells and GalNAcβ1-4Gal, GalNAcβ1-3Gal, and GlcNAc glycoconjugate receptors on type II pneumocytes [120-122]. *S. pneumoniae* binds poorly to ciliated airway cells but it does bind to components of the basement membrane, such as fibronectin, which are exposed after respiratory epithelium damage caused by *S. pneumoniae* pneumolysin or influenza virus [123-125]. It has also been shown to adhere to the platelet activating factor receptor (PAFr) on activated cells, resulting in increased internalization of *S. pneumoniae* and translocation across the endothelial barrier [65, 121]. The phosphorylcholine of the pneumococcal cell wall is able to bind to PAFr in a similar manner as PAF, thereby anchoring the *S. pneumoniae* to the cell [65].

Pneumococcal virulence factors/surface proteins

Capsule. The polysaccharide composition of the pneumococcal capsule was first described by Avery et al. in 1925 [126, 127]. The polysaccharide is the primary virulence factor for the pneumococcus, and *S. pneumoniae* strains which are not encapsulated are avirulent and do not colonize the nasopharynx [79, 128]. Although the capsule does not contribute directly to inflammation or adherence [129, 130], it has been show to inhibit phagocytosis [112] and bacterial killing via the classical complement pathway [80]. The effectiveness of phagocytosis is dependent on the composition of the capsule and not the amount of capsule present [131]. *S. pneumoniae* exists in two forms based on the phenotype of its capsule: opaque or transparent [92]. The transparent form, which displays less capsule, adheres more readily to the nasopharynx and aids in colonization while the opaque phenotype, which displays more capsule, less choline, and more PspA, plays a role in survival in the bloodstream [92, 132-134]. Both phenotypes adhere equally to resting cultured human type II pneumocytes [65].

Cell Wall. Unlike the capsule, the pneumococcal cell wall, which contains lipoteichoic acid, teichoic acid, and C polysaccharide, has been shown to be highly inflammatory [79, 89, 135, 136]. Both lipoteichoic acid and C polysaccharide activate the alternative complement pathway and bind C-reactive protein [137, 138]. The lipoteichoic acid contains phosphorylcholine, which allows *S. pneumoniae* to adhere to the platelet activating factor (PAF) receptor on activated human endothelial and epithelial

cells [121]. Phosphorylcholine also anchors *S. pneumoniae* surface proteins known as choline binding proteins [139].

Choline Binding Proteins. *S. pneumoniae* has several choline binding proteins, including CbpA, CbpD, CbpE, CbpF, LytA, LytB, LytC, PcpC, PcpA, and PspA [140]. These proteins have a conserved binding domain [141] which anchor them to the choline found in cell wall teichoic acid or lipoteichoic acid [142]. The functions of CbpD, PcpA, and PcpC are not known [140].

Pneumococcal surface protein A (PspA) is an antigenically variable virulence protein found on the surface of every known serotype of *S. pneumoniae* [79, 143]. PspA binds lactoferrin [144] and inhibits complement activation [145]. Its role in the virulence of *S. pneumoniae* has been shown by deleting the PspA gene and by passive immunization with anti-PspA antibodies, with both effects resulting in attenuated virulence [146, 147].

Choline-binding protein A (CbpA), the most abundant CBP [78], is a pneumococcal surface protein which plays an important role in adherence of *S. pneumoniae* to cytokine-activated type II pneumocytes and endothelial cells [148]. It has also been shown to bind to the IgA secretory component, leading to decreased function of IgA [149]. In addition, it binds to the polymeric immunoglobulin receptor (pIgR) molecules on nasopharyngeal epithelial cells, which then translocate the bacteria across the epithelial barrier to the bloodstream [150].

Autolysin (LytA) is a protein found in the cell envelope which is normally inactive. However, when cell wall biosynthesis stops during stationary phase or when

penicillin is present, the enzyme becomes active and degrades the peptidoglycan within the cell wall, leading to autolysis of the *S. pneumoniae* and release of cell wall components such as peptidoglycan and teichoic acid [78, 79]. This release of cell wall components leads to an inflammatory response and eventual tissue damage [79, 89]. In addition, bacterial lysis releases cytoplasmic contents such as pneumolysin, a powerful toxin which is normally not secreted by *S. pneumoniae* [79, 151]. *S. pneumoniae* mutants which lack autolysin are significantly less virulent, with rapid clearance of *S. pneumoniae* from the lung, no invasion of the bloodstream, and no generation of an inflammatory response [152]. Protection from *S. pneumoniae* can be generated through immunization with autolysin, but not to a greater extent than the protection generated from immunization with pneumolysin administered either alone or in conjunction with autolysin, indicating that protection through autolysin vaccination is due to inhibition of release of pneumolysin [153].

Other Virulence Factors**.** The pneumococcus contains an IgA1 protease which may allow *S. pneumoniae* to evade the local mucosal immune response [79]. Two forms of superoxide dismutase (SOD) exist in the pneumococcus: MnSOD and FeSOD [148]. MnSOD has been shown to play a role in virulence through the use of MnSOD mutants, which produce a more localized neutrophil response than wild type *S. pneumoniae* [154].

Pneumococcal surface adhesin A (PsaA) is a 37 kDa surface protein which also plays a role in the virulence of *S. pneumoniae* [79]. Its main function is hypothesized to be to transport Mn^{+} and Zn^{2+} into the cytoplasm [155]. Mutant strains which lack PsaA

are avirulent and adhere less readily to type II pneumocytes [156]. Protection from *S. pneumoniae* infection has been shown after immunization with PsaA [157].

Neuraminidase (NA) is a protein found on all serotypes of *S. pneumoniae* which cleaves the terminal sialic acid residues of glycoproteins and glycolipids, which may enable the *S. pneumoniae* to disrupt the mucus layer and colonize the respiratory epithelium [158]. In addition, NA may expose *S. pneumoniae* receptors by altering the structure of surface carbohydrates, leading to increased *S. pneumoniae* adherence to the respiratory epithelium [159, 160]. *S. pneumoniae* has at least two separate neuraminidase genes, *nanA* and *nanB* [79, 161-163]. NanA is a surface protein which plays a role in *S. pneumoniae* virulence by allowing *S. pneumoniae* to survive and replicate within the lung [79]. The function of NanB is not yet known.

The *S. pneumoniae* surface protein hyaluronate lyase (Hyl) is a hyaluronidase enzyme which degrades hyaluronic acid found in the connective tissue [76]. This enzyme may facilitate the invasion of *S. pneumoniae* into the bloodstream as well as allow *S. pneumoniae* to access the respiratory epithelium [79]. Hly has been shown to play a role in allowing *S. pneumoniae* to cross the blood-brain barrier, leading to bacterial meningitis, and in survival of *S. pneumoniae* in the bloodstream [79, 164].

Pneumolysin (Ply) is a 53 kDa pore-forming toxin found in the cytoplasm of *S. pneumoniae* which has a variety of effects on the host system [65, 79]. It is found in all serotypes of *S. pneumoniae* [165] with little variation between serotypes [166]. Since Ply is cytoplasmic and is not secreted, it depends on the actions of autolysin for its release from the cytoplasm [76]. Ply has both cytotoxic and proinflammatory properties,

resulting in an influx of neutrophils into the lungs during pulmonary infection [70]. It has been shown to inhibit the respiratory burst of neutrophils [167], to stimulate human monocytes and macrophages to produce nitric oxide, interleukin-1, and tumor necrosis factor [168, 169], to activate the classical complement pathway [170], to stimulate phospholipase A in endothelial cells [171], and to inhibit ciliary beating of the respiratory epithelium, thereby facilitating the spread of *S. pneumoniae* [172]. It has been shown to be an important virulence factor in *in vivo* infections as shown by studies using a Ply-deficient mutant [79, 152]. It is hypothesized that Ply exerts its toxic effects on cells by binding to cholesterol on cell membranes and inserting itself into the membrane, where it then oligomerizes to form pores in the cellular membrane, leading to cell lysis [79]. It also activates complement by binding the Fc portion of immunoglobulin, leading to activation of the classical complement pathway [79, 173]. It has been shown that both complement activation and lytic activity by Ply are necessary for full virulence of *S. pneumoniae* [79]. The lytic activity of Ply is important during the initial stages of infection, leading to acute lung injury and bacterial growth, while complement activation by Ply is important for bacterial growth and bacteremia during the later stages of infection [79].

Effects of Influenza Infection on Susceptibility to *S. pneumoniae* Infection

Clinical, epidemiological, and experimental evidence indicates that a prior influenza infection increases susceptibility to a secondary *S. pneumoniae* infection, with maximal susceptibility occurring around one week after the onset of the influenza

infection [174-178]. There are a variety of mechanisms proposed to account for the increased susceptibility to *S. pneumoniae* infection after an influenza infection. One of the two main hypotheses to explain this increase in susceptibility is that influenza-induced tissue damage, resulting in denudation of the respiratory epithelium and exposure of the basement membrane, leads to the exposure of novel cells and/or receptors to which the *S. pneumoniae* are able to adhere more readily. The other main hypothesis is that the influenza virus affects neutrophil function, thereby reducing their ability to kill and eliminate *S. pneumoniae*. Evidence for both hypotheses exists, but the overall contribution of each of these mechanisms has not yet been fully elucidated.

Tissue Damage

Evidence from the 1918 influenza pandemic indicates that secondary bacterial pneumonias were responsible for many of the deaths during this pandemic [179, 180]. During the 1957-1958 influenza pandemic, some of the patients who died from secondary bacterial pneumonia had *S. aureus* adherent to the denuded epithelium of the tracheo-bronchial tree [78, 181]. Since the ciliated epithelial cells which normally remove invading bacteria were destroyed, the bacteria were able to gain access to and adhere to the denuded epithelium [181]. During the pandemics of 1918 and 1968-1969, *S. pneumoniae* was the most commonly isolated organism from patients with secondary bacterial pneumonia infections [179, 180, 182, 183]. *In vitro* studies have shown that a prior adenovirus infection of human respiratory epithelium increased *S. pneumoniae* adherence [184]. In addition, exposure to influenza virus or rhinovirus has been shown to

increase *S. pneumoniae* adherence to cultured primary and immortalized epithelial cells [185, 186].

During an influenza infection, viral-infected ciliated epithelial cells of the respiratory tract are lysed by the virus, leading to exposure of the basement membrane [124]. As the tissue repairs itself, several other cell types are expressed, including basal cells, undifferentiated epithelial cells, microvilli-covered cells, goblet cells, differentiating epithelial cells, and differentiated epithelial cells [124, 187, 188]. *S. pneumoniae* may adhere to receptors which are exposed during this process, either on the basement membrane or on the cells responsible for repairing the damaged epithelium. *In vitro* adherence assays showed *S. pneumoniae* binds to fibronectin, which is a protein of the extracellular matrix in the basement membrane [123]. In addition, virus-infected cells may express viral proteins such as hemagglutinin or viral-induced cellular proteins which may be receptors for *S. pneumoniae* [63]. Several studies have shown that influenza viral neuraminidase increases susceptibility to a secondary *S. pneumoniae* infection through cleavage of sialic acid residues, thereby exposing potential *S. pneumoniae* receptors [189-191].

Plotkowski et al. examined *S. pneumoniae* adherence to the tracheal epithelium of mice which had been infected *in vivo* with influenza virus and found that a prior influenza infection of 6 days increased *S. pneumoniae* adherence [124]. They found that *S. pneumoniae* bound preferentially to areas where the epithelium was denuded and the basement membrane was exposed, presumably due to the influenza infection [124]. In other *S. pneumoniae* adherence studies, *S. pneumoniae* was found bound to the mucus

layer but never to healthy, ciliated respiratory epithelium, indicating that the mucus layer contains *S. pneumoniae* receptors [192]. An influenza infection has also been shown to impair tracheal ciliary function, which may decrease the effectiveness of *S. pneumoniae* removal thereby increasing the number of *S. pneumoniae* which can adhere to the damaged epithelium [174]. Since *S. pneumoniae* can migrate from the nasopharynx to the lungs via the trachea [193], alterations in the morphology and/or function of the tracheal respiratory epithelium caused by the influenza virus could drastically alter the outcome of an influenza infection by allowing *S. pneumoniae* into the lungs. These studies provide evidence that influenza-induced tissue damage increases susceptibility to a secondary *S. pneumoniae* infection, but the overall contribution to this increase in susceptibility has not yet been fully elucidated.

Neutrophil Function

Neutrophils are the primary cells involved in eliminating a bacterial infection and preventing invasion into the bloodstream [194]. An influenza infection may alter the local immune response by interfering with neutrophil function, thereby decreasing phagocytosis of *S. pneumoniae* resulting in increased growth of *S. pneumoniae* [195]. Studies using neutrophils infected with the influenza virus *in vitro* have shown that the influenza virus suppresses the respiratory burst activity of neutrophils after stimulation with either FMLP or PMA [196]. This suppression of respiratory burst can be seen following 5 minutes of incubation time with influenza virus [197]. The influenza virus has been shown to increase neutrophil apoptosis by increasing neutrophil expression of Fas antigen and Fas ligand [198] and by inducing DNA fragmentation [199]. In addition,

neutrophil apoptosis and hydrogen peroxide production have been shown to both be increased after incubation of neutrophils with influenza virus followed by *S. pneumoniae in vitro* compared to neutrophils incubated with influenza virus or *S. pneumoniae* alone [200]. Other studies with chinchillas have shown that an *in vivo* influenza infection resulted in decreased neutrophil chemotaxis [195]. Martin et al. showed that neutrophil chemotaxis in influenza-infected human subjects was impaired [201]. Abramson et al. showed that neutrophils infected with influenza virus and stimulated with FMLP or *S. aureus* released less lactoferrin than those not infected with influenza virus [202]. In addition, blood neutrophils collected from chinchillas infected with influenza virus for 6 days had decreased bactericidal activity against *S. pneumoniae* and decreased phagocytosis of *S. pneumoniae* [203].

Influenza virus can also affect structural components of neutrophils. Wheeler et al. showed that neutrophils incubated with influenza virus had alterations in the structure and distribution of their cytoskeletons as well as alterations in the phosphorylation of cytoskeletal proteins [204]. The influenza virus has also been shown to affect neutrophil function by inhibiting lysosome-phagosome fusion, resulting in decreased bactericidal activity [205]. Neutrophils which were previously exposed to influenza virus had decreased phosphorylation of several membrane and cytosolic proteins after stimulation with FMLP or PMA [206]. Together, these studies indicate that the influenza virus affects neutrophil function. The extent to which influenza-induced neutrophil dysfunction contributes to increased susceptibility to a *S. pneumoniae* infection is not yet known.

Other Possible Mechanisms of Increased Susceptibility

An influenza virus infection has also been shown to affect macrophage and monocyte function in that it decreases monocyte chemotaxis and accumulation of macrophages at the site of infection and decreases their antibacterial defenses [207-209]. It has been shown that an influenza infection suppresses alveolar macrophage phagocytic ability [210]. Warshauer et al. showed that a prior influenza infection reduced the ingestion of *Staphylococcus epidermidis* by alveolar macrophages and allowed for growth of the bacteria within the macrophages after they had been ingested, indicating intracellular killing of the bacteria was impaired [211]. Alveolar macrophages are vital elements of the pulmonary response against pathogens such as *S. pneumoniae*, and they have been shown to be critical in the immune response to pneumococcal pneumonia in that they eliminate both bacteria and apoptotic neutrophils [212]. Apoptotic neutrophils which are not phagocytosed by alveolar macrophages eventually leak out their cytotoxic components, such as reactive oxygen species, defensins, granule proteases, and lysozyme, which can lead to amplified inflammation and tissue damage [212].

Another possible mechanism by which a prior influenza infection may increase susceptibility to a secondary *S. pneumoniae* infection is through the cytokine response. Previous *in vitro* studies have shown that epithelial cells incubated with inflammatory cytokines such as TNF-α and IL-1α, which are produced during an influenza infection [213], supported increased adherence and invasion of *S. pneumoniae*, indicating that cytokines may upregulate *S. pneumoniae* receptors such as the platelet-activating factor receptor [121]. Recently, IL-10 has been shown to be an important player in affecting

susceptibility to a secondary *S. pneumoniae* infection [214]. In mice infected with influenza for 14 days, IL-10 levels were elevated despite the lack of virus in the lungs. This elevation of IL-10 resulted in increased susceptibility to *S. pneumoniae* infection compared to mice with no prior influenza infection [214]. When IL-10 levels were decreased, so was susceptibility to *S. pneumoniae* infection, indicating that elevated levels of IL-10 are partially responsible for secondary *S. pneumoniae* infections [214]. However, the onset of IL-10 expression after an influenza infection and how this expression affects susceptibility to *S. pneumoniae* have not yet been determined. The cells responsible for this increased production of IL-10 have not yet been determined. In addition, other inflammatory cytokines may be elevated during an influenza infection, thereby contributing to the initiation of a secondary *S. pneumoniae* infection.

Hypothesis

Since evidence exists indicating that both influenza-induced tissue damage and altered neutrophil function contribute to increased susceptibility to *S. pneumoniae* infection, we wanted to determine the relative contributions of each of these alterations in function to the overall increase in susceptibility. We hypothesized that influenza-induced tissue damage is the primary mechanism by which a prior influenza infection increases susceptibility to a secondary *S. pneumoniae* infection. However, we did not want to ignore alterations in susceptibility due to changes in neutrophil function. Therefore, we examined the roles both neutrophil dysfunction and tissue damage after an influenza

infection had in this increased susceptibility. In order to elucidate the contributions of each of these mechanisms, we used a variety of *in vivo*, *in vitro*, and *ex vivo* techniques.

BOTH INFLUENZA-INDUCED NEUTROPHIL DYSFUNCTION AND NEUTROPHIL-INDEPENDENT MECHANISMS CONTRIBUTE TO INCREASED SUSCEPTIBILITY TO A SECONDARY *STREPTOCOCCUS PNEUMONIAE* INFECTION

Introduction

A prior influenza infection increases susceptibility to a secondary bacterial pneumonia infection, which has been associated with an increase in morbidity and mortality during influenza epidemics and pandemics [29, 35, 57, 78, 175-178, 183, 189-191, 215-221]. Bacteria commonly causing pneumonia in the most severely ill influenza patients are *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*. Together, influenza and secondary bacterial pneumonia were the most common cause of infectious death in the United States in 2002 [64]. With the ever-increasing bacterial antibiotic resistance and the possibility of another influenza pandemic in the near future, understanding and controlling these infections is crucial.

Evidence from research, clinical studies, and epidemiological studies have shown a positive correlation between the increase in morbidity and mortality during influenza epidemics and pandemics, and the associated increase in secondary *S. pneumoniae* infections [29, 35, 57, 78, 175-178, 183, 189-191, 215-221]. There are currently two hypotheses to explain the increase in susceptibility to secondary *S. pneumoniae* infections after an influenza infection. One hypothesis is that influenza infection alters neutrophil function, thereby reducing the effectiveness of phagocyte-mediated killing of the

bacteria. The other hypothesis is that the tissue damage caused by influenza virus alters the epithelial surface of the respiratory tract, thereby exposing different surface receptors to which the *S. pneumoniae* adhere. Influenza-induced tissue damage may also increase the affinity of the *S. pneumoniae* for its receptors, which may result in increased growth and decreased neutrophil killing of *S. pneumoniae* in the respiratory tract.

The hypothesis that influenza-induced neutrophil dysfunction is the primary means of increased susceptibility to *S. pneumoniae* infection after influenza infection has been supported by several groups using both *in vitro* and *in vivo* models of influenza infection [194, 195, 197, 201-206, 222-224]. Previous studies by our laboratory have shown neutrophils are important in resistance to *S. pneumoniae* infection independently of an influenza infection [225]. The three major properties of neutrophils which are crucial for bacterial clearance (chemotactic response, phagocytosis, and intracellular killing) have been shown to be altered by influenza virus, thereby potentially increasing susceptibility to *S. pneumoniae* infection after influenza infection due to decreased phagocytosis and killing of the bacteria by neutrophils [197, 198, 201, 223]. While there have been extensive *in vitro* studies to support this hypothesis, the only studies examining the effects of an *in vivo* influenza infection on neutrophil function used blood neutrophils from an influenza-infected mouse [195, 203] or whole lungs from mice infected with both influenza and *S. pneumoniae in vivo* [176, 226]. The effects of an *in vivo* influenza infection on lung neutrophil function independently of an *S. pneumoniae* infection have not yet been determined. Therefore, the extent that compromise of neutrophil function contributes to influenza-induced increased susceptibility to *S. pneumoniae* is not known.

Influenza-induced tissue damage may also play a role in increasing susceptibility to *S. pneumoniae* infection after influenza infection. During the initial stage of influenza infection in which viral replication occurs, the virus may alter the surface receptors expressed on the epithelial cells, such as the platelet-activating factor (PAF) receptor, to which the *S. pneumoniae* can adhere [65, 121, 227]. In addition, expression of viral glycoproteins, such as neuraminidase and hemagglutinin, on influenza-infected cells can be induced by the virus and may increase *S. pneumoniae* adherence [63]. The influenza virus can also expose novel *S. pneumoniae* receptors by cleaving sialic acid on glycoproteins of host respiratory cells with its neuraminidase protein, resulting in increased pneumococcal adherence [159, 189-191, 228, 229]. After replication, the virus lyses the infected cells, denuding the epithelium and exposing the basement membrane and proteins from the extracellular matrix, such as fibronectin, to which the *S. pneumoniae* can potentially adhere [63, 124]. In addition to altering the cell surface receptors, influenza virus also causes the exposure of different cell types as the epithelium repairs itself [124]. Initially, basal cells migrate to cover the surface of the exposed basement membrane, where they then differentiate into ciliated epithelial cells (33). During the entire influenza infection period, different cell types and receptors are exposed on the respiratory epithelium. The *S. pneumoniae* may adhere more tightly to these receptors and/or cell types, making it more difficult for neutrophils to phagocytose and kill the bacteria, thereby increasing the severity of the infection as the *S. pneumoniae* continue to replicate without the consequence of neutrophil-mediated killing. In addition, influenza-induced tissue damage leading to denudation of the tracheal respiratory

epithelium has been shown to affect mucociliary clearance, which may allow *S. pneumoniae* to adhere more readily to the trachea and eventually infect the lungs, resulting in pneumonia [124, 174]. Recently, it has been shown that IL-10 is increased in lungs of mice infected with influenza 14 days prior to a *S. pneumoniae* infection, and this increased IL-10 expression increases susceptibility to a secondary *S. pneumoniae* infection [214]. However, neither the initial onset of IL-10 production after an influenza infection, the cells responsible for this increase in IL-10 produciton, nor the effects of the initial IL-10 expression on susceptibility to a *S. pneumoniae* infection have yet been determined.

In this study, we examined the contributions of influenza-induced changes in neutrophil-dependent and –independent mechanisms in increased susceptibility to a secondary *S. pneumoniae* infection.

Materials and Methods

Infectious Agents

Influenza virus A/PR/8/34 (PR8) was cultured and stored in allantoic chicken egg fluid at -80°C. *Streptococcus pneumoniae* Type 4 (ATCC 6304) was cultured in Todd Hewitt broth supplemented with 0.5% yeast extract (THY) at 37°C and 5% CO_2. Stock cultures in logarithmic growth were frozen in 10% glycerol and stored at -80°C. CFUs were determined by plating on 5% sheep blood agar plates and optical density readings.

Mice

Female C57BL/6 mice 6 to 12 weeks of age from NCI-Frederick and Charles River Laboratories were used for all experiments. Mice were housed at the Animal Resource Center at Montana State University. All procedures were performed following Institutional Animal Care and Use Committee approved protocols.

Infection Model

Mice were anesthetized with isoflurane and infected intranasally (i.n.) with 600 plaque forming units (PFUs) PR8 influenza virus in 100 µl of sterile HBSS (50 µL/nare). At days 3 or 6 after influenza infection, mice were challenged i.n. with 10^7 CFUs of *S. pneumoniae* type 4 in 100 µl of sterile DPBS (Dulbecco's PBS) (50 µL/nare) for 12 or 24 hours. *S. pneumoniae*-infected only mice were mock-influenza infected i.n. with 100 µl HBSS. Influenza-infected only mice were mock-*S. pneumoniae* infected i.n. with 100 µl DPBS. Uninfected control mice were mock-influenza infected i.n. with HBSS and mock-*S. pneumoniae* infected i. n. with DPBS. For neutrophil depletion studies, half of the mice in each treatment group were depleted of neutrophils by i.p. injection of 500 µg RB6-8C5 monoclonal antibody (which recognizes the granulocyte-differentiation antigen (Gr-1) on neutrophils) 16 hours prior to *S. pneumoniae* infection [225, 230-232]. The RB6-8C5 mAb was grown from a hybridoma cell line. The other half of the mice in each treatment group were mock-neutrophil depleted with 500 µg isotype-matched rat IgG (Sigma) i.p.

Tissue Preparation

At the specified timepoints, mice were euthanized by deep pentobarbital anesthesia followed by exsanguination. Bronchial alveolar lavage fluids (BALF) were collected for differential cell counts and to confirm neutrophil depletions as previously described [225, 232]. Lungs were collected, placed in the BALF, snap frozen in liquid nitrogen, and stored at -80°C. To enumerate *S. pneumoniae* CFUs, lungs were homogenized, serially diluted in 10-fold dilutions, and plated on sheep blood agar plates with neomycin using the drop plate method [233]. Plates were incubated for 24 hours at 37°C, 5% CO_2 before counting CFUs. PFUs were enumerated using the plaque assay method with Madin-Darby Canine Kidney cells [234].

Neutrophil ROS Generation and
S. pneumoniae Association/Phagocytosis

Mice were infected with PR8 influenza virus as described above for 3 or 6 days. Control mice (mock-influenza infected) received 100 µL HBSS i.n. 6 days before they were sacrificed. LPS-stimulated mice were placed in an aerosolization chamber (made at The Trudeau Institute, Saranac Lake, NY) and LPS (10 mg/mL in 5 mL HBSS) was nebulized for 15 minutes using a Hudson RCI® "T" UP-DRAFT® Hand-Held Nebulizer. In a separate experiment, mice were infected similarly except influenza-infected mice were also LPS-stimulated as described above to control for any effects LPS may have on neutrophil function. All mice were then sacrificed 8 hrs after the LPS challenge. ROS generation by neutrophils and *S. pneumoniae* association with neutrophils were then

measured using two previously described methods adapted for this study as described below [235, 236].

S. pneumoniae were washed twice in DPBS, resuspended to 1×10^9 CFUs/mL in 5 mL DPBS, and labeled with DiI (Molecular Probes V22889). Unlabeled *S. pneumoniae* were also prepared. For opsonization, bacteria were washed twice in DPBS and resuspended at 1×10^9 CFUs/mL in RPMI-H (RPMI with 10 mM HEPES) containing 25% fresh mouse serum, prepared as described below. Bacteria were then incubated at 37°C for 30 minutes with mild agitation. *S. pneumoniae* were washed twice in DPBS and resuspended at 10^8 CFUs/mL in RPMI-H for bone marrow cells or 10^7 CFUs/mL in RPMI-H for BALF cells. Serial dilutions were plated to confirm actual CFUs, and the efficiency of labeling was checked using flow cytometry and microscopy with unlabeled bacteria and DiI labeled bacteria.

Fresh mouse serum was collected, and it was used for *S. pneumoniae* opsonization and to coat sterile 96-well flat bottom plates. Plates for the phagocytosis and ROS generation assays were coated with 20% normal mouse serum diluted in DPBS for one hour at room temperature and washed twice with DPBS before adding cells.

Bone marrow cells were flushed from the femurs and tibias with 5 mL of HBSS with 3 mM EDTA and BALF cells were collected by lavaging the lungs with 5 mL of HBSS with 3 mM EDTA. All samples were kept at room temperature until noted otherwise to prevent neutrophil activation by temperature changes. Cell counts and differential cell counts were done. Lungs were removed after lavaging, placed in 5 mL HBSS, snap frozen in liquid nitrogen, and stored at -80ºC for influenza plaque assays.

RBCs were removed from bone marrow and BALF cells by hypotonic lysis, washed, and resuspended to 10^7 cells/mL (bone marrow) or 10^6 cells/mL (BALF) in RPMI-H. In order to obtain sufficient cell numbers, samples were combined in sets of 2 at this point with 3 sets of two per treatment for a final *n* of 3 per group. An aliquot of each sample was removed and stained with FITC-labeled RB6-8C5 antibody to identify neutrophils. The remainder of bone marrow samples not used in these assays were snap frozen and stored at -80ºC for plaque assays.

To detect ROS, the ROS indicator 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, C-2938) was added to the samples to a final concentration of 0.2 μM from a 10 mM stock made up in DMSO, as previously described [236]. For negative ROS controls, no ROS indicator was added. Samples were incubated in the dark at room temperature for 20 minutes before being transferred to ice.

To measure *S. pneumoniae* association/phagocytosis, cells were added (10^6 cells/100 μL for bone marrow and 10^5 cells/100 μL for BALF) to the wells of sterile 96-well plates previously coated with serum and kept on ice, as previously described [236]. DiI-labeled *S. pneumoniae* were added to the wells (10^7 CFUs/100 μL for bone marrow and 10^6 CFUs/100 μL for BALF). For positive ROS generation controls, PMA was added to wells (100 ng/mL). The plates were centrifuged at 400 x *g* for 7 minutes at 4°C. Samples that represented the start of the assay (time = 0 minutes) were placed on ice in the dark. The remaining samples were incubated at 37°C with 5% CO_2 for 60 minutes. At the end of the designated time, samples were transferred to flow cytometry tubes and kept on ice. ROS generation and *S. pneumoniae* association were then measured using

flow cytometry. To quench extracellular *S. pneumoniae* and measure phagocytosis, 100 μL trypan blue was added and the sample was reanalyzed as previously described [236]. Neutrophils were specifically analyzed by gating for RB6-8C5 positively-stained cells using the aliquots of cells described above, which did not have ROS indicator or *S. pneumoniae* added.

<u>Lung Cytokine Levels</u>

Homogenized, unperfused lungs were centrifuged at 300 × *g* for 15 minutes and the supernatants were removed and analyzed using the BD Cytokine Bead Array Mouse Inflammation Kit following the manufacturer's instructions. Samples were analyzed by flow cytometry on a BD FACScan and data was analyzed using the BD Cytokine Bead Array software.

<u>Statistical Analysis</u>

Data were analyzed using GraphPad Prism 4 software. *S. pneumoniae* CFUs and influenza PFUs data were analyzed using the Mann-Whitney test. Cytokines were analyzed using one-way analysis of variance followed by the Bonferroni post test. All other data were analyzed using an unpaired, two-tailed t test. The minimal value of significance was set at $p < 0.05$.

Results

Peak Susceptibility to Secondary *S. pneumoniae* Infection is at 6 Days after Influenza Infection

Previous studies have found that susceptibility to *S. pneumoniae* infection is greatest at 6-7 days after influenza infection [175, 177, 193, 237]. To validate our co-infection model, *S. pneumoniae* was inoculated into uninfected mice or mice infected with influenza for 3 or 6 days, and *S. pneumoniae* growth over 12 and 24 hours was measured to determine when susceptibility to *S. pneumoniae* infection was greatest (Figure 2.1). We found that susceptibility to secondary *S. pneumoniae* infection was greatest at 6 days after influenza infection. Six day co-infected mice had 485.5- and 149.4-fold higher lung *S. pneumoniae* CFUs at 12 and 24 hours, respectively, after *S. pneumoniae* infection compared to mice infected only with *S. pneumoniae* ($p < 0.0001$; Figure 2.2*A* and *B*). At 3 days after influenza infection, when influenza viral replication is at its peak, there was no significant difference in susceptibility to *S. pneumoniae* infection between co-infected mice compared to *S. pneumoniae*-infected only mice at either 12 or 24 hours after *S. pneumoniae* infection ($p = 0.3109$ and 0.1832, respectively; Figure 2.2*A* and *B*).

We quantitated influenza PFUs in the lungs of both non-influenza infected mice and mice infected with influenza for either 3 or 6 days to measure the growth of infectious virus particles. In the lungs of mice infected with influenza for 3 days, there were 2.3-fold more PFUs compared to mice infected with influenza for 6 days ($p = 0.029$; Figure 2.2*C*). In non-influenza infected mice, no influenza PFUs were found (data not

shown). Together, these data indicate that susceptibility to *S. pneumoniae* infection is greatest at 6 days after influenza infection. At 3 days after influenza infection, when viral replication was at its peak, susceptibility to *S. pneumoniae* infection was not increased compared to mice infected with *S. pneumoniae* only. These results agree with previous studies [176, 177] and serve to validate our co-infection model.

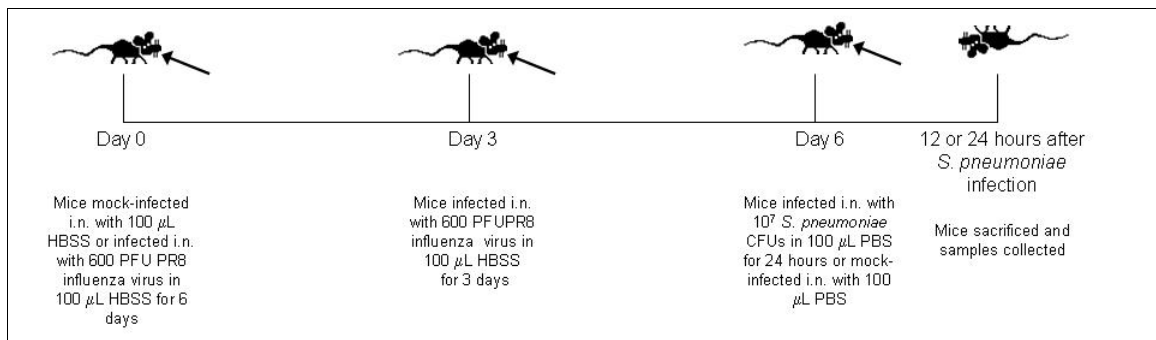

Figure 2.1. Timeline of influenza and/or *S. pneumoniae* infections for *in vivo* co-infection studies. Arrows indicate route of infection (intranasal).

Effects of RB6-8C5 Depletion on Blood
and BALF Neutrophil Numbers

In order to determine the roles of neutrophil-dependent and –independent mechanisms of increased susceptibility to *S. pneumoniae* during an influenza infection, we depleted uninfected and influenza-infected mice of neutrophils by injecting the RB6-8C5 (RB6) antibody intraperitoneally 16 hours before *S. pneumoniae* infection. To validate our neutrophil depletion method, we quantified the number of neutrophils present in peripheral blood and bronchial alveolar lavage fluids (BALF) of depleted and undepleted mice. Control mice which were RB6-depleted (uninfected and influenza-infected only) had significant reductions in blood neutrophils, indicating that our

depletion model was adequate for our studies. Uninfected depleted mice had a 94.4% reduction in blood neutrophils compared to undepleted mice ($p < 0.001$; Table 2.1) while depleted mice infected with influenza for 3 or 6 days had reductions of 72.7% and 91.4% in peripheral blood neutrophils, respectively, compared to undepleted mice ($p < 0.01$ for both; Table 2.1). In the BALF of uninfected depleted mice, no reductions were seen in neutrophil numbers since few to no neutrophils were present in the lungs of uninfected mice whether or not they were depleted of neutrophils (Table 2.1). Depleted mice infected with influenza for 3 days had an 85.0% reduction in BALF neutrophils ($p < 0.01$) while those infected with influenza for 6 days had a 25.9% reduction in BALF neutrophils ($p > 0.05$) compared to undepleted mice with similar infections (Table 2.1).

Neutrophil depletion resulted in a significant decrease in the number of peripheral blood neutrophils compared to undepleted mice for all groups after 12 hours of *S. pneumoniae* infection, with reductions of 89.3% for *S. pneumoniae*-infected only, 81.4% for 3 day co-infected, and 61.9% for 6 day co-infected mice ($p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively; Table 2.1). At 24 hours after *S. pneumoniae* infection, mice infected only with *S. pneumoniae* had an 88.2% reduction and 3 day co-infected mice had a 58.5% reduction in blood neutrophils ($p < 0.01$; Figure 2.3*A*). However, in mice infected with influenza for 6 days followed by *S. pneumoniae* for 24 hours, there was not a significant difference in blood neutrophils between depleted and undepleted mice, although there was a 31.3% reduction ($p = 0.2954$; Figure 2.3*A*). The overall reduction in blood neutrophils at 24 hours after *S. pneumoniae* infection could be due to the overwhelming

infections in the lungs recruiting all available neutrophils from the blood, resulting in temporary neutropenia.

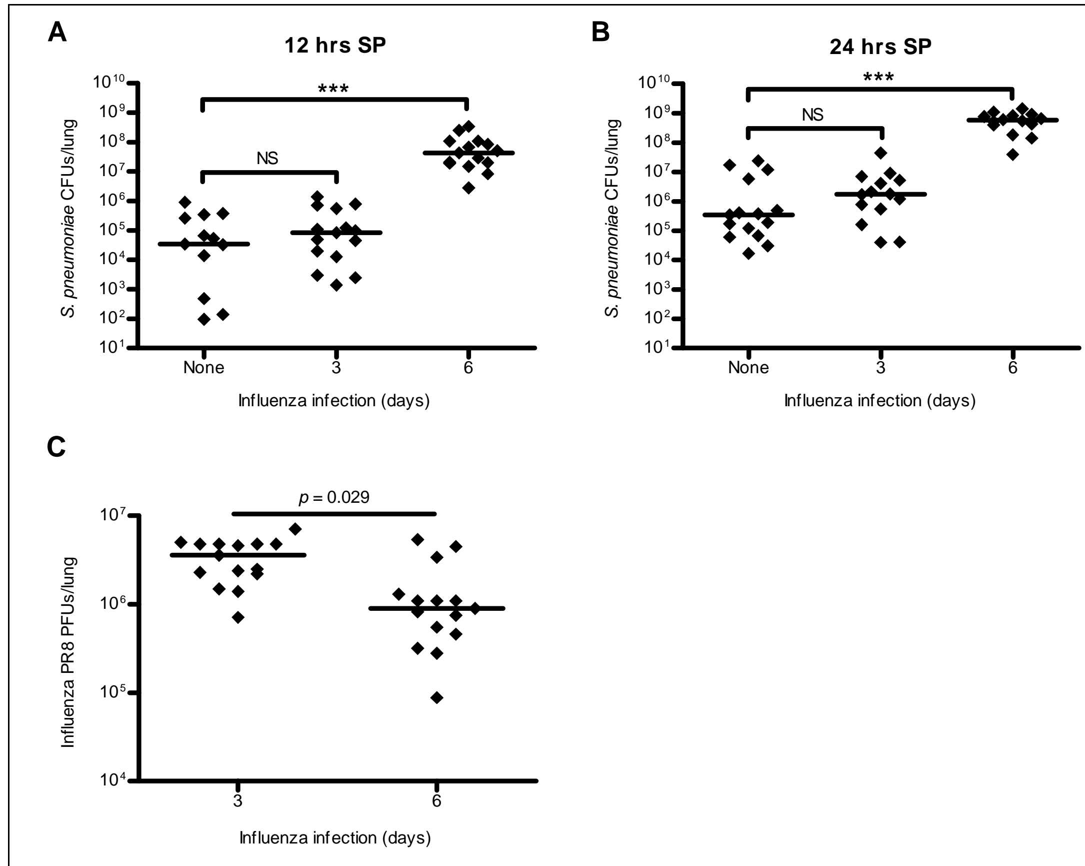

Figure 2.2. *S. pneumoniae* CFUs (*A* and *B*) and influenza PFUs (*C*) in lungs after 12 (*A*) or 24 (*B* and *C*) hours of *S. pneumoniae* infection for mice infected with *S. pneumoniae* only or infected with *S. pneumoniae* after 3 or 6 days of influenza infection. Data pooled from 3 independent experiments with 4-5 mice per group per experiment. Significant differences between mice infected with *S. pneumoniae* only and mice co-infected with influenza and *S. pneumoniae* are indicated by ***, $p < 0.001$; NS, $p > 0.05$. Line represents median value for each infection. Data analyzed using Mann-Whitney U test.

TABLE 2.1. Peripheral blood and BALF neutrophil numbers 16 hours after RB6-8C5 neutrophil depletion.[a]

Influenza and/or *S. pneuemoniae* infection(s)	Peripheral blood neutrophils (% of total leukocytes)		BALF total neutrophils	
	Undepleted	RB6 depleted	Undepleted	RB6-depleted
No influenza, no *S. pneumoniae*	14.4 ± 5.7	0.8 ± 1.3 ***	$1.6 \times 10^3 \pm 2.4 \times 10^3$	$1.6 \times 10^3 \pm 2.9 \times 10^3$ NS
Influenza 3 days, no *S. pneumoniae*	32.6 ± 10.8	2.8 ± 4.3 **	$3.8 \times 10^5 \pm 1.6 \times 10^5$	$5.7 \times 10^4 \pm 2.7 \times 10^4$ **
Influenza 6 days, no *S. pneumoniae*	25.6 ± 8.8	7.0 ± 4.9 **	$2.7 \times 10^5 \pm 1.9 \times 10^5$	$2.0 \times 10^5 \pm 1.0 \times 10^5$ NS
No influenza, *S. pneumoniae* 12 hrs	56.3 ± 11.7	6.0 ± 2.2 ***	$9.5 \times 10^5 \pm 6.0 \times 10^5$	$1.6 \times 10^5 \pm 1.7 \times 10^5$ *
Influenza 3 days, *S. pneumoniae* 12 hrs	50.6 ± 10.0	9.4 ± 2.1 **	$1.0 \times 10^6 \pm 5.2 \times 10^5$	$1.3 \times 10^5 \pm 5.0 \times 10^4$ **
Influenza 6 days, *S. pneumoniae* 12 hrs	53.2 ± 9.6	20.3 ± 4.8 ***	$1.9 \times 10^6 \pm 1.1 \times 10^6$	$3.5 \times 10^5 \pm 1.7 \times 10^5$ *

[a] Values represent mean ± standard deviation (4-5 mice/group, similar results seen with separate experiment). Mice were either uninfected, infected with influenza only, infected with *S. pneumoniae* only for 12 hours, or infected with influenza virus for 3 or 6 days followed by *S. pneumoniae* for 12 hours. Depleted mice were neutrophil-depleted with the RB6-8C5 mAb 16 hours before *S. pneumoniae* infection. Data analyzed using unpaired Student's *t* test. Significant reductions in neutrophils compared to undepleted mice are indicated by *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, $p > 0.05$.

In the BALF of *S. pneumoniae*-infected only mice, neutrophil depletion resulted in lung neutrophil reductions of 83.3% and 69.4% at 12 and 24 hours after *S. pneumoniae* infection ($p < 0.05$, respectively; Table 2.1 and Figure 2.3*B*). Neutrophil-depleted mice that were infected with influenza for 3 days had lung neutrophil reductions of 87.7% and 90.3% at 12 and 24 hours after *S. pneumoniae* infection ($p < 0.01$ and 0.001, respectively; Table 2.1 and Figure 2.3*B*). For 6 day co-infected mice, lung neutrophils were reduced in the depleted mice by 81.3% and 89.2% at 12 and 24 hours after *S. pneumoniae* infection ($p < 0.05$ and 0.01, respectively; Table 2.1 Figure 2.3*B*). In addition, there were no significant differences in neutrophil numbers in undepleted, *S. pneumoniae*-infected mice

whether or not they had a previous influenza infection, indicating that a prior influenza infection of 3 or 6 days does not affect neutrophil migration into the lungs ($p > 0.05$; Figure 2.3*B*). Together, these data indicate that our RB6-8C5 neutrophil depletion model can be used to study neutrophil-dependent and –independent mechanisms of increased susceptibility to *S. pneumoniae* infection after an influenza infection.

Neutrophil-Dependent Mechanisms of Increased Susceptibility to *S. pneumoniae* Infection

In order to determine whether neutrophil depletion affects susceptibility to *S. pneumoniae* infection, we compared *S. pneumoniae* CFUs in the lungs of depleted mice to undepleted mice with the same infection(s). Neutrophil-depleted mice infected with *S. pneumoniae* only had 10.5- and 384.5-fold higher lung *S. pneumoniae* CFUs at 12 and 24 hours, respectively, after *S. pneumoniae* infection compared to undepleted mice (p = 0.0159 and 0.0079, respectively; Figure 2.3*C* and *D*). In 3 day co-infected mice, depleted mice had 26.8- and 108.9-fold more lung *S. pneumoniae* CFUs at 12 and 24 hours, respectively, after *S. pneumoniae* infection compared to undepleted mice ($p = 0.0079$ for both; Figure 2.3*C* and *D*). However, after 6 days of influenza infection, there was no significant difference in susceptibility to *S. pneumoniae* in the lungs of mice infected with *S. pneumoniae* for 12 or 24 hours whether or not they were neutrophil depleted (p = 0.7302 and 0.0635, respectively; Figure 2.3*C* and *D*).

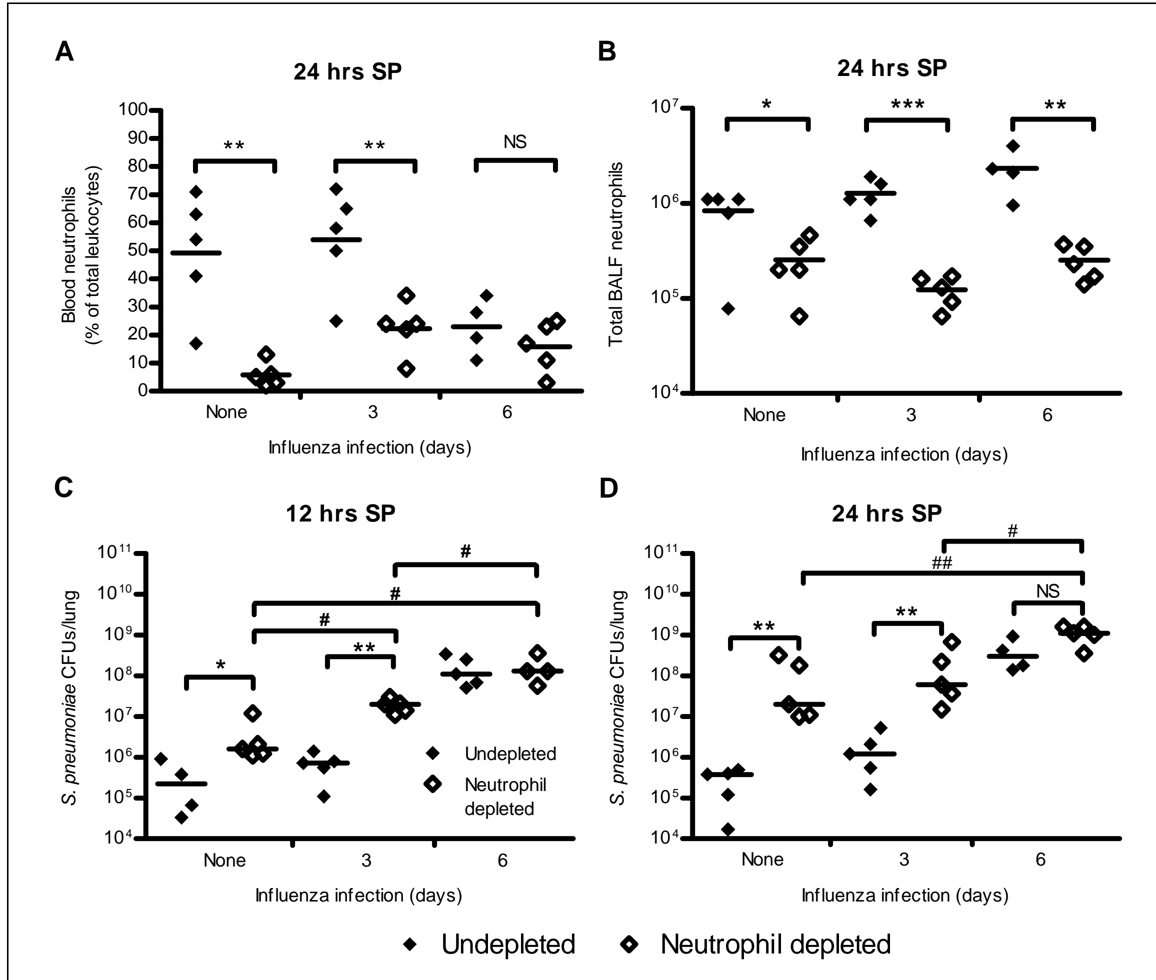

Figure 2.3. Percentage of neutrophils in peripheral blood relative to total peripheral leukocytes (*A*), total number of neutrophils in BALF (*B*), and lung *S. pneumoniae* CFUs (*C* and *D*) of undepleted and RB6-depleted mice at 12 (*C*) and 24 (*A, B,* and *D*) hours after *S. pneumoniae* infection. Mice were infected with *S. pneumoniae* for 12 or 24 hours after 3 or 6 days of influenza infection, or were only infected with *S. pneumoniae* for 12 or 24 hours. Data are representative of two independent experiments with 4-5 mice per group per experiment. Significant differences between neutrophil-depleted mice and undepleted mice are indicated by *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, $p > 0.05$. Significant differences between neutrophil-depleted mice infected with *S. pneumoniae* only and neutrophil-depleted mice infected with influenza for 3 or 6 days followed by *S. pneumoniae* are indicated by #, $p < 0.05$; ##, $p < 0.01$. Lines represent mean (A and B) or median values (C) for each infection. Data analyzed using unpaired *t* test (A and B) or Mann-Whitney U test (C).

In summary, our depletion studies indicate that neutrophils play an important role in resistance to a *S. pneumoniae* infection in uninfected mice or mice with an influenza infection for 3 days. When neutrophils were depleted in these groups, susceptibility was increased. However, in mice infected with influenza for 6 days, neutrophil depletion did not affect resistance to *S. pneumoniae*, suggesting that neutrophil function may be compromised by the influenza infection to the point similar to that caused by neutrophil depletion. These results suggest the possibility that influenza-induced effects have compromised the neutrophils so much by day 6 after an influenza infection (but not at day 3) that they are no longer a significant factor in protection.

Influenza-Induced Changes in Neutrophil-Independent Mechanisms Increase Susceptibility to *S. pneumoniae* Infection

To determine whether a prior influenza infection affected susceptibility to *S. pneumoniae* independently of neutrophil function, we compared *S. pneumoniae* lung CFUs of neutrophil-depleted mice infected with *S. pneumoniae* only to depleted mice infected with influenza for 3 or 6 days. After 12 hours of *S. pneumoniae* infection, both 3 and 6 day co-infected depleted mice had 5.3- and 46.9-fold more lung *S. pneumoniae* CFUs, respectively, than *S. pneumoniae*-infected only mice depleted of neutrophils (p = 0.0159 for both; Figure 2.3*C*). In addition, 6 day co-infected depleted mice had 8.8-fold more *S. pneumoniae* CFUs than 3 day co-infected depleted mice (p = 0.0159; Figure 2.3*C*). After 24 hours of *S. pneumoniae* infection, 3 day co-infected depleted mice had only 1.9-fold more *S. pneumoniae* CFUs (p = 0.4206) while 6 day co-infected depleted mice had 10.5-fold more *S. pneumoniae* CFUs compared to *S. pneumoniae*-infected only

depleted mice (*p* = 0.0079; Figure 2.3*D*). In addition, depleted mice infected with influenza for 6 days had 5.6-fold higher *S. pneumoniae* CFUs compared to 3 day co-infected depleted mice after 24 hours of *S. pneumoniae* infection (*p* = 0.0159; Figure 2.3*D*). Thus, in mice depleted of neutrophils, an influenza infection still caused increased susceptibility to a secondary *S. pneumoniae* infection. Together, these data suggest that a prior influenza infection of 6 days increases susceptibility to a *S. pneumoniae* infection by neutrophil-independent mechanisms.

Neutrophil Function Assays

To determine whether influenza virus causes defects in neutrophil function *in vivo*, thereby increasing susceptibility to a secondary *S. pneumoniae* infection, we measured the amount of *S. pneumoniae* associated (either attached or phagocytosed) with either BALF or bone marrow neutrophils and reactive oxygen species (ROS) production by neutrophils from the lungs and bone marrow of uninfected, LPS-stimulated, and 3 or 6 day influenza-infected mice. In a separate experiment, we stimulated influenza-infected mice with LPS to determine if any changes in neutrophil function were due to the effects of LPS or influenza.

Lung and bone marrow neutrophils were analyzed at 0 and 60 minutes after the addition of *S. pneumoniae* to compare the resting and activated levels of both *S. pneumoniae* association with neutrophils and ROS generation by neutrophils. Since normal, healthy lungs contain few, if any, neutrophils, it was necessary to induce neutrophil migration in order to compare lung neutrophils from uninfected mice to those from influenza-infected mice. LPS was aerosolized for 20 minutes to induce a strong

neutrophil influx into the lungs of uninfected mice, and BALF was collected 8 hours later when neutrophil accumulation was at its peak (Figure 2.4). For the bone marrow neutrophils, uninfected mice were also used in the analysis.

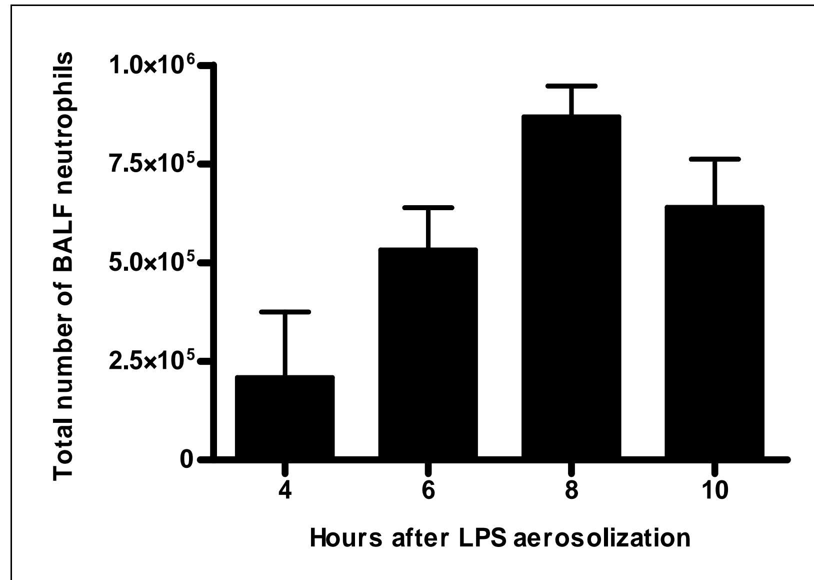

Figure 2.4. Number of total BALF neutrophils at 4, 6, 8, or 10 hours after LPS aerosolization. Data represent 3-5 mice/group.

In the lungs, there were no significant differences in the total numbers of neutrophils between LPS-stimulated, 3 day influenza-infected, and 6 day influenza-infected mice (Figure 2.5*A*). Mice infected with influenza for 3 or 6 days and stimulated with LPS had 2.4- and 2.5-fold more lung neutrophils, respectively, compared to mice stimulated with LPS only (p <0.0001 for both; Figure 2.5*B*), indicating the neutrophil reservoir was not depleted during the influenza infection. In the bone marrow, there were no significant differences in neutrophil numbers between any of the groups except there was a 1.6-fold increase in neutrophils in the 3 day influenza-infected mice compared to the LPS-stimulated mice (p = 0.0096; Figure 2.5*C*), although this difference was not

seen in a repeat of the experiment (Figure 2.5*D*). For mice infected with influenza for 3 or 6 days followed by LPS stimulation, there were no significant differences in the numbers of bone marrow neutrophils between any of the groups (Figure 2.5*D*).

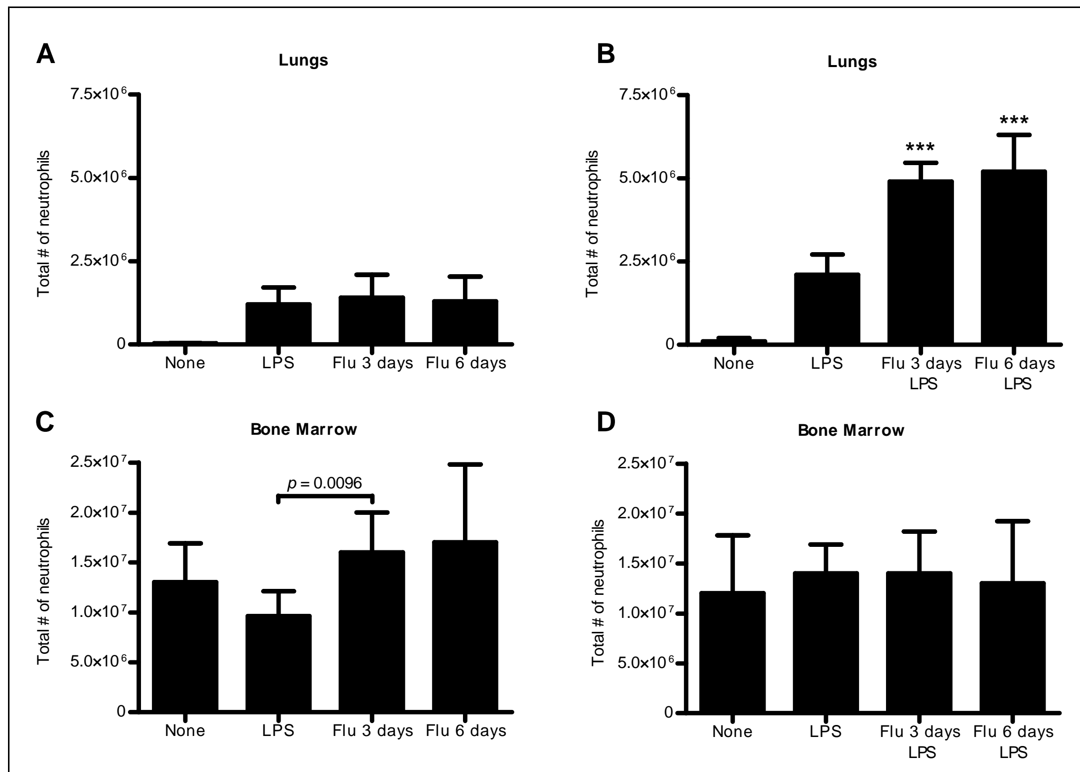

Figure 2.5. Neutrophil counts for BALF (*A* and *B*) and bone marrow (*C* and *D*) samples from uninfected mice, LPS-stimulated mice, or mice infected with influenza for 3 or 6 days with (*B* and *D*) or without (*A* and *C*) LPS stimulation. Data represent two independent experiments with 3 sets of 2 mice per group. Significant differences between LPS-stimulated mice and mice with both influenza infection and LPS stimulation are indicated by ***, $p < 0.001$. Data expressed as mean ± SD. Data analyzed using unpaired *t* test.

Influenza infection reduces
<u>*S. pneumoniae* association with neutrophils</u>

Association of *S. pneumoniae* with neutrophils was measured by incubating neutrophils with DiI-labeled *S. pneumoniae* for 0 or 60 minutes. The mean

fluorescence in the FL-2 red channel was then measured to determine the relative number of *S. pneumoniae* associated with neutrophils, either bound to the surface or phagocytosed. In the lungs, there were 68.2% and 71.2% reductions in the initial (0 minutes) *S. pneumoniae* association with neutrophils from mice infected with influenza for 3 or 6 days compared to those from LPS-stimulated mice (p = 0.0057 and 0.0049, respectively; Figure 2.6*A*). After 60 minutes incubation with *S. pneumoniae*, for lung neutrophils from 3 and 6 day influenza-infected mice, the amount of associated *S. pneumoniae* was 70.6% and 79.8%, respectively, of that seen with neutrophils from LPS-stimulated mice ($p < 0.0001$ and $p = 0.0001$, respectively; Figure 2.6*A*). After 60 minutes incubation with *S. pneumoniae*, the level of *S. pneumoniae* association with influenza-infected neutrophils only reached levels comparable to those seen initially with neutrophils from LPS-stimulated mice at 0 minutes incubation (Figure 2.6*A*).

Studies using trypan blue to quench the fluorescence of extracellular *S. pneumoniae* indicated that most of the *S. pneumoniae* association with lung neutrophils after 60 minutes incubation with *S. pneumoniae* was the result of phagocytosis of *S. pneumoniae* rather than adherence to the neutrophils (Table 2.2).

In the lungs of mice infected with influenza for 3 or 6 days followed by LPS stimulation, there was a significant decrease in initial *S. pneumoniae* association with neutrophils compared to mice stimulated with LPS only (p = 0.0464 and 0.0175, respectively; Figure 2.6*B*). A similar decrease was seen after 60 minutes incubation (p = 0.0031 and 0.0035, respectively; Figure 2.6*B*). These data indicate that the differences seen in *S. pneumoniae* association are due to the effects of influenza infection and not

LPS stimulation. Thus, a prior influenza infection of 3 or 6 days decreases the ability of lung neutrophils to associate with *S. pneumoniae*.

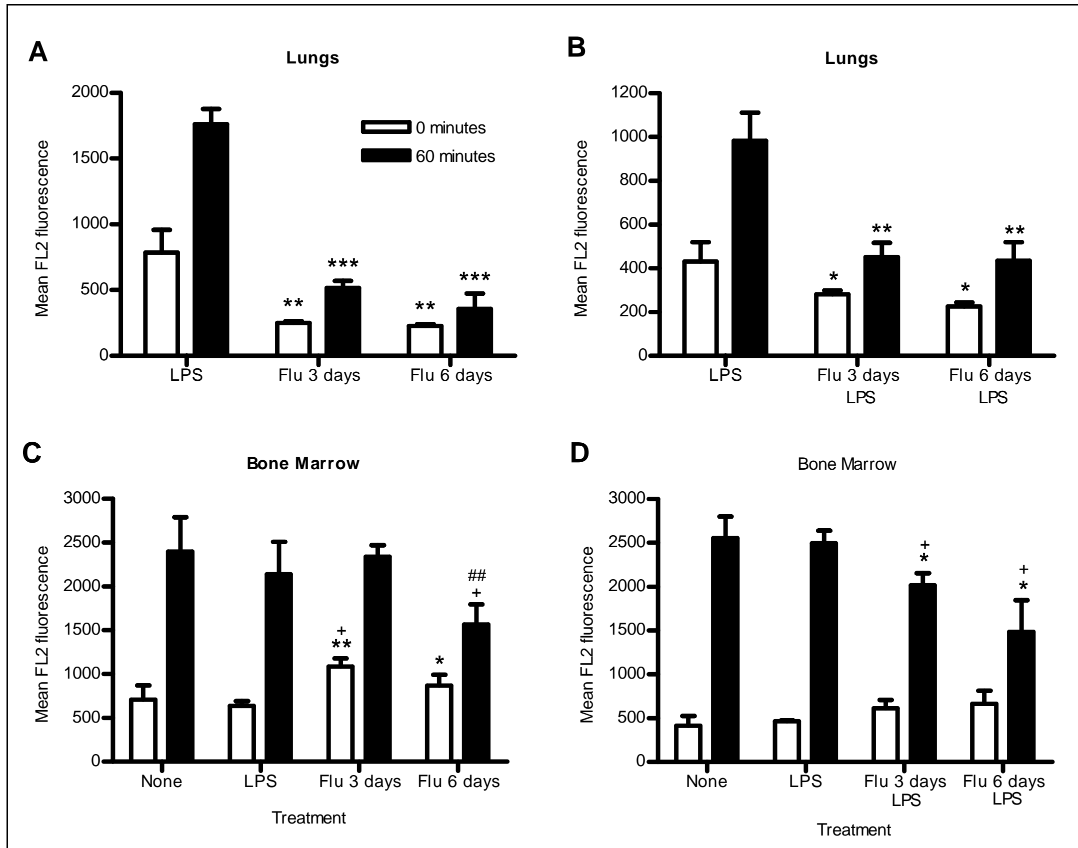

Figure 2.6. *S. pneumoniae* association after 0 or 60 minutes incubation with lung (*A* and *B*) or bone marrow (*C* and *D*) neutrophils from uninfected mice, LPS-stimulated mice, or mice infected with influenza for 3 or 6 days with (*B* and *D*) or without (*A* and *C*) subsequent LPS stimulation. Data represent one (*B* and *D*) or two (*A* and *C*) independent experiments with 3 sets of 2 mice per group. Significance is indicated by *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to LPS-stimulated mice; +, $p < 0.05$ compared to uninfected (none) mice; ##, $p < 0.01$ compared to 3 day influenza-infected mice. Data expressed as mean ± SD. Data analyzed using unpaired *t* test.

Table 2.2. Trypan blue staining of BALF neutrophils to determine % of phagocytic neutrophils and % of phagocytic neutrophils that are producing ROS. [a]

Influenza infection or LPS treatment	% of neutrophils that were phagocytic		% of phagocytic neutrophils that were producing ROS	
	0 minutes	60 minutes	0 minutes	60 minutes
LPS 8 hours	43.3 ± 4.2	67.7 ± 17.8	56.5 ± 1.5	80.6 ± 1.5
Influenza 3 days	95.1 ± 19.9*	82.2 ± 5.7	31.3 ± 3.8*	62.8 ± 5.2
Influenza 6 days	78.1 ± 24.7	91.1 ± 8.4	10.4 ± 9.0***	35.9 ± 16.8***,†

[a] Values represent means ± standard deviation of one experiment with 3 sets of 2 mice/group. Neutrophils were collected from mice and incubated with DiI-labeled *S. pneumoniae* for 0 or 60 minutes to measure phagocytosis and ROS generation. Statistical differences between groups were calculated using the one-way ANOVA followed by the Bonferroni post test. Differences between groups with the same incubation time are indicated by *, $p < 0.05$; ***, $p < 0.001$ compared to neutrophils from LPS-challenged mice and †, $p < 0.05$ compared to neutrophils from mice infected with influenza for 3 days.

In the bone marrow, there were significantly more *S. pneumoniae* initially associated with bone marrow neutrophils from mice infected with influenza for 3 days compared to bone marrow neutrophils from uninfected or LPS-stimulated mice, with increases of 53.7% and 69.9%, respectively ($p = 0.0261$ and 0.0020, respectively; Figure 2.6*C*). However, by 60 minutes, there were no significant differences in *S. pneumoniae* association with bone marrow neutrophils after 3 days of influenza infection compared to uninfected or LPS-stimulated mice ($p = 0.8293$ and 0.4234, respectively; Figure 2.6*C*). Bone marrow neutrophils from mice infected with influenza for 6 days showed 35.2% greater initial *S. pneumoniae* association than LPS-stimulated bone marrow neutrophils ($p = 0.0419$; Figure 2.6*C*). By 60 minutes incubation with *S. pneumoniae*, there were significantly fewer *S. pneumoniae* associated with bone marrow neutrophils from 6 day

influenza-infected mice than those from uninfected mice or 3 day influenza-infected mice, with reductions of 34.7% and 33.1% ($p = 0.0343$ and 0.0071, respectively; Figure 2.6*C*). There was no significant difference in *S. pneumoniae* association at 60 minutes between 6 day influenza-infected mice or LPS-stimulated mice, although there was a 26.8% reduction in *S. pneumoniae* association ($p = 0.0855$; Figure 2.6*C*). Similar results were seen when influenza-infected mice were stimulated with LPS (Figure 2.6*D*).

Trypan blue quenching showed that initially, 43.2% ± 6.9% of bone marrow neutrophils associated with *S. pneumoniae* from all groups studied had phagocytosed *S. pneumoniae* (Table 2.3). By 60 minutes, the level of phagocytosis had increased to 79.9% ± 10.5% (Table 2.3). After 60 minutes of incubation with *S. pneumoniae*, bone marrow neutrophils from 6 day influenza-infected mice had a lower percentage of phagocytic neutrophils than those from LPS-stimulated mice ($p < 0.01$; Table 2.3).

Thus, an influenza infection of 6 days duration compromised bone marrow neutrophil function but not to the extent of suppression seen in lung neutrophil function. These data show that an influenza infection of 6 days can have slight systemic effects on the ability of bone marrow neutrophils to associate with *S. pneumoniae*.

Influenza infection reduces ROS production by neutrophils

ROS production by neutrophils after 0 or 60 minutes incubation with *S. pneumoniae* was measured using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, which fluoresces in the FL-1 green channel. As a positive control, cells were incubated with PMA, which activates neutrophils to produce high levels of ROS. The initial

amount of ROS produced after *S. pneumoniae* stimulation was significantly reduced by 69.2% and 85.2% in lung neutrophils from 3 and 6 day influenza infected mice compared to those from LPS-stimulated mice ($p < 0.0001$; Figure 2.7*A*). Similar results were seen with lung neutrophils incubated with PMA, with reductions of 63.0% and 80.2% for mice infected with influenza for 3 or 6 days ($p < 0.0001$; Figure 2.7*B*). After 60 minutes of incubation with *S. pneumoniae*, lung neutrophils from both 3 and 6 day influenza-infected mice produced significantly less ROS than those from LPS-stimulated mice, with reductions of 68.1% and 82.9% ($p = 0.0002$ and < 0.0001, respectively; Figure 2.7*A*). With PMA-stimulation, similar results were seen with reductions of 73.2% and 85.4% for mice infected with influenza for 3 or 6 days ($p = 0.0002$ and 0.0001, respectively; Figure 2.7*B*). As seen with *S. pneumoniae* association, the levels of ROS produced by lung neutrophils from influenza-infected mice after 60 minutes incubation with either *S. pneumoniae* or PMA were comparable to that seen initially at 0 minutes by neutrophils from LPS-stimulated mice (Figure 2.7*A* and *B*).

Trypan blue quenching of extracellular fluorescence showed that a lower percentage of phagocytic lung neutrophils from 3 or 6 day influenza-infected mice were initially producing ROS compared to phagocytic neutrophils from LPS-stimulated mice ($p < 0.05$ and 0.001, respectively; Table 2.2). In addition, after 60 minutes of incubation with *S. pneumoniae*, a lower percentage of phagocytic lung neutrophils from 6 day influenza-infected mice were producing ROS compared to phagocytic lung neutrophils from 3 day influenza-infected mice and LPS-stimulated mice ($p < 0.05$ and 0.001, respectively; Table 2.2). Thus, in the lungs of 6 day influenza-infected mice, fewer

neutrophils were associated with *S. pneumoniae*, and although most of these neutrophils were phagocytic, they were not able to produce ROS in response to *S. pneumoniae* as readily as those neutrophils from LPS-stimulated mice or mice infected with influenza for 3 days.

Table 2.3. Trypan blue staining of bone marrow neutrophils to determine % of phagocytic neutrophils and % of phagocytic neutrophils that are producing ROS.[a]

Influenza infection or LPS treatment	% of neutrophils that were phagocytic		% of phagocytic neutrophils that were producing ROS	
	0 minutes	60 minutes	0 minutes	60 minutes
None	38.5 ± 4.3	76.3 ± 5.5	41.1 ± 16.0*	93.7 ± 3.4
LPS 8 hours	36.7 ± 2.6	92.9 ± 5.5	64.4 ± 4.4	95.9 ± 1.8
Influenza 3 days	50.7 ± 4.3	78.5 ± 6.9	55.5 ± 13.9	94.7 ± 1.3
Influenza 6 days	46.9 ± 3.9	71.8 ± 8.4**	23.6 ± 6.4*,†	88.3 ± 2.4

[a] Values represent means ± standard deviations from one experiment with 3 sets of 2 mice/group. Neutrophils were collected from mice and incubated with DiI-labeled *S. pneumoniae* for 0 or 60 minutes to measure phagocytosis and ROS generation. Statistical differences between groups were calculated using the one-way ANOVA followed by the Bonferroni post test. Differences between groups with the same incubation time are indicated by *, $p < 0.05$; **, $p < 0.01$ compared to neutrophils from LPS-challenged mice and †, $p < 0.05$ compared to neutrophils from mice infected with influenza for 3 days.

Influenza-infected mice stimulated with LPS showed similar results. Initial ROS production by neutrophils after 0 minutes incubation with *S. pneumoniae* was significantly reduced in mice infected with influenza for 3 or 6 days followed by LPS simulation compared to mice stimulated with LPS only ($p = 0.0024$ and 0.0009, respectively; Figure 2.7*C*). Similar decreases were seen initially with PMA ($p = 0.0038$ and 0.0013, respectively; Figure 2.7*C*). After 60 minutes incubation with *S. pneumoniae*,

lung neutrophils from mice infected with influenza for 3 or 6 days followed by LPS stimulation produced significantly less ROS than neutrophils from mice stimulated with LPS only ($p = 0.0016$ and 0.0004, respectively; Figure 2.7*C*). Similar results were seen after 60 minutes incubation with PMA ($p < 0.0001$ for both; Figure 2.7*D*). These data show that the changes in ROS production are due to influenza-induced effects and not the effects of LPS stimulation. In summary, a prior influenza infection of 3 or 6 days reduces the ability of lung neutrophils to produce ROS after stimulation with either *S. pneumoniae* or PMA.

In the bone marrow, initial ROS production induced by *S. pneumoniae* stimulation was reduced by 52.6% and 60.0% in 6 day influenza-infected bone marrow neutrophils compared to those from LPS-stimulated or 3 day influenza-infected mice ($p = 0.0069$ and 0.0019, respectively; Figure 2.7*E*). After 60 minutes incubation with *S. pneumoniae*, bone marrow neutrophils from 6 day influenza-infected mice still produced less ROS than LPS-stimulated or 3 day influenza-infected mice, with reductions of 43.4% and 43.9% ($p = 0.0212$ and 0.0034, respectively, Figure 2.7*E*). Bone marrow neutrophils from 6 day influenza-infected mice also showed reductions of 54.5% and 46.7% in initial ROS production after incubation with PMA compared to LPS-stimulated and 3 day influenza-infected neutrophils ($p = 0.0007$ and 0.0068, respectively; Figure 2.7*F*). However, unlike the results seen after 60 minutes incubation with *S. pneumoniae*, there were no significant differences in ROS production after 60 minutes incubation with PMA between 6 day influenza-infected bone marrow neutrophils and LPS-stimulated or 3 day influenza-infected neutrophils ($p = 0.0536$ and 0.1389, respectively; Figure 2.7*F*). There

were no significant differences in ROS production by 3 day influenza-infected bone marrow neutrophils compared to uninfected or LPS-stimulated neutrophils after 0 or 60 minutes incubation with *S. pneumoniae* or PMA (Figure 2.7*E* and *F*).

Trypan blue quenching of extracellular fluorescence associated with *S. pneumoniae* bound to the surface of bone marrow neutrophils showed that initially there was some variation between groups in the percentages of phagocytic neutrophils which were producing ROS (Table 2.3). However, after 60 minutes incubation with SP, no differences between any of the groups were seen (Table 2.3).

These results indicate that an influenza infection of 6 days has systemic effects on the ability of bone marrow neutrophils to produce ROS initially and after stimulation with *S. pneumoniae* for 60 minutes. However, with PMA stimulation, any differences in initial ROS production are overcome by 60 minutes incubation with PMA.

Effects of Influenza and/or *S. pneumoniae* Infection(s) on Lung Cytokine Production

Since our neutrophil-depletion studies indicated that influenza-induced changes in neutrophil-independent mechanisms also contributed to the increased susceptibility to *S. pneumoniae* infection, we measured cytokine levels in the lungs of uninfected mice, mice infected with *S. pneumoniae* only, mice infected with influenza for 3 or 6 days, and mice infected with influenza for 3 or 6 days followed by *S. pneumoniae* for 24 hours to determine whether changes in cytokine expression levels were responsible for this increase in susceptibility. In addition, we measured cytokine levels in the lungs of neutrophil-depleted mice in each of the infection groups to determine whether the

increases in susceptibility to *S. pneumoniae* seen in depleted mice were due to altered cytokine production.

No significant differences in IL-12p70 levels were seen between any of the groups examined ($p > 0.05$; data not shown). Mice infected with *S. pneumoniae* only for 24 hours had elevated levels of TNF-α, IFN-γ, MCP-1, IL-10, and IL-6 compared to uninfected mice, although none of these increases were statistically significant ($p > 0.05$; Figure 2.8*A-E*). Mice infected with influenza only for 3 or 6 days had elevated levels of TNF-α, IFN-γ, MCP-1, IL-10, and IL-6 compared to uninfected mice, however, only IFN-γ was significantly increased after 6 days of influenza infection compared to uninfected mice ($p < 0.05$; Figure 2.8*A-E*). Together, these data indicate that an influenza infection of 3 or 6 days or a *S. pneumoniae* infection of 24 hours do not have significant effects on lung cytokine levels, except for IFN-γ at 6 days after influenza infection.

A previous influenza infection of 6 days followed by a *S. pneumoniae* infection increased lung cytokine production. Undepleted mice co-infected with influenza for 6 days followed by *S. pneumoniae* had significantly more TNF-α ($p < 0.001$), IFN-γ ($p < 0.01$), MCP-1 ($p < 0.001$), and IL-10 ($p < 0.001$) than undepleted mice infected only with influenza for 6 days (Figure 2.8*A-D*). These co-infected mice also had significantly more TNF-α ($p < 0.001$), IFN-γ ($p < 0.001$), MCP-1 ($p < 0.001$), and IL-10 ($p < 0.001$) than undepleted mice infected with *S. pneumoniae* only and uninfected mice. Compared to undepleted mice co-infected with influenza for 3 days followed by *S. pneumoniae*, these co-infected mice had significantly more TNF-α ($p < 0.01$), IFN-γ ($p < 0.001$), MCP-1 ($p < 0.001$), and IL-10 ($p < 0.001$) (Figure 2.8*A-D*). In addition, IL-6 was slightly

increased, although not significantly ($p > 0.05$), in these co-infected mice compared to undepleted groups of mice which were uninfected, infected with *S. pneumoniae* only, co-infected with influenza for 3 days followed by *S. pneumoniae*, or infected with influenza only for 3 or 6 days (Figure 2.8*E*). Together, these data indicate that an influenza infection of 6 days followed by a *S. pneumoniae* infection increases lung cytokine production compared to uninfected mice, mice infected with influenza for 3 or 6 days, and mice co-infected with influenza for 3 days followed by an *S. pneumoniae* infection.

Neutrophil depletion also affected the amounts of lung cytokines. After neutrophil depletion, the concentration of TNF-α was increased, although not significantly, for mice co-infected with influenza for 3 days followed by *S. pneumoniae* (Figure 2.8*A*). Depleted mice infected with *S. pneumoniae* only had significantly more TNF-α than depleted uninfected mice ($p < 0.05$). There were no significant differences in TNF-α between all depleted groups which were infected with *S. pneumoniae* only or co-infected with influenza for 3 or 6 days followed by *S. pneumoniae* infection ($p > 0.05$; Figure 2.8*A*). In addition, depleted mice co-infected with influenza for 6 days followed by *S. pneumoniae* had significantly more TNF-α than depleted uninfected mice ($p < 0.05$; Figure 2.8*A*).

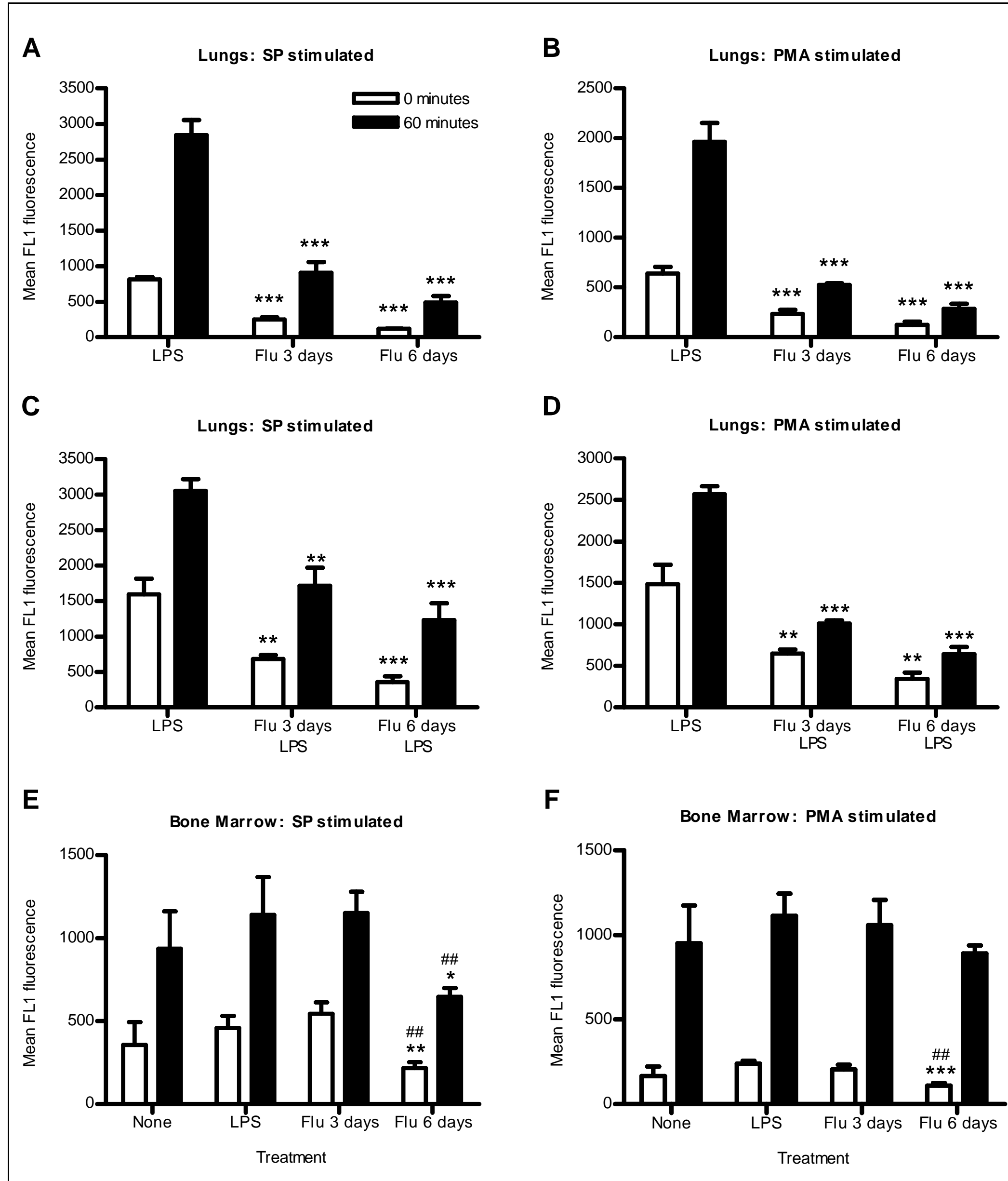

Figure 2.7. ROS generation by lung (*A*-*D*) and bone marrow (*E* and *F*) neutrophils from uninfected mice, LPS-stimulated mice, or mice infected with influenza for 3 or 6 days with (*C* and *D*) or without (*A, B, E,* and *F*) subsequent LPS stimulation. Neutrophils were incubated with *S. pneumoniae* (*A, C,* and *E*) or PMA (*B, D,* and *F*) for 0 or 60 minutes. Data represent one (*C* and *D*) or two (*A, B, E,* and *F*) independent experiments with 3 sets of 2 mice per group. Significance is indicated by *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to LPS-stimulated mice; ##, $p < 0.01$ compared to 3 day influenza-infected mice. Data expressed as mean ± SD. Data analyzed using unpaired *t* test.

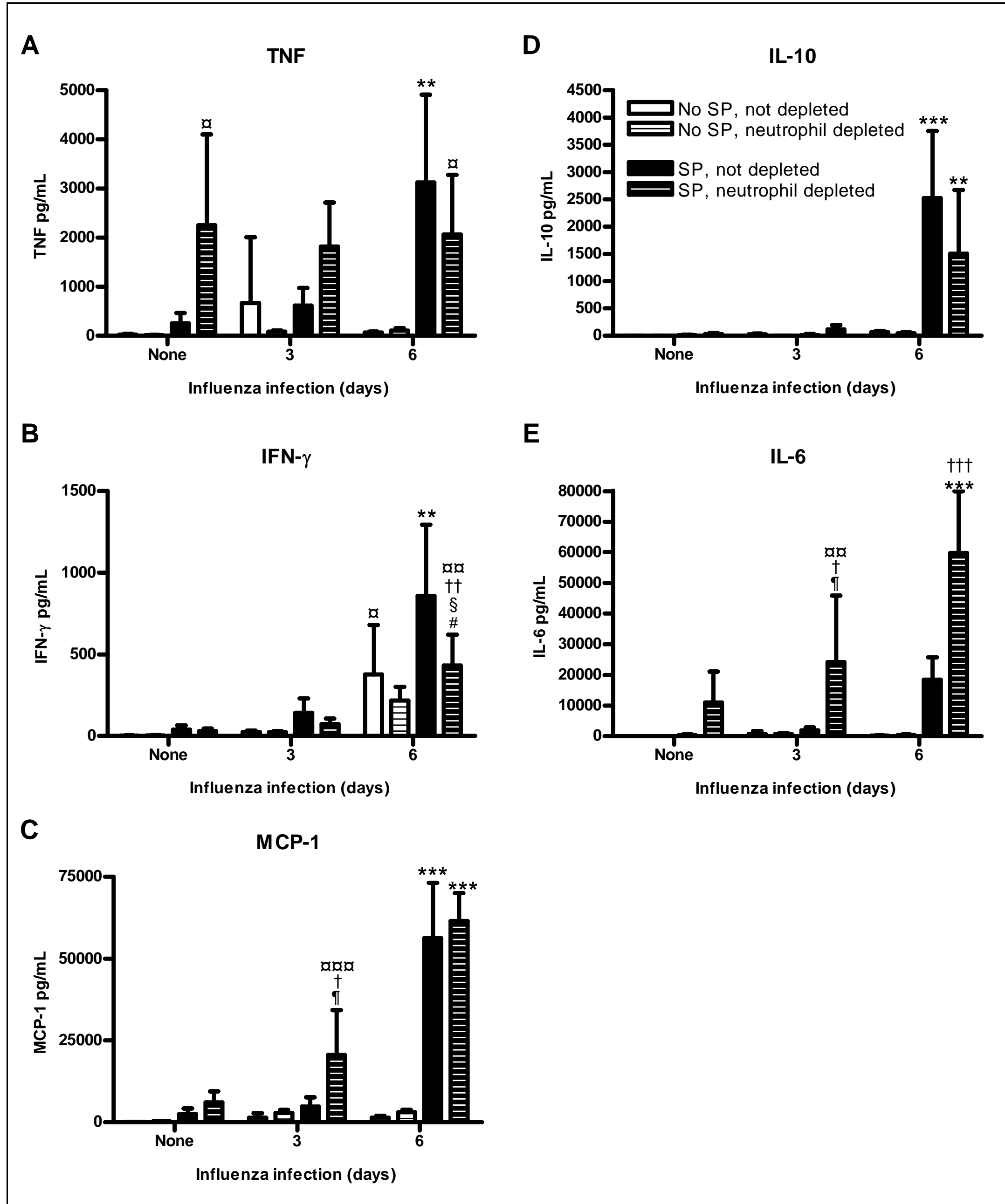

Figure 2.8. Lung cytokine levels in neutrophil-depleted and undepleted mice with or without *S. pneumoniae* for 24 hours and/or influenza virus for 3 or 6 days. TNF-α (A), IFN-γ (B), MCP-1, IL-10, and IL-6 levels were measured in undepleted and neutrophil-depleted mice which were uninfected, infected with *S. pneumoniae* only for 24 hours, infected with influenza only for 3 or 6 days, or infected with influenza for 3 or 6 days followed by *S. pneumoniae* for 24 hours. Significance is indicated by *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for mice infected with influenza virus for 6 days followed by *S. pneumoniae* compared to uninfected mice, mice infected with *S. pneumoniae* only, mice

infected with influenza virus only, and mice infected with influenza virus for 3 days followed by *S. pneumoniae* with the same depletion status; ¶, $p < 0.05$ for mice with the same influenza infection and depletion status to determine the effects of a *S. pneumoniae* infection; #, $p < 0.05$ for mice with the same *S. pneumoniae* infection and depletion status to determine the effects of an influenza infection; †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ for mice with the same influenza and *S. pneumoniae* infections to determine the effects of neutrophil depletion; §, $p < 0.05$ for mice with the same *S. pneumoniae* infection and depletion status to determine the effects of 3 days versus 6 days of influenza infection; ¤, $p < 0.05$; ¤¤, $p < 0.01$; ¤¤¤, $p < 0.001$ for mice with the same depletion status to determine the effects of a co-infection or *S. pneumoniae* infection versus no infection. Data represent one experiment with 5 mice/group. Data expressed as mean ± SD. Data analyzed using one-way analysis of variance followed by the Bonferroni post test.

Neutrophil depletion tended to slightly decrease the amount of IFN-γ compared to undepleted mice, although insignificantly except for mice which were co-infected with influenza for 6 days followed by *S. pneumoniae* ($p < 0.01$; Figure 2.8*B*). In addition, these co-infected mice had significantly more IFN-γ than depleted mice which were uninfected ($p < 0.01$), infected with *S. pneumoniae* only ($p < 0.05$), or co-infected with influenza for 3 days followed by *S. pneumoniae* ($p < 0.05$) (Figure 2.8*B*). Neutrophil depletion also affected the levels of MCP-1. Depleted mice infected only with *S. pneumoniae* had a slight, though insignificant, increase in MCP-1 compared to undepleted mice ($p > 0.05$; Figure 2.8*C*). In depleted mice co-infected with influenza for 3 days followed by *S. pneumoniae*, there was significantly more MCP-1 than undepleted mice co-infected with influenza for 3 days followed by *S. pneumoniae* ($p < 0.05$), depleted mice infected with influenza only for 3 days ($p < 0.05$), and depleted uninfected mice ($p < 0.001$) (Figure 2.8*C*). Depleted mice co-infected with influenza for 6 days followed by *S. pneumoniae* had significantly more MCP-1 and IL-6 than depleted groups of mice infected with *S. pneumoniae* only ($p < 0.001$), co-infected with influenza for 3 days followed by *S. pneumoniae* ($p < 0.001$), infected with influenza only for 6 days ($p <$

0.001), and uninfected ($p < 0.001$) (Figure 2.8*C* and *E*). Neutrophil depletion significantly increased the amount of IL-6 in mice co-infected with influenza for 3 days ($p < 0.05$) or 6 days ($p < 0.001$) followed by *S. pneumoniae* compared to undepleted mice (Figure 2.8*E*). Depleted mice infected only with *S. pneumoniae* had a slight, though insignificant, increase in IL-6 production compared to undepleted mice. In addition, depleted mice co-infected with influenza for 3 days followed by *S. pneumoniae* had significantly more IL-6 than depleted mice which were uninfected ($p < 0.01$) or infected with influenza only for 3 days ($p < 0.05$) (Figure 2.8*E*). Depleted mice co-infected with influenza for 6 days followed by *S. pneumoniae* had significantly more IL-10 than depleted groups of mice infected with *S. pneumoniae* only ($p < 0.001$), co-infected with influenza for 3 days followed by *S. pneumoniae* ($p < 0.01$), infected with influenza only for 6 days ($p < 0.001$), and uninfected ($p < 0.001$) (Figure 2.8*E*). In summary, neutrophil depletion affected the cytokine production in infected lungs, with elevated levels of TNF-α, MCP-1, and IL-6 in depleted mice infected with *S. pneumoniae* only or co-infected with influenza for 3 days followed by *S. pneumoniae* compared to mice with the same infection treatment which were not depleted of neutrophils.

Discussion

In order to establish a time course of susceptibility to *S. pneumoniae* infection after influenza infection, mice infected with influenza virus for 3 or 6 days were challenged with *S. pneumoniae*. We found that susceptibility to *S. pneumoniae* was the greatest at 6 days after influenza infection and was not increased at 3 days after influenza

infection. Several other groups have found similar results, and clinical evidence supports this time frame for maximum susceptibility [175, 177, 193, 237]. Influenza-induced tissue damage is greatest after 6 days of influenza infection [174], and Plotkowski et al. found that adherence of *S. pneumoniae* to influenza-infected tracheas was greatest after 6 days of influenza infection [124]. In addition, influenza-induced neutrophil dysfunction has also been shown to be greatest around 6 days after influenza infection [195, 203]. We found that when influenza titers within the lungs were at their peak (3 days after infection), there was no significant difference in susceptibility to *S. pneumoniae* with or without a prior influenza infection. Gerone et al. showed similar results, with no increased susceptibility to *S. pneumoniae* infection until after 3 days of influenza infection [175]. McCullers and Rehg found that mortality to *S. pneumoniae* infection was greatest after 7 days of influenza infection, with all mice dying within 24 hours, but at 3 days after influenza infection, the rate of mortality was decreased, with all mice dying 3.3 days after *S. pneumoniae* infection [177]. Although our own data of the kinetics of influenza infection and susceptibility to *S. pneumoniae* infection agree with previously published results, none of these studies determined the relative contributions of both neutrophil-dependent and –independent mechanisms to increased susceptibility to *S. pneumoniae* during an influenza infection.

Earlier neutrophil depletion studies done in our laboratory showed that neutrophils play an important role in resistance to *S. pneumoniae* infection in the lungs [225]. By using similar neutrophil depletion models, other studies have shown neutrophils play an important role in controlling infections such as herpes simplex virus

type I, *Chlamydophila abortus*, *Toxoplasma gondii*, and *Listeria monocytogenes* [238-242]. We used neutrophil depletions to determine whether neutrophils play a role in resistance to *S. pneumoniae* infection in mice infected with *S. pneumoniae* only or mice previously infected with influenza for 3 or 6 days. The present study confirmed previous findings by our group in that neutrophils are important to *S. pneumoniae* resistance in non-influenza infected mice [225]. In addition, we showed that they are important in *S. pneumoniae* resistance in mice infected with influenza for 3 days. However, although neutrophils accumulate in the lungs of 6 day influenza-infected mice, they do not function in resistance to *S. pneumoniae*. It is likely that in the influenza-infected, undepleted mice, lung neutrophil bactericidal functions were suppressed, making the mice as susceptible to *S. pneumoniae* as those which had been neutrophil-depleted.

Since the RB6-8C5 mAb used in our neutrophil depletion studies can recognize other cell types, such as CD8 memory T cells and plasmacytoid dendritic cells, that express the Gr-1 antigen [243, 244], we may have also eliminated cell types other than neutrophils which may contribute to eliminating *S. pneumoniae*. Plasmacytoid dendritic cells play an important role in eliminating viral infections by secreting type I interferons and also play a role in initiating the adaptive immune response to both viral and bacterial pathogens [245]. Depletion of plasmacytoid dendritic cells may have altered the initial innate immune response to the *S. pneumoniae* infection since they are important during the initiation of the immune response to pathogens [245]. Since we did not deplete mice until the last 16 hours of the influenza infection and the *S. pneumoniae* infection was not long enough for the adaptive immune response to respond, we believe that any effects

RB6-8C5 depletion had on cell typesof the acquired immune response most likely did not significantly contribute to the results found with our neutrophil depletion studies. Although this model of neutrophil depletion may deplete cell types other than neutrophils, it is currently the best model available for neutrophil depletion.

Effective phagocytosis and killing of *S. pneumoniae* is necessary for elimination of *S. pneumoniae* from the lungs [246]. Studies have found that neutrophils are affected within 30 minutes of an *in vitro* influenza infection [197-199, 202, 205, 206, 223], with some effects seen as rapidly as 5 minutes after incubation with influenza virus [197]. Influenza-induced changes in neutrophils include decreased protein phosphorylation, accelerated apoptosis, decreased respiratory burst activity, altered cytoskeleton, depressed bactericidal capacity, decreased chemotactic ability, decreased adherence, decreased release of lactoferrin into phagosomes, and inhibition of lysosome-phagosome fusion [197-199, 202, 205, 206, 223]. Changes seen in blood neutrophils from mice infected with influenza *in vivo* include decreased chemotactic, chemiluminescent, and bactericidal activities [195, 203]. These studies, along with the results from our studies, show that the effects of influenza virus on neutrophil function are not limited to the lungs, indicating that the effects are systemic despite a lack of viremia [195, 203]. Studies examining the function of lung neutrophils infected with influenza *in vivo* have measured the amount of superoxide and myeloperoxidase produced after infection with *S. pneumoniae* [176, 226]. While these previous neutrophil studies provide evidence of influenza-induced neutrophil dysfunction, they did not examine the effects of an *in vivo* influenza infection on lung neutrophil function independently of an *S. pneumoniae*

infection. In addition, the previous studies did not examine neutrophil function directly since they did not select for neutrophils and therefore were measuring the responses of other lung cells such as macrophages and lymphocytes, whose function may also be affected during an influenza infection.

In the present study, the function of neutrophils that had accumulated in the lungs and were exposed to influenza virus in the lungs was investigated. The function of BALF neutrophils as opposed to BALF macrophages was determined by flow cytometry using neutrophil-specific staining to gate the neutrophil population. The effects of influenza on these neutrophils in response to an *S. pneumoniae* infection could be measured independently of other changes to the host response since the *S. pneumoniae* was added *in vitro*. Since we were interested in determining whether influenza was affecting neutrophil function locally at the site of infection and/or systemically, we measured the ability of neutrophils isolated from the lungs and bone marrow of uninfected (LPS-stimulated) and influenza-infected mice (3 or 6 days) to associate with *S. pneumoniae* (either through binding or phagocytosis) and produce reactive oxygen species. Our method allows for a direct measurement of any changes in neutrophil function in the lungs during an *in vivo* influenza infection prior to an *S. pneumoniae* infection since the neutrophils were exposed to influenza virus *in vivo* and *S. pneumoniae in vitro*.

Lungs from normal, healthy mice contain primarily alveolar macrophages and few to no neutrophils. In order to compare lung neutrophils from healthy mice to those from influenza-infected mice, we used LPS to elicit a rapid neutrophil migration into the lungs of healthy mice. The peak of neutrophil migration and accumulation in the lungs

was seen 8 hours after LPS stimulation. We compared these neutrophils to those from mice infected with influenza for 3 or 6 days to determine whether an influenza infection alters neutrophil function, and if so, whether this alteration was greatest at 6 days after influenza, resulting in increased susceptibility to *S. pneumoniae* infection at 6 days, but not 3 days, after influenza infection. We found that both *S. pneumoniae* association and ROS production by lung neutrophils were significantly decreased at both 3 and 6 days after influenza infection compared to LPS-stimulated mice. This decrease was seen after initial contact with *S. pneumoniae* and after 60 minutes incubation with *S. pneumoniae*. In addition, lung neutrophils from mice infected with influenza for 6 days which were phagocytosing bacteria were not producing ROS as well as phagocytic neutrophils from LPS-stimulated mice. These data indicate that lung neutrophil function is affected at both 3 and 6 days after influenza infection. However, there may be neutrophil functions, such as bacterial killing, which we did not examine that are altered more at 6 days than 3 days after influenza, resulting in the difference in susceptibility seen between 3 and 6 days after influenza infection.

In the bone marrow of mice infected with influenza for 6 days, we observed an initial decrease in ROS production after initial contact with both *S. pneumoniae* and PMA. This decrease was seen after 60 minutes incubation with *S. pneumoniae*. However, after 60 minutes incubation with PMA, there was no decrease in ROS production by neutrophils. *S. pneumoniae* phagocytosis by bone marrow neutrophils was also decreased after 60 minutes incubation with *S. pneumoniae*, but not initially. The decrease in bone marrow neutrophil function may be due to systemic viral effects.

Another possible explanation is the proportion of immature neutrophils may be greater in mice infected with influenza for 6 days compared to LPS-stimulated mice or mice infected with influenza for 3 days since neutrophils are recruited from the bone marrow during an influenza infection. However, Boxio et al. found that murine bone marrow neutrophils function relatively homogenously and there is a large population of competent neutrophils which can be recruited during infections [247].

Since the LPS challenge may have altered the neutrophil function in the lungs of otherwise healthy mice, we challenged influenza infected mice with LPS to determine whether the changes in neutrophil function seen previously were due to influenza or LPS effects. In the lungs, there was a significant increase in the number of neutrophils after LPS stimulation, indicating that the neutrophil reservoir had not been depleted by the influenza infection. The LPS challenge is similar to a secondary bacterial infection, and therefore indicates that fresh neutrophils can enter the lungs in response to a secondary infection. After LPS stimulation in influenza-infected mice, we found similar reductions in *S. pneumoniae* association with and ROS production by lung neutrophils as observed with mice infected with influenza only, indicating that the effects on neutrophil function seen were due to the effects of influenza infection and not LPS stimulation. Additionally, the new neutrophils recruited into the lungs by LPS were not able to overcome the previous deficit in neutrophil function caused by the influenza infection. This may be due to the relatively short period of time in contact with influenza virus (5-30 minutes) necessary to induce neutrophil dysfunction, as seen by others after an *in vitro* influenza infection [197-199, 202, 205, 206, 223]. The neutrophils recruited to the lungs by LPS

may be inhibited by influenza just as rapidly as those in *in vitro* infections since infectious influenza particles were seen in the lungs at both 3 and 6 days after influenza infection, indicating that influenza infection affects neutrophil function locally, possibly by binding directly to the freshly recruited neutrophils. In the bone marrow, the effects of influenza infection and LPS stimulation on neutrophil function do not appear to be as significant as those seen in the lungs.

Our results indicate that an influenza infection of either 3 or 6 days in neutrophil-depleted mice increased susceptibility to *S. pneumoniae* infection compared to depleted mice infected with *S. pneumoniae* only, with the greatest increase at 6 days after influenza infection. These data indicate that an influenza infection also increases susceptibility to *S. pneumoniae* by a mechanism other than neutrophil dysfunction, such as altered cytokine production in the lungs. We found that mice which were infected by influenza for 6 days followed by *S. pneumoniae* for 24 hours had elevated levels of TNF-α, IFN-γ, MCP-1, IL-10, and IL-6. Previous studies have shown that IFN-γ, TNF-α, and IL-6 levels are increased when staphylococcal enterotoxin B or LPS are added to influenza-infected mice, indicating that a prior influenza infection primed influenza-infected cells for rapid cytokine production upon secondary challenge with bacterial components [50, 248]. In addition, LeVine et al. showed that TNF-α and IL-1 are significantly increased in mice infected with influenza for 7 days followed by *S. pneumoniae* for 48 hours [176]. Influenza infection is well known to lyse epithelial cells, leading to tissue damage, and this elevation in both pro- and anti-inflammatory cytokines

may lead to increased tissue damage and pathogenesis during influenza virus and *S. pneumoniae* infections.

Studies by McCullers et al. have shown that viral influenza neuraminidase increases *S. pneumoniae* adherence in the lungs by cleaving sialic acid residues, exposing receptors to which *S. pneumoniae* adhere [189]. Hirano et al. examined the changes in carbohydrates exposed after sialic acid residues are removed by neuraminidase during an influenza infection, which may allow for increased *S. pneumoniae* adherence [249]. Plotkowski et al. documented the influenza-induced changes in the respiratory epithelium throughout the course of an influenza infection and adherence of *S. pneumoniae* to influenza-infected tissue using scanning electron microscopy [124]. Throughout the course of an influenza infection, different cell types and surface molecules are exposed, to which *S. pneumoniae* may adhere more readily. As shown previously, influenza-induced tissue damage is greatest around 6 days after influenza infection [174], which correlates with our data showing increased susceptibility to *S. pneumoniae* infection at 6 days after influenza, indicating that tissue damage likely plays a role in increased susceptibility.

IL-10 has recently been shown to be elevated in post-influenza pneumococcal pneumonia, leading to increased susceptibility to *S. pneumoniae* long after an influenza infection has been resolved [214]. This increase in susceptibility 14 days after a primary influenza infection is believed to be due to IL-10 inhibiting neutrophil function, which results in increased bacterial growth in the lungs and increased mortality [214]. Our data indicate that the onset of this increase in IL-10 production is as early as 6 days after an

influenza infection, which also correlates with the peak of susceptibility shown in our study. IL-10 levels were not significantly elevated at 3 days after an influenza infection when we did not see an increase in susceptibility to *S. pneumoniae* infection.

Since depleted mice infected with *S. pneumoniae* only or influenza for 3 days followed by *S. pneumoniae* were more susceptible to *S. pneumoniae* than undepleted mice, we measured the cytokine levels in these mice to determine whether an elevated cytokine response may be responsible for this increase in susceptibility. We found that TNF-α, MCP-1, and IL-6 all showed slight increases in depleted mice compared to undepleted mice. In addition, TNF-α levels were similar between depleted groups which were infected with *S. pneumoniae* only or influenza for 3 or 6 days followed by *S. pneumoniae*. These data indicate that elevated cytokine production in depleted mice may increase susceptibility to *S. pneumoniae* infection. Further investigation into the role of these cytokines in secondary bacterial pneumonia is needed to determine the contributions of these cytokines to increased susceptibility to *S. pneumoniae* after influenza.

Another possible explanation for the mechanism of increased susceptibility to *S. pneumoniae* infection after influenza infection is alterations in alveolar macrophage function. Several studies have demonstrated that influenza infection decreases the functions of alveolar macrophages, such as bacterial phagocytosis and killing [210, 211]. Astry and Jakab found the suppression of alveolar macrophage phagocytosis by influenza was greatest after 7 to 10 days of influenza infection [210], which correlates with our data for increased susceptibility to *S. pneumoniae* infection after 6 days of influenza infection.

Alveolar macrophages have been shown to have a protective anti-inflammatory role during *S. pneumoniae* infections through the elimination of apoptotic neutrophils [212]. If apoptotic neutrophils are not effectively cleared from the lungs, their cytotoxic components eventually leak into the alveolar space, leading to increased tissue damage [212, 250]. Since alveolar macrophages are important in the defense against bacterial infections, a change in their ability to eliminate *S. pneumoniae* and apoptotic neutrophils could alter the severity of the infection.

In summary, our neutrophil depletion studies indicated that both neutrophil-dependent and –independent mechanisms are responsible for increased susceptibility to a secondary *S. pneumoniae* infection after an influenza infection. While an influenza infection may cause other changes to the host immune response which we did not examine, our data indicate that both neutrophil dysfunction and elevated cytokine production contribute to the increased susceptibility to a secondary *S. pneumoniae* infection. These effects are cumulative, with the greatest susceptibility to *S. pneumoniae* infection at 6 days after influenza infection. At 3 days after influenza infection, viral replication is at its peak and tissue damage is minimal. At this time, lung neutrophil function was depressed, yet there was no significant increase in susceptibility to *S. pneumoniae* infection compared to mice infected with *S. pneumoniae* only. In addition, neutrophil depletion studies showed that after 3 days of influenza infection, neutrophil depletion increased susceptibility to *S. pneumoniae* infection, indicating that neutrophils from 3 day influenza-infected mice can still eliminate *S. pneumoniae*. However, even at 3 days after influenza infection, neutrophil depletion studies showed evidence of

neutrophil-independent mechanisms for increased susceptibility. After 6 days of influenza infection, when susceptibility to *S. pneumoniae* infection is at its peak, viral replication is decreased and tissue damage is at its greatest [124, 174]. Neutrophil function was also suppressed after 6 days of influenza infection. Neutrophils in mice infected with influenza for 6 days, which already have a decreased phagocytic capability, may not be able to eliminate *S. pneumoniae in vivo* as readily as neutrophils from 3 day influenza-infected mice due to the increased burden of phagocytosing cellular debris from influenza-induced tissue damage and apoptotic neutrophils. In addition, there may be more influenza-induced changes in neutrophil function at 6 days than 3 days which we did not examine. Cytokine levels were also increased in mice co-infected with influenza for 6 days followed by *S. pneumoniae*, indicating an elevated cytokine response may be responsible for the increased morbidity and mortality seen during influenza and *S. pneumoniae* infections. A better understanding of how the influenza virus affects both tissue damage and host bacterial responses (such as neutrophil function and cytokine expression) will facilitate the rational design of therapies to prevent secondary *S. pneumoniae* infections and the associated increase in morbidity and mortality.

USE OF A NOVEL *EX VIVO* MURINE TRACHEAL EXPLANT SYSTEM TO MODEL INFLUENZA-INDUCED TISSUE DAMAGE AND SUBSEQUENT *STREPTOCOCCUS PNEUMONIAE* INFECTION

Introduction

Secondary bacterial infections such as pneumonia caused by *Streptococcus pneumoniae* are associated with an increase in morbidity and mortality during influenza epidemics and pandemics [78, 175-178, 183, 189-191, 215-218]. In 2002, the combined infections of influenza and pneumonia were the number one cause of infectious death in the United States [64]. With the threat of an influenza pandemic in the near future and rapidly increasing antibiotic resistance, it is critical to understand how a prior influenza infection increases susceptibility to *S. pneumoniae* infection. Several possible mechanisms may account for the increase in pneumococcal pneumonia associated with influenza infection, including influenza-induced damage to the tracheal epithelium and/or alterations in host cellular responses to bacterial pathogens [174, 188, 211]. While the exact mechanism(s) for increased susceptibility to secondary *S. pneumoniae* infections after influenza infection are not known, two competing hypotheses have been advanced to explain concurrent increased susceptibility. The first hypothesis is that influenza-induced phagocytic dysfunction (such as impaired neutrophil chemotaxis and bactericidal activities) increase susceptibility to *S. pneumoniae* infections [195, 196, 199, 203, 207]. The other hypothesis states that influenza-induced damage to the respiratory epithelium

alters the cellular characteristics of the epithelium by exposing surface molecules and cell receptors to which the *S. pneumoniae* more readily adhere [63, 124, 177, 251, 252].

Previous studies have used *in vitro* cell culture assays to measure the adherence of *S. pneumoniae* to influenza-infected lung and bronchial epithelial cells [185, 186, 189]. However, a limitation of this method is that undifferentiated or immortalized cells in monolayers may not express the same surface molecules as differentiated cells of the intact respiratory epithelium and therefore may not represent cell types present during *in vivo* influenza infections. Several different cell types are exposed during the course of an influenza virus infection, including ciliated pseudostratified columnar epithelial cells, microvilli-covered cells, goblet cells, basal cells, undifferentiated epithelial cells, and differentiating epithelial cells [124, 187, 188]. Influenza virus infection is cytolytic, resulting in denudation of the epithelium and exposure of the basement membrane [124]. Consequently, *S. pneumoniae* may adhere more readily to components of the basement membrane such as fibronectin [123] and/or different cell types or proteins exposed or expressed during an influenza infection [63].

Tracheal explant systems, such as tracheal rings or whole organ explants from a variety of animal species, including chicken and duck embryos, hamsters, mice, rats, ferrets, pigs, baboons, guinea pigs, and chinchillas, have been widely used as an alternative to *in vitro* cell culture assays [228, 253-277]. Tracheal explants have been used to investigate the effects of toxins such as mineral dusts, titanium dioxide, air pollution particles, and cigarette smoke on tracheal function [256, 259, 260, 275]. In addition, the interactions of several bacterial and viral pathogens, such as *Mycoplasma*

spp., *Bordetella spp.*, influenza virus, cytomegalovirus, and *S. pneumoniae*, with the respiratory epithelium have been studied [228, 253-255, 257, 258, 261-274, 277]. In particular, *S. pneumoniae* adherence has been studied using chinchilla tracheal explants [228, 274]. A distinct advantage of a tracheal explant system is that the differentiated tissue more accurately reproduces influenza and respiratory epithelial cell interactions with *S. pneumoniae* than do *in vitro* cellular monolayers.

In the present study, using an *in vivo* model of co-infection, we found that a prior influenza infection significantly increased *S. pneumoniae* adherence to the mouse trachea compared to uninfected tracheas, suggesting that influenza-induced tissue damage may be responsible for this increase. However, because influenza infection affects other host immune factors, such as neutrophil function, it is difficult to determine the extent of influenza-induced tissue damage as a contributing factor to the susceptibility to a *S. pneumoniae* infection *in vivo* [195, 196, 199, 203, 207]. Therefore, we developed an *ex vivo* murine tracheal explant system to mimic influenza-induced tissue damage seen *in vivo* in order to determine how different stages of an influenza infection (including the initial presence of virus-infected cells, influenza-induced damage of the respiratory epithelium and resultant exposure of the basement membrane, and repair of the respiratory epithelium) affect *S. pneumoniae* adherence to the tracheal respiratory epithelium. Specifically, the tracheal explant model enables the effects of influenza-induced tissue damage on *S. pneumoniae* adherence to be determined independently of influenza-induced effects on the immune system, such as neutrophil dysfunction. Additionally, this model allows us to simulate physiological shear conditions that may

occur *in vivo* by adjusting the flow of media through the tracheas. We found that a prior *ex vivo* influenza infection, resulting in exposure of the tracheal basement membrane, contributes minimally to increased *S. pneumoniae* adherence. The increase in *S. pneumoniae* adherence after an influenza infection *in vivo* may be due to influenza-induced defects in mucociliary clearance, leading to increased numbers of *S. pneumoniae* within the tracheal lumen and increased adherence of *S. pneumoniae* to the denuded epithelium. We have developed a novel murine tracheal explant system which can be used to study bacterial adherence to the respiratory epithelium of uninfected and influenza-infected tracheal explants.

Materials and Methods

Mice

Female C57BL/6N mice ages 10-16 weeks old from the National Cancer Institute (Frederick, MD) were used for all experiments. Animals were housed at the Montana State University Animal Research Center. All animal protocols were approved by the Montana State University Institute for Animal Care and Use Committee.

Infectious Agents

Influenza virus A/PR8/8/34 (PR8; H1N1) from the Trudeau Institute (Saranac Lake, NY) was used for all influenza infections. The virus was grown in the allantoic fluid of embryonated chick eggs and stored at -80°C. *Streptococcus pneumoniae* type 4 (ATCC 6304; American Tissue Type Collection, Manassas, VA) was grown in Todd Hewitt broth (Becton Dickinson and Company, Sparks, MD) supplemented with 0.5% yeast

extract (THY) (Fisher Scientific, Fair Lawn, NJ) at 37°C with 5% CO_2 until mid-log phase of growth. Stock cultures in THY with 10% glycerol (Sigma, St. Louis, MO) were snap frozen in liquid nitrogen and stored at -80°C until needed. Before use, stock cultures were washed twice at 16,000 × *g* with Dulbecco's PBS (DPBS; Gibco, Grand Island, NY) and resuspended to the appropriate concentration. *S. pneumoniae* colony forming units (CFUs) were approximated using the OD at 450_{nm}. CFUs were confirmed by plating serial dilutions on neomycin blood agar plates (30 μg/mL neomycin; Becton Dickinson Microbiology Systems, Cockeysville, MD) using the drop plate method as previously described [233].

In vivo Infection Model

Mice were lightly anesthetized with isoflurane and infected intranasally (i.n.) with 600 plaque forming units (PFUs) PR8 influenza virus in 100 μl of sterile HBSS (50 μL/nare). At days 3 or 6 after influenza infection, mice were challenged i.n. with 10^7 CFUs of *S. pneumoniae* in 100 μl of sterile DPBS (50 μL/nare) for 2 hours. *S. pneumoniae*-infected only mice were mock-influenza infected i.n. with 100 μl HBSS. Influenza-infected only mice were mock-*S. pneumoniae* infected i.n. with 100 μl DPBS. Uninfected control mice were mock-influenza infected i.n. with HBSS and mock-*S. pneumoniae* infected i.n. with DPBS. Two hours after *S. pneumoniae* infection, mice were euthanized by deep pentobarbital anesthesia followed by exsanguination, and the tracheas were removed, placed in 2 mL HBSS, snap frozen in liquid nitrogen, and stored at -80°C. *S. pneumoniae* CFUs and influenza PFUs were enumerated as described below.

Tracheal Explant Media

Tracheal explants were maintained with continuous media flow through the tracheal lumen of RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), HEPES (20 mM; Gibco), neomycin (30 μg/mL; Cellgro, Herndon, VA), and gentamicin (5 μg/mL; Gibco) (cRPMI) at a flow rate of 0.1 mL/min. Prior to influenza infection, tracheal explants were incubated with continuous flow of cRPMI supplemented with penicillin (200 units/mL) and streptomycin (200 μg/mL) (cRPMI-pen/strep; Gibco) at a flow rate of 0.1 mL/min to remove normal bacterial flora from the explants.

Tracheal Explant System Setup

Mice were anesthetized intraperitoneally with sodium pentabarbitol and exanguinated. For each tracheal explant, the trachea was separated from the esophagus and cut below the larynx. The trachea, lungs, and heart were removed *en bloc* and placed in a sterile Petri dish where the heart was removed. The lungs were transected below the bronchial bifurcation, leaving part of the lungs attached to the trachea for added weight. The excised trachea was then placed in cRPMI. A blunted 18-gauge needle (Becton Dickinson and Company) was inserted through an opening in the filter cap of a sterile 0.2 μm vented cap 162 cm^2 cell culture flask (Corning Inc., Corning, NY) and the cap was removed from the flask. The needle was then inserted into the trachea, and the cap and attached trachea were placed back onto the flask.

To sterilely maintain medium influent, two holes were burned into the lid of a sterile 1000 mL filter unit receiver (Nalge Nunc International, Rochester, NY) with a 45

mm neck using a heated 1/8” slotted screwdriver. Two 100-200 μL Drummond Microdispenser replacement tubes (Drummond Scientific Company, Broomall, PA) were placed into each of the holes and sealed using a silicone adhesive sealant (Henkel Corporation, Rocky Hill, CT). To allow filtered air into the system to aerate the medium, 5.5 cm of 1.85 mm ID silicone tubing (Cole-Parmer Instrument Company, Vernon Hills, IL) was attached to the exterior end of one of the glass tubes and a 0.2 μm 10 mm membrane filter (Whatman Inc., Clifton, NJ) was attached to the tubing using a 1/16” male luer fitting (Cole-Parmer). To the interior end of the other glass tube, 22 cm of 2.29 mm ID silicone tubing (Cole-Parmer) was attached and placed in the medium to allow for media flow. The exterior end of the tube was attached to 83 cm of 1.52 mm ID silicone tubing (Cole-Parmer), which was attached to the yellow end of 2-stop tubing (1.52 mm ID yellow/blue; Cole-Parmer) with a straight 1/16” connector (Cole-Parmer). The blue end of the 2-stop tubing was attached to 107 cm of 1.52 mm ID silicone tubing with a straight 1/16” connector. This tubing was connected to a 5” microbore extension set with a “T” connector and pre-pierced injection site (needle port) (Abbott Laboratories, North Chicago, IL) using a male 1/16” luer fitting. The microbore extension kit was then inserted into the 18 gauge needle in the flask with the trachea as described above. The 2-stop tubing was connected to an Ismatec® IPC High Precision Multichannel Dispenser pump (Cole-Parmer) and the flow rate was calibrated to 0.1 mL/minute. Before use, the medium reservoir lid and all attached glass tubes and silicone tubing were autoclaved for sterility.

<u>Tracheal Explant Infection Model</u>

Tracheal explants were incubated at 37°C and 5% CO_2 for 24 hours with cRPMI-pen/strep at a continuous flow rate of 0.1 mL/min to remove normal bacterial flora. After 24 hours, the medium was changed to cRPMI without pen/strep. To assess viability of tracheal explants, uninfected tracheas were incubated for up to 8 days. Trypan blue (MP Biomedicals, LLC, Aurora, OH) was used to measure the volume needed to fill both the needle and the tracheal lumen. For influenza-infected tracheas, medium flow was stopped and approximately 5×10^5 PFUs of PR8 in 100 µL HBSS were added by injecting a 23-gauge needle (Becton Dickinson and Company) into the needle port. To allow for viral attachment, tracheal explants were incubated with PR8 for one hour before resuming flow with medium. Tracheal explants were then incubated for up to 5 days with or without PR8 at 37°C and 5% CO_2.

S. pneumoniae was added to the tracheal explants at designated timepoints after influenza infection. Frozen stocks of *S. pneumoniae*, as described above, were used for all experiments. The inoculum was prepared by washing the *S. pneumoniae* twice in D-PBS and resuspending the bacteria with D-PBS to the desired concentration. To confirm actual CFUs of the inoculum, serial dilutions of the inoculum were plated.

For *S. pneumoniae* adherence assays, medium flow was stopped and one mL of inoculum with approximately 5×10^7 CFUs *S. pneumoniae* (approximately 10^6 CFUs within tracheal lumen) was injected into the needle port with a 23-gauge needle. Tracheal explants were then incubated for 1 hour with *S. pneumoniae* to allow for *S. pneumoniae* adherence. Medium flow was then resumed for 30 minutes to remove non-

adherent *S. pneumoniae*. To measure adherence based on initial inoculum size of *S. pneumoniae*, tracheal explants were incubated with *S. pneumoniae* as described above except with the following concentrations of *S. pneumoniae*: 5×10^3, 5×10^5, 5×10^6, 5×10^7, or 5×10^8 CFUs/mL/trachea. The approximate numbers of *S. pneumoniae* CFUs within the tracheal lumen were 10^2, 10^3, 10^5, 10^6, and 10^7, respectively.

Quantification of PFUs and CFUs

At designated time points, the remaining lung tissue from the explants was removed and the tracheas were homogenized through a sterile wire screen. Samples were serially diluted and plated using the drop plate method [233] to enumerate *S. pneumoniae* CFUs. Plates were incubated overnight at 37°C and 5% CO_2. For PFU enumeration, the remainder of each sample was snap frozen in liquid nitrogen and stored at -80°C. Samples were serially diluted and incubated with Madin-Darby canine kidney cells using the plaque assay method as previously described [234].

Histological Examination

Ciliary beating was visualized microscopically using a Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss Microimaging, Thornwood, NY) and an Olympus CK2 inverted microscope (Olympus America, Inc., Melville, NY). Tracheas were instilled with 3% agarose and cut into 750 μm rings using a Leica VT1000S vibratome (Leica Microsystems Nussloch GmbH, Germany). The agarose plugs were removed from the rings using forceps and the rings were placed in cRPMI for microscopy.

For morphological examination of the respiratory epithelium, tracheal explants were fixed at various timepoints after incubation with or without influenza infection for at least 24 hours in 10% phosphate-buffered formalin (Fisher Scientific), embedded in paraffin, and cut into 5 μM sections. Sections were then stained using hemotoxylin and eosin (H & E), and morphology of the respiratory epithelium was examined using a Nikon Eclipse E800 microscope (Nikon, Inc., Melville, NY).

For visualization of influenza virus, tracheal explants were instilled with Tissue-Tek® OCT (Sakura Finetek, Torrence, GA) and snap frozen in liquid nitrogen. Frozen tracheal explants were cut into serial 5 μm sections and air-dried. For visualization of tissue structure in adjacent sections, tissue sections were stained using hematoxylin and eosin. For immunofluorescence, tissue sections were fixed in acetone/alcohol (75/25) at room temperature for 5 minutes then washed 3 times for 3-5 minutes each in D-PBS. Sections were blocked with avidin solution (Vector Laboratories Inc., Burlingame, CA) in 5% normal goat serum (ICN Biomedicals, Inc., Aurora, OH) and 5% normal donkey serum (Chemicon International, Temecula, CA) (NG/NDS) for 15 minutes, rinsed with D-PBS/0.2% bovine serum albumin (Serologicals Corporation, Norcross, GA) (D-PBS/BSA), blocked with biotin solution (Vector Laboratories, Inc.) in NG/NDS for 15 minutes, and rinsed with D-PBS/BSA. All antibodies were diluted in 2.5% NG/NDS and all incubations were 30 minutes at room temperature followed by rinses with D-PBS/BSA. For influenza detection, slides were incubated with 4 μg/mL biotin conjugated goat anti-influenza virus A H1N1 (Accurate Chemical & Scientific Corporation, Westbury, NY). Control slides were incubated with 4 μg/mL biotinylated

goat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). All slides were incubated with 10 μg/mL streptavidin Alexa Fluor® 488 conjugate (Molecular Probes, Invitrogen Corporation, Carlsbad, CA) followed by three rinses with pure D-PBS. Slides were coverslipped with Immu-Mount™ mounting medium (Thermo Shandon, Pittsburgh, PA). Slides were viewed using the Nikon Eclipse E800 microscope and imaged using the Nikon Digital Camera DXM1200 and MetaVue software (Molecular Devices Corporation, Downingtown, PA).

Statistical Analysis

Data were analyzed using the Mann-Whitney Test or unpaired *t* test (GraphPad Prism Software, San Diego, CA). Significant differences were reported for P values of < 0.05.

Results

In vivo S. pneumoniae Adherence

To determine whether a prior *in vivo* infection increases adherence of *S. pneumoniae* to the tracheal epithelium, we compared *S. pneumoniae* adherence to tracheas from uninfected mice to that of tracheas from mice infected with influenza for 3 or 6 days. A prior influenza infection of 3 or 6 days significantly increased *S. pneumoniae* adherence to influenza-infected tracheas compared to uninfected tracheas (Figure 3.1*A*). Tracheas from mice infected with influenza for 3 days had a 98.7-fold increase in the number of adherent *S. pneumoniae* compared to tracheas from uninfected mice ($p < 0.0001$; Figure 3.1*A*). In tracheas from mice infected with influenza for 6 days, there was a 206.9-fold increase in *S. pneumoniae* adherence compared to tracheas from

uninfected mice ($p < 0.0001$; Figure 3.1*A*). In addition, tracheas from mice infected with influenza for 6 days had 2.1-fold more adherent *S. pneumoniae* than those from mice infected with influenza for 3 days prior to the *S. pneumoniae* infection ($p = 0.0184$; Figure 3.1*A*). Tracheas from mice infected with influenza for 3 or 6 days had similar influenza PFUs while those from uninfected mice had none (Figure 3.1*B*). These data suggest that a prior influenza infection of 3 or 6 days significantly increases *S. pneumoniae* adherence to the tracheal respiratory epithelium, with maximal adherence occurring at 6 days after influenza infection.

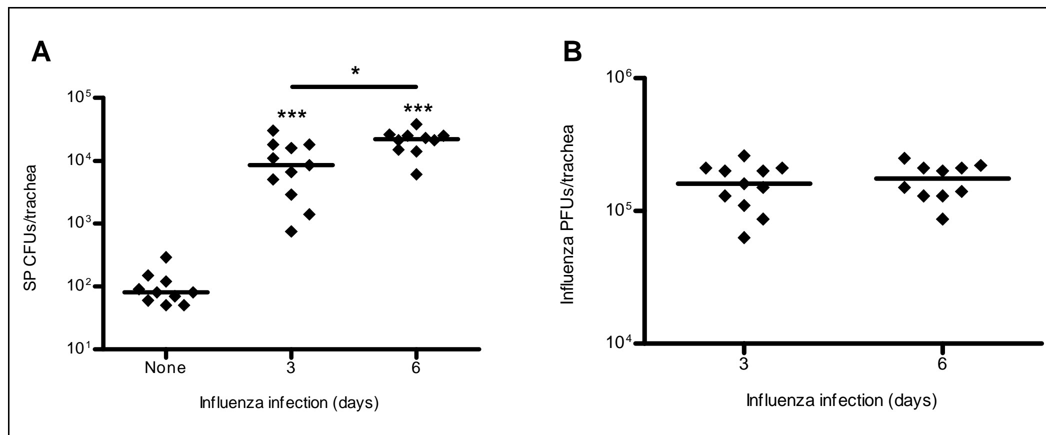

Figure 3.1. *S. pneumoniae* adherence after 2 hours *in vivo* infection to tracheas from uninfected mice or mice infected with influenza for 3 or 6 days. *S. pneumoniae* CFUs (*A*) and influenza PFUs (*B*). Significant differences between mice infected with SP only and mice co-infected with influenza and SP are indicated by ***, $p < 0.001$. Significant differences between mice co-infected with influenza for 3 or 6 days followed by SP are indicated by *, $p < 0.05$. Line represents median value for 10-11 mice for each infection. Data analyzed using Mann-Whitney U test.

Tracheal Explant System Setup

We developed a murine whole tracheal explant system to examine *S. pneumoniae* adherence independently of other changes in the host immune response. Any changes in *S. pneumoniae* adherence could then be solely attributed to the tissue damage caused by the influenza infection. Tracheas were removed and attached to needles of the tracheal explant system (Figure 3.2). A portion of the lung was left attached to the trachea to add weight to the trachea, allowing for complete exposure of the respiratory epithelium (Figure 3.2). Fresh medium (cRPMI) was pumped through the tracheal explants at a constant flow rate of 0.1 mL/minute in order to keep the tissue evenly hydrated and supplied with nutrients. Infectious agents were injected through the needle port of the microbore extension set. After incubation with influenza virus or *S. pneumoniae*, flow was resumed to remove unattached bacteria. Spent medium (waste) was collected in the tissue culture flask and discarded before it reached the tracheal explant (Figure 3.2).

Viability of Uninfected Tracheal Explants

To determine how *ex vivo* culture affected the viability and morphology of tracheal explants, we used both confocal and light microscopy. Ciliary beating was determined using both an inverted microscope and a confocal microscope as a measure of viability. The cellular morphology of tracheal explants was examined using H & E stained sections at various timepoints of *ex vivo* culture (0-8 days). We found that tracheal explants remained viable for the 8 day period studied. The cilia and respiratory epithelium appeared normal with a ciliated pseudostratified columnar epithelium present

in uninfected tracheas (Figure 3.3). In addition, ciliary beating could be observed during the entire 8 days of culture (data not shown).

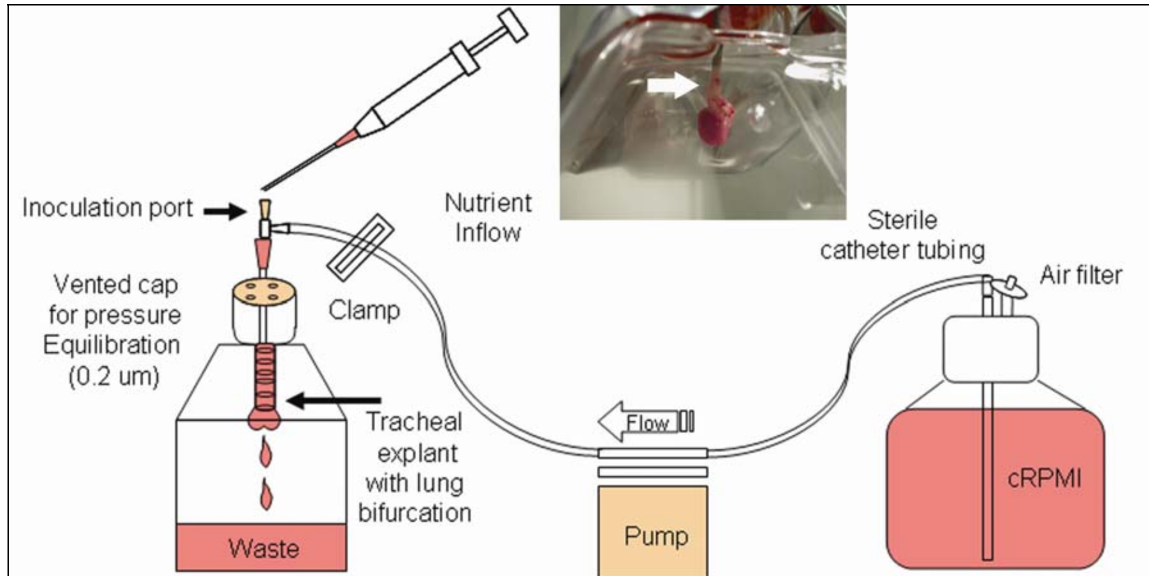

Figure 3.2. Diagram of tracheal explant system showing media reservoir connected to pump and flask with tracheal explant. Tracheas were suspended from blunt-ended 18-gauge needles, which were connected to silicone tubing through a pump with a constant media flow rate of 100 µL/min. Media (cRPMI) and explants were incubated at 37°C in 5% CO_2. Inset: picture of tracheal explant and portion of lung connected to needle inside flask. The arrow indicates trachea attached to needle.

Establishment of an Influenza
<u>Infection in Tracheal Explants</u>

We injected trypan blue into tracheal explants through the needle port to determine the minimum volume needed to fill the tracheal lumen. We found a volume of 40 µL filled the lumen, and therefore used volumes of 100 µL for influenza and 1 mL for *S. pneumoniae* infections in order to ensure complete coverage of the tracheal epithelium by the inoculum. Tracheal explants were infected with PR8 influenza virus for 0.5-5 days and viral PFUs were enumerated at each timepoint after infection. Influenza PFUs

were detected as early as 12 hours after influenza infection ($3.5 \times 10^1 \pm 1.6 \times 10^1$ PFUs/trachea) and could be detected for up to 5 days (Figure 3.4). Influenza PFUs increased to a peak of $2.0 \times 10^3 \pm 4.5 \times 10^3$ PFUs/trachea at 3 days in culture and gradually decreased in number to $1.9 \times 10^2 \pm 4.1 \times$x 10^2 PFUs/trachea by 5 days of culture (Figure 3.4). These results demonstrate that an influenza infection can be initiated and maintained with infectious influenza virus particles present in our tracheal explant system for up to 5 days.

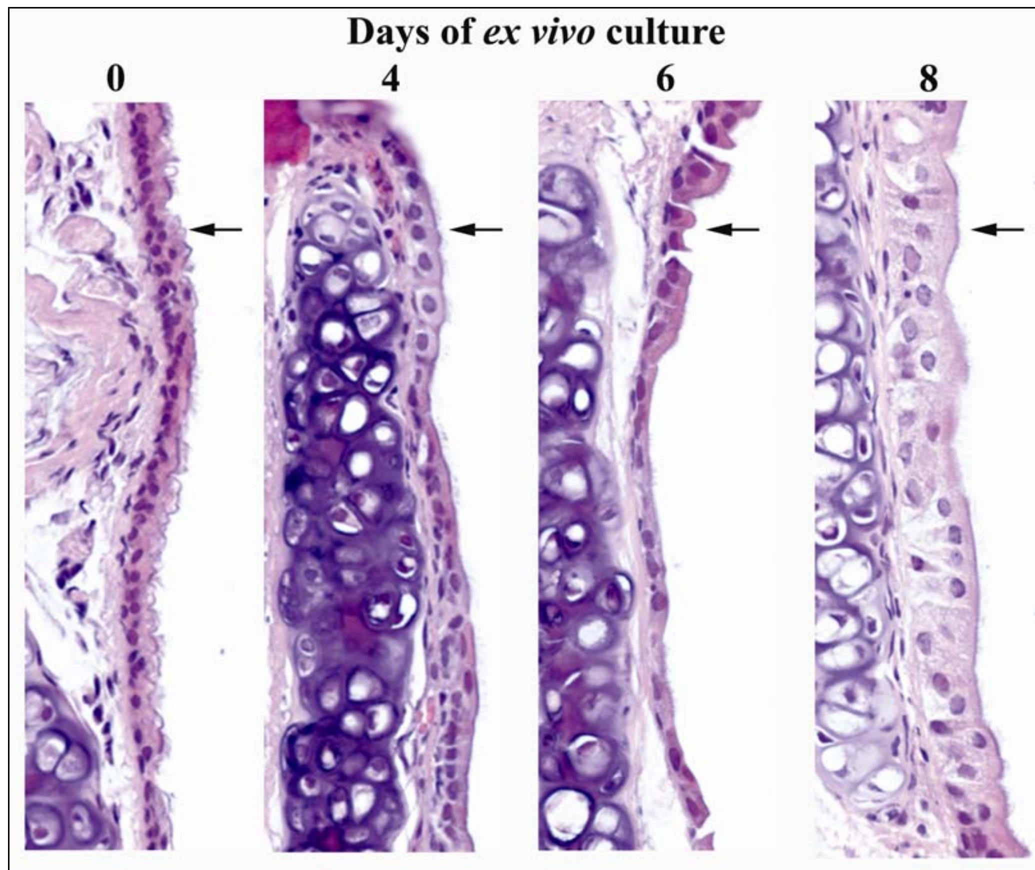

Figure 3.3. Uninfected tracheal explants at 0, 4, 6, and 8 days after *ex vivo* culture stained with H & E (pictures representative of 5 tracheas for each day). Intact cilia are present at all timepoints. Arrows point to tracheal respiratory epithelium. 400X magnification.

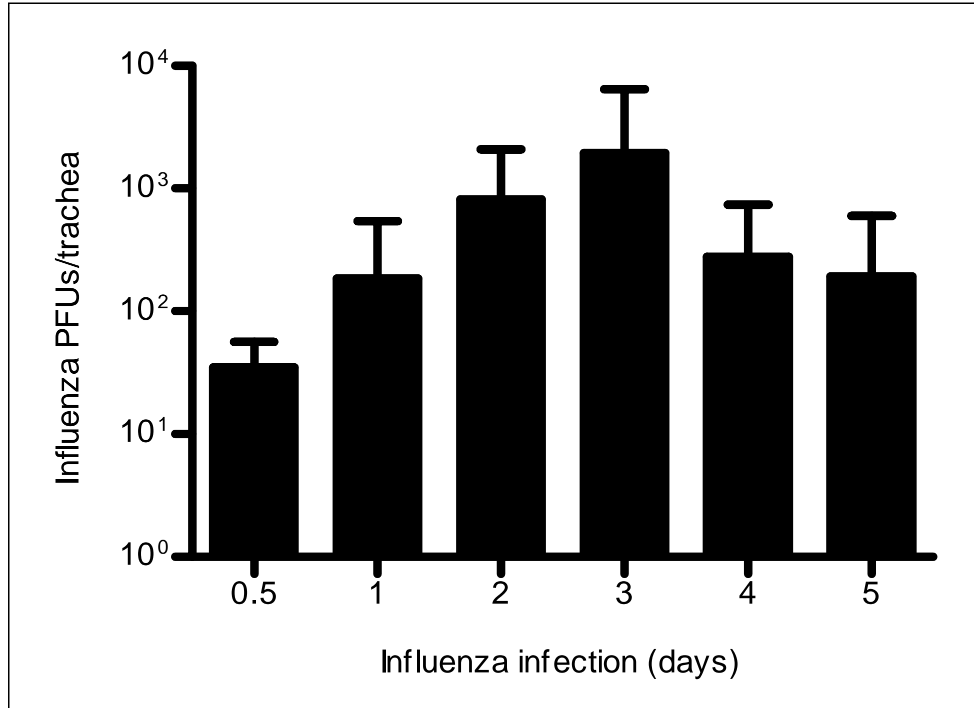

Figure 3.4. Influenza PR8 virus PFUs in tracheal explants at various timepoints after *ex vivo* influenza infection. Data expressed as mean ± SD. Results represent 12-15 tracheas per day of influenza infection.

Effects of Influenza Virus Infection on
Respiratory Epithelium of Tracheal Explants

Influenza-induced tissue damage and influenza-infected cells were visualized using tracheal explants after 1-5 days *ex vivo* culture (Figure 3.5*A*). Influenza virus particles were visualized in influenza-infected tissues using immunofluorescence. Influenza-induced tissue damage was shown using H & E stained sections and uninfected tracheal explants were stained for comparison (Figures 3.3 and 3.5*A*). Influenza-infected tracheal explants were also stained with a negative control antibody (biotinylated goat IgG) (Figure 3.5*B*). At 1 day after influenza infection, influenza-induced damage to the epithelium was evident and influenza-infected cells were present as shown by

immunofluorescence (Figure 3.5 *A*). At 2 days of influenza infection, most of the epithelium was denuded, exposing the basement membrane and undifferentiated basal cells (Figure 3.5 *A*). Influenza-infected epithelial cells were also seen using immunofluorescence. At 3 days after infection, basal cells were seen migrating over the exposed basement membrane, evidenced by the intense violet H&E staining of the tracheal epithelium, and some influenza-infected cells were visible by IFA (Figure 3.5 *A*). At 4 and 5 days after infection, the epithelium appeared almost normal, with some influenza-antigen positive cells present at 4 days but few to none present at 5 days (Figure 3.5 *A*). Thus, the tracheal explant system enabled examination of both influenza-infected cells and influenza-induced tissue damage, as well as resolution of viral infection and tissue repair.

S. pneumoniae Adherence to Uninfected and Influenza-Infected Tracheal Explants

To determine the effect of *S. pneumoniae* inoculum size on initial adherence to tracheal explants with and without influenza infections, tracheas were infected with 5×10^3, 5×10^5, 5×10^6, 5×10^7, or 5×10^8 *S. pneumoniae* CFUs in 1 mL of PBS. In both influenza-infected and non-influenza infected tracheas, there were no detectable viable *S. pneumoniae* adherent to the tracheal explants with an inoculum of 5×10^3 CFUs/mL (Figure 3.6). Pneumococci adhered to the tracheal explants in a dose-dependent manner, with maximal adherence occurring with an inoculum of 5×10^8 *S. pneumoniae* CFUs/mL ($3.1 \times 10^4 \pm 2.5 \times 10^4$ CFUs/trachea) (Figure 3.6). *S. pneumoniae* adherence to influenza-infected tracheas was not significantly increased compared to adherence to

non-influenza infected tracheas at inoculum sizes of 5×10^5, 5×10^6, and 5×10^7 *S. pneumoniae* CFUs/mL (Figure 3.6). Unless otherwise noted, the inoculum for the remainder of tracheal explants was 5×10^7 *S. pneumoniae* CFUs/mL. These data suggest that *S. pneumoniae* adherence to both uninfected and influenza-infected tracheal explants occurs in a dose-dependent manner.

We measured adherence of *S. pneumoniae* to uninfected tracheas and tracheas infected with influenza for up to 5 days in *ex vivo* culture to determine whether influenza virus affected initial adherence of *S. pneumoniae* in the tracheal explants. There was a 3.2-fold increase in *S. pneumoniae* adherence to tracheal epithelium at 1 day after influenza infection compared to non-influenza infected tracheas ($p = 0.047$; Figure 3.7). However, no other time points showed significant increases in adherence with a prior influenza infection, and 3 and 5 day influenza-infected tracheal explants showed an insignificant decrease in adherence when compared to tracheas without a prior influenza infection (Figure 3.7). These data indicate that a prior influenza infection facilitates *S. pneumoniae* adherence early in an *ex vivo* influenza infection when influenza-infected cells are present and influenza-induced damage to the respiratory epithelium has begun. However, adherence is not increased during the later stages of an *ex vivo* influenza infection when the epithelium is denuded and the basement membrane is exposed or during the initial stages of epithelial repair.

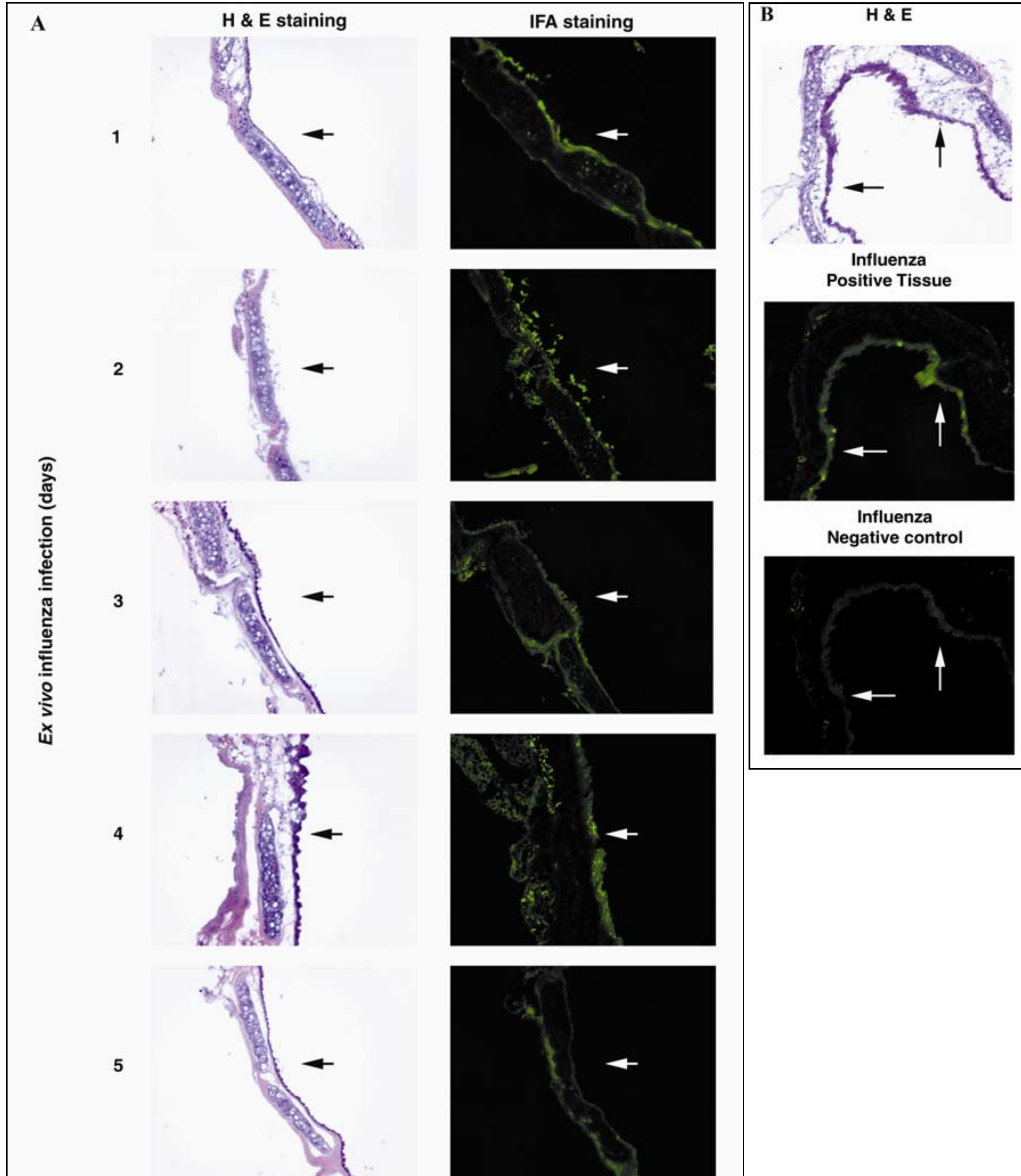

Figure 3.5. Tracheal explants infected *ex vivo* with influenza virus for 1-5 days (*A*) and control IFA staining for tracheal explant infected with influenza virus for 1 day (*B*, lower panel). Hemotoxylin and eosin (H & E) staining and corresponding influenza immunofluorescence (IFA) staining of tracheal explants at various time points after *ex vivo* influenza infection. 200X magnification. Tracheas shown represent 5 tracheas per time point. Arrows point to tracheal respiratory epithelium.

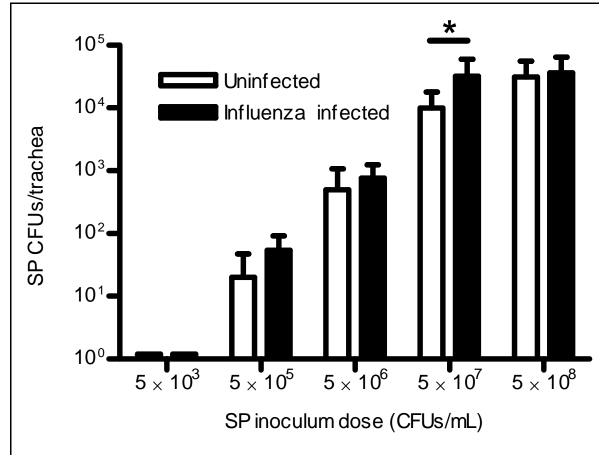

Figure 3.6. Effect of *S. pneumoniae* inoculum dose on SP adherence to uninfected and influenza-infected (1 day) tracheal explants. Tracheas were inoculated with 5×10^3, 5×10^5, 5×10^6, 5×10^7, or 5×10^8 SP CFUs in 1 mL of PBS for one hour to allow for adherence. Data expressed as mean ± SD. Results represent 4-5 tracheas/dose except 8 tracheas were used for the 5×10^7 dose.

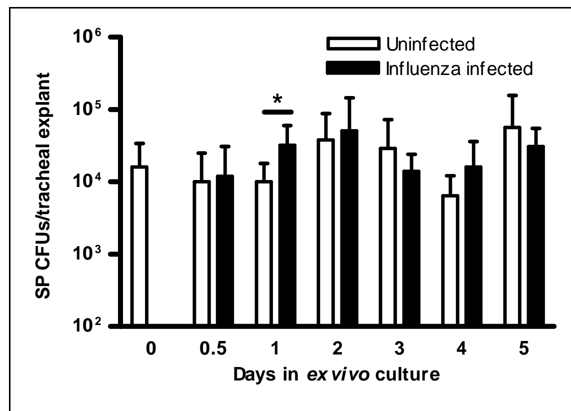

Figure 3.7. Effect of influenza infection on SP adherence to tracheal explants at various times after influenza infection (0.5-5 days). Data expressed as mean ± SD. Data analyzed using unpaired *t* test. Significant differences in SP adherence between uninfected and influenza-infected tracheas are indicated by *, $p < 0.05$. Results represent 8-10 tracheas/timepoint, except 0 and 0.5 days with and without influenza, which have 14 and 15 tracheas each, respectively.

Discussion

Our *in vivo* co-infection results indicate that a prior influenza infection increases *S. pneumoniae* adherence to the tracheal epithelium at both 3 and 6 days after an influenza infection, with maximal adherence at 6 days after influenza infection. We used an infection time of 2 hours to measure the amount of *S. pneumoniae* initially adherent to the tracheas in order to allow for mucociliary clearance of non-adherent *S. pneumoniae* and to avoid increased counts due to replication of the *S. pneumoniae*. This increase in *S. pneumoniae* in influenza-infected tracheas could be due to either increased adherence or decreased mucociliary clearance [124, 174]. Our results confirm those of Plotkowski et al. who showed that tracheas from mice infected *in vivo* with influenza and infected with *S. pneumoniae in situ* for 90 minutes had more *S. pneumoniae* adherent than non-influenza infected tracheas [124]. This increase in adherence was greatest at 6 days after influenza infection when tissue damage to the tracheal respiratory epithelium was the greatest [124]. However, since these tracheas were infected with influenza virus *in vivo*, it is difficult to conclude that influenza-induced tissue damage was responsible for this increase in *S. pneumoniae* adherence since adherence may have been affected by other influenza-induced effects on the immune system, such as neutrophil dysfunction [195, 196, 199, 203, 207]. An influenza infection has also been shown to affect the mucociliary clearance of bacteria from the trachea, which may lead to increased numbers of *S. pneumoniae*, and therefore increased *S. pneumoniae* adherence, in the tracheas of influenza-infected mice and an increased likelihood of spread to the lungs resulting in pneumonia [174].

The trachea is thought to play a role in the progression of pneumococcus from the nasal mucosa where it asymptomatically colonizes the nasal epithelium to the lungs, resulting in pneumonia [174, 193]. However, increased adherence to the trachea after an influenza infection may not be the only factor in increasing susceptibility to a *S. pneumoniae* infection. Changes to the trachea induced by influenza virus may increase susceptibility to a secondary *S. pneumoniae* infection by decreasing the clearance of *S. pneumoniae* via the mucociliary escalator of the trachea since many of the ciliated cells are destroyed [124, 174]. This impairment of clearance and enhanced progression of *S. pneumoniae* due to an influenza infection would allow more *S. pneumoniae* to reach the lungs [174]. In other studies examining susceptibility of mice to *S. pneumoniae* following an influenza infection in the lungs, such an increase in susceptibility to *S. pneumoniae* was seen when influenza-induced tissue damage of the tracheal respiratory epithelium is greatest [124, 176, 177, 187, 278].

Influenza virus infection causes the spread of a *S. pneumoniae* infection from the upper respiratory tract to the lungs via the trachea [193] and we found that a prior *in vivo* influenza infection of both 3 and 6 days increased *S. pneumoniae* adherence to influenza-infected tracheas compared to uninfected tracheas. Thus, we wanted to determine whether increased pneumococcal adherence was due to influenza-induced damage to the tracheal respiratory epithelium. To achieve this objective, we developed a novel murine tracheal explant method to study how an influenza infection directly affects the tracheal respiratory epithelium and subsequent *S. pneumoniae* adherence. A murine system was developed since most *in vivo* co-infection studies are done in mice [124, 176, 177]. With

a tracheal explant system, the effects of an influenza-infection on subsequent *S. pneumoniae* adherence due to epithelial tissue damage can be more readily determined without the contribution of influenza-mediated suppression of neutrophil function.

Our study is the first to examine how an *ex vivo* influenza infection affects *S. pneumoniae* adherence using murine tracheal explants. Plotkowski et al. used an *in vivo* infection model to show that after an influenza infection, *S. pneumoniae* adhered preferentially to areas where the epithelium was denuded and the basement membrane was exposed, indicating that the damage caused by influenza increased *S. pneumoniae* adherence [124]. However, previous studies using explant models to study *S. pneumoniae* adherence have looked only at adherence to normal epithelium [228, 279]. One of these studies measured *S. pneumoniae* adherence of 34 strains of *S. pneumoniae* representing 15 serotypes to murine tracheal explants and found the geometric mean of adherence was 8.93×10^4 CFU/ml, with D-galactose blocking adhesion by up to 95.5% [279]. Tong et al. found that *S. pneumoniae* adherence to chinchilla tracheal explants occurred within 30 minutes, was receptor-specific, and could be inhibited with lacto-N-neotetraose and asialoganglioside-GM1 [228]. Our results demonstrating a dose dependent effect of inoculum on pneumococcal adherence also supports the hypothesis that *S. pneumoniae* adherence to both uninfected and influenza-infected tracheal explants was receptor-specific.

The explant model allowed us to examine *S. pneumoniae* adherence to uninfected respiratory epithelium as well as influenza-infected respiratory epithelium during all stages of influenza infection, from initial changes in cellular receptors (0.5 days), to

epithelial damage and exposure of the basement membrane (1-2 days), to initial repair and regeneration of the epithelium (3-5 days). Specifically, we found that a prior influenza infection increased *S. pneumoniae* adherence to the tracheal epithelium only at the stage when influenza-infected cells were present and denudation of the epithelium had just begun. Adherence of *S. pneumoniae* was not increased significantly at other times after an influenza infection when the epithelium was nearly completely denuded (2 days), thereby exposing the basement membrane, or during the initial stages of repair of the epithelium when basal cells and undifferentiated epithelial cells were present (3-5 days). Previous studies of *in vivo* influenza infections have shown a similar time frame for damage and repair of the tracheal epithelium after an influenza infection [124, 187, 278]. Ramphal et al. showed evidence of desquamation as early as 24 hours and complete desquamation by 3 days after influenza infection, with repair starting at day 5 and completion by day 14 after influenza infection [187]. Azoulay-Dupuis et al. used a guinea pig model of influenza infection and found that tracheal epithelial damage occurred as early as 48 hours after infection and lasted up to 7 days, with repair taking 2 weeks [278].

Results from our *in vivo* infection demonstrated a significant increase in *S. pneumoniae* adherence to influenza-infected tracheas at both 3 and 6 days after influenza infection compared to uninfected tracheas (98.7-fold and 206.9–fold, respectively). In contrast, results from the tracheal explant model showed a significant increase (3.2-fold) in *S. pneumoniae* adherence only after one day of influenza infection when influenza-infected cells and the early stages of tissue damage are present. These differences in

adherence between our *in vivo* and our *ex vivo* models of co-infection may be due to the ability of the trachea to remove *S. pneumoniae* via the mucociliary escalator. In a normal, healthy trachea with an intact respiratory epithelium, *S. pneumoniae* is removed by ciliary beating and the resulting movement of mucus. However, during an *in vivo* influenza infection, ciliated epithelial cells are destroyed and this clearance process is disrupted, which may allow *S. pneumoniae* to adhere to the damaged epithelium more readily as seen with the studies done by Plotkowski et al. [124, 174]. Other studies have shown that *S. pneumoniae* adheres primarily to the mucus layer and not the respiratory epithelium [192, 280]. The mucus layer may have been removed from our system, thereby leading to ineffective mucociliary clearance of *S. pneumoniae* and similar *S. pneumoniae* adherence patterns for both uninfected and influenza-infected tracheal explants. The compromise of mucociliary clearance mechanisms may account for the increase in *S. pneumoniae* adherence to uninfected tracheas *ex vivo* compared to *S. pneumoniae* adherence to uninfected tracheas *in vivo*.

The tracheal explant model we developed has several advantages over other methods, such as cell culture or tracheal rings, for studying interactions between bacteria and host cells. One advantage is this model uses an intact tissue complete with a fully differentiated respiratory epithelium and basement membrane whereas cell culture methods rely on only one individual cell type, making it difficult to reproduce the actual respiratory tissue. Another advantage is our tracheal explant system allows for a controlled, synchronous infection and tissue damage throughout the trachea. In addition, the infection process is expedited compared to an *in vivo* infection since the inoculum is

concentrated only in the trachea rather than distributed through the entire respiratory tract. With an *in vivo* infection, the infection is difficult to synchronize since it spreads throughout the entire respiratory tract, resulting in different stages of infection throughout the respiratory tract [281].

A third advantage of our tracheal explant model is that only specific adhesion to the tracheal epithelium is measured since there is no cutting involved and it is a closed system. This method eliminates non-specific adherence to cut areas and the trachea adventitia, which can occur with the tracheal ring model. By using a closed system, we can also control the amount of medium, influenza virus, and/or bacteria that each trachea receives and flow can be adjusted to simulate shear forces found *in vivo*. In addition, non-adherent influenza virus particles and *S. pneumoniae* are efficiently removed from the system by resuming the flow of medium. Finally, since influenza virus has also been shown to affect neutrophil function [195, 196, 199, 203, 207], another advantage of the explant model is that systemic and confounding effects of influenza infection that are present *in vivo*, such as changes in neutrophil function, are eliminated allowing for the direct examination of influenza's effects on bacterial adherence due specifically to tissue damage.

In summary, we found that an *in vivo* influenza infection of 3 or 6 days increased adherence of *S. pneumoniae* to the tracheal epithelium indicating that influenza-induced changes in the respiratory epithelium increase *S. pneumoniae* adherence. To further investigate this result, we developed a novel murine tracheal explant system that can be used to study a variety of cellular processes, such as viral and/or bacterial infections and

mechanisms of epithelial repair. We used this model to study the effects of influenza virus on the respiratory epithelium of the trachea and subsequent *S. pneumoniae* adherence. We showed that tracheal explants remained viable for at least 8 days, influenza infections can be maintained for at least 5 days, and *S. pneumoniae* adherence increased at 1 day after an *ex vivo* influenza infection when influenza-infected cells are present and damage to the respiratory epithelium is evident. However, *S. pneumoniae* adherence did not increase compared to uninfected tracheas after 2 days of an *ex vivo* influenza infection when epithelial damage was greatest and the basement membrane was maximally exposed. In addition, no differences in *S. pneumoniae* adherence between influenza-infected and uninfected tracheas were seen during the initial stages of repair seen after 3 days of an *ex vivo* influenza infection, when basal cells were migrating to cover the exposed basement membrane and undifferentiated epithelial cells were present. At 4 and 5 days after influenza infection, the epithelium was covered by undifferentiated and differentiating epithelial cells which did not increase *S. pneumoniae* adherence compared to uninfected tracheas.

Together, our *in vivo* and *ex vivo* data indicate that influenza-induced tissue damage to the tracheal epithelium results in increased *S. pneumoniae* adherence (which may be due to ineffective mucociliary clearance of *S. pneumoniae*) and represents one mechanism of increased susceptibility to a secondary *S. pneumoniae* infection after an influenza infection. At the initial stage of *S. pneumoniae* infection we examined (2 hours), any increases in the number of *S. pneumoniae* associated with the trachea would be due to either increased *S. pneumoniae* adherence and/or decreased mucociliary

clearance of *S. pneumoniae*. Our *ex vivo* results indicate that influenza-induced exposure of the basement membrane contributes minimally to increased *S. pneumoniae* adherence. The increased numbers of *S. pneumoniae* found *in vivo* in influenza-infected tracheas compared to uninfected tracheas may be due to reduced mucociliary clearance of *S. pneumoniae*, resulting in increased numbers of *S. pneumoniae* remaining in the trachea and therefore increased adherence to the damaged epithelium. This tracheal explant model can be further used to identify cellular receptors and bacterial adhesins that increase adherence after an influenza infection, which will allow us to develop therapeutic inhibitors to prevent the spread of *S. pneumoniae* to the lungs via the trachea, and thereby reduce the mortality associated with influenza infections due to secondary *S. pneumoniae* infections.

SUMMARY AND CONCLUSIONS

Susceptibility to a *S. pneumoniae* infection has been shown to be increased after a primary influenza infection. There are two primary hypotheses as to how an influenza infection results in this increased susceptibility to a secondary bacterial infection. One hypothesis is that influenza-induced damage to the respiratory epithelium exposes new receptors to which the *S. pneumoniae* adhere. The other hypothesis is that influenza-induced alterations in neutrophil function result in decreased killing, and therefore, increased growth of *S. pneumoniae*. The purpose of the present studies was to determine the role that each of these mechanisms play in increasing susceptibility to *S. pneumoniae* infection.

The goal of the studies described in chapter two was to elucidate how influenza-induced changes in neutrophil-dependent and –independent mechanisms affect susceptibility to a *S. pneumoniae* infection. We found that susceptibility to *S. pneumoniae* was greatest at 6 days after influenza infection and there was no increase in susceptibility after 3 days of influenza infection compared to mice infected with *S. pneumoniae* only. We depleted mice of neutrophils and showed that neutrophils were functioning in the resistance to *S. pneumoniae* infection in mice infected with *S. pneumoniae* only or influenza for 3 days prior to *S. pneumoniae* infection, but not after 6 days of influenza. To determine whether influenza affected neutrophil function locally and/or systemically, we measured *S. pneumoniae* association with neutrophils and reactive oxygen species (ROS) production by neutrophils from the lungs and bone marrow of uninfected and influenza-infected mice. We found that neutrophil function

was suppressed following influenza infection in the lungs and, to a lesser extent, in the bone marrow. In addition, neutrophil-depleted mice infected with influenza for 3 or 6 days were more susceptible to *S. pneumoniae* infection than depleted mice infected with *S. pneumoniae* only, indicating there is a neutrophil-independent mechanism of increased susceptibility. We measured pulmonary cytokine levels and found that mice infected with influenza for 6 days followed by *S. pneumoniae* had elevated levels of TNF-α, IFN-γ, MCP-1, IL-10, and IL-6. In addition, depleted mice infected with either *S. pneumoniae* only or influenza for 3 days followed by *S. pneumoniae* had elevated lung cytokine levels. Together, these data indicate that both neutrophil dysfunction and elevated production of both pro-inflammatory and anti-inflammatory cytokines contribute to the increased susceptibility to *S. pneumoniae* infection seen after a primary influenza infection.

The goal of the studies described in chapter three was to determine whether influenza-induced damage to the tracheal respiratory epithelium increased adherence of *S. pneumoniae*. Using an *in vivo* infection model, we found that a prior influenza infection increased the number of *S. pneumoniae* associated with the tracheas after a 2 hour incubation period. However, it was not possible to determine whether this increase was due to either increased adherence or decreased mucociliary clearance, resulting in increased numbers of *S. pneumoniae*. In order to further examine how adherence was affected after an influenza infection, we developed a novel *ex vivo* tracheal explant system which kept tracheas intact and viable for up to 8 days in *ex vivo* culture. Influenza infections in the explants were established as early as 12 hours after inoculation and were

maintained over a period of 5 days with a peak viral titer at 3 days after infection. Influenza-induced tissue damage and subsequent repair as well as influenza-infected tissue were visualized using immunofluorescence and immunohistochemistry. We found that damage to the epithelium occurred as early as 1 day after infection, with denudation of the epithelium leading to the exposure of the basement membrane by 2 days after influenza infection. At 3 days after influenza infection, the initial stages of repair were evident in that basal cells were migrating to cover the exposed basement membrane. The epithelium was completely covered by undifferentiated cells at 4 and 5 days after influenza virus infection. We infected these influenza-infected tracheal explants with *S. pneumoniae* for one hour to examine how different stages of influenza infection from initial damage to repair affect subsequent adherence. We found the only increase in *S. pneumoniae* adherence after a prior influenza infection occurred at 1 day after an influenza infection, which was when influenza-infected cells were initially present and tissue damage had just begun. However, at the other times we examined, we did not see any increases in adherence. These data indicate that the increases in *S. pneumoniae* association within the tracheas seen after an *in vivo* infection may be due to decreased mucociliary clearance rather than increased adherence due to exposure of the basement membrane.

Together, the results from the present studies indicate that multiple mechanisms account for the increased susceptibility to a secondary *S. pneumoniae* infection due to a primary influenza virus infection. Our studies indicate that susceptibility to a *S. pneumoniae* infection was greatest at 6 days after an influenza virus infection. However,

at 3 days after an influenza infection, when viral titers are at the greatest levels, no differences in susceptibility were seen compared to mice infected with bacteria only. We found that neutrophils play an important role in controlling the *S. pneumoniae* infection, and the influenza virus infection alters ROS production and phagocytosis of *S. pneumoniae*. Both of these processes are critical in controlling a *S. pneumoniae* infection. In addition, we found that a prior influenza infection of 6 days altered the expression of cytokines within the lungs, which may also contribute to the increased susceptibility. Our tracheal explant system showed that a prior influenza infection significantly increases subsequent *S. pneumoniae* adherence only after 1 day of an *ex vivo* influenza infection, indicating that increased susceptibility to *S. pneumoniae* seen *in vivo* at 6 days after influenza infection may be due to other mechanisms such as decreased mucociliary clearance. By understanding more about how the influenza virus alters the host immune response and the respiratory epithelium, we can gain insight into how to better treat an influenza infection to prevent a subsequent secondary bacterial infection.

FUTURE STUDIES

Our neutrophil assays indicated that ROS production and bacterial phagocytosis were reduced after an influenza infection. Another important function of neutrophils which we did not examine is bactericidal activity. Future studies should determine whether neutrophils infected with influenza virus *in vivo* have a decreased ability to kill *S. pneumoniae* after phagocytosis.

Since we saw differences in cytokine levels in mice that were infected with influenza virus for 6 days followed by *S. pneumoniae*, further examination is needed to determine which of these cytokines play a critical role in increasing susceptibility to secondary bacterial infections. IL-10 has been shown to be important in increasing susceptibility at 14 days after an influenza infection [214]. Mice lacking IL-10 could be used with our co-infection model to determine if it also plays a significant role in increasing susceptibility to bacterial infections at earlier timepoints after an influenza infection. In addition, other cytokines such as TNF-α and IL-6, which were elevated in the 6 day co-infected mice, may contribute to the increased susceptibility.

For our tracheal explant system, we measured adherence of one of the 90 serotypes of *S. pneumoniae* to uninfected and influenza-infected tracheal explants. It would be interesting to examine if different serotypes adhere more readily to influenza-infected explants. In addition, it would be useful to also measure adherence of clinical samples of *S. pneumoniae* since they may have properties which make them more likely to adhere to the tracheal epithelium. Another possibility for examining differences in *S. pneumoniae* adherence to uninfected and influenza-infected tracheal explants is to use

mutants of *S. pneumoniae* which lack surface proteins, such as CbpA, which are important in adherence to cytokine-activated type II pneumocytes in the lungs [148]. With our tracheal explant system, it would also be interesting to look at later timepoints after the influenza infection to determine whether the epithelial cells can fully differentiate into ciliated-epithelial cells to complete the repair process and whether these later timepoints affect adherence of *S. pneumoniae*.

In conclusion, these studies would contribute to the current knowledge about this co-infection model and how both influenza-induced neutrophil dysfunction as well as influenza-induced damage to the respiratory epithelium increase susceptibility to *S. pneumoniae* infection. As the likelihood of another influenza pandemic increases and the number of elderly people continues to increase, it is critical to better understand both of these diseases and their interactions in order to prevent the increased mortality rate seen with secondary bacterial pneumonias due to primary influenza infections.

REFERENCES

1. Lamb, R.A. and R.M. Krug, *Orthomyxoviridae: The Viruses and Their Replication*, in *Fundamental Virology*, D.M. Knipe and P.M. Howley, Editors. 2001, Lippincott Williams & Wilkins: Philadelphia. p. 725-769.

2. Julkunen, I., et al., *Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression.* Cytokine Growth Factor Rev, 2001. **12**(2-3): p. 171-80.

3. Fouchier, R.A., et al., *Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls.* J Virol, 2005. **79**(5): p. 2814-22.

4. Yuen, K.Y. and S.S. Wong, *Human infection by avian influenza A H5N1.* Hong Kong Med J, 2005. **11**(3): p. 189-99.

5. Cox, N.J. and K. Subbarao, *Influenza.* Lancet, 1999. **354**(9186): p. 1277-82.

6. Yasuda, J., et al., *Molecular assembly of influenza virus: association of the NS2 protein with virion matrix.* Virology, 1993. **196**(1): p. 249-55.

7. Richardson, J.C. and R.K. Akkina, *NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells.* Arch Virol, 1991. **116**(1-4): p. 69-80.

8. Ligon, B.L., *Avian influenza virus H5N1: a review of its history and information regarding its potential to cause the next pandemic.* Semin Pediatr Infect Dis, 2005. **16**(4): p. 326-35.

9. Murphy, B.R. and R.G. Webster, *Orthomyxoviruses*, in *Fields virology*, B.N. Fields, D.M. Knipe, and P.M. Howley, Editors. 1996, Lippincott-Raven Publishers: Philadelphia. p. 1397-1445.

10. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiol Rev, 1992. **56**(1): p. 152-79.

11. Lamb, R.A., *Genes and proteins of the influenza viruses*, in *The influenza viruses*, R.M. Krug, Editor. 1989, Plenum: New York. p. 1-87.

12. Lamb, R.A., C.J. Lai, and P.W. Choppin, *Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins.* Proc Natl Acad Sci U S A, 1981. **78**(7): p. 4170-4.

13. Lamb, R.A., S.L. Zebedee, and C.D. Richardson, *Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface.* Cell, 1985. **40**(3): p. 627-33.

14. Zebedee, S.L. and R.A. Lamb, *Growth restriction of influenza A virus by M2 protein antibody is genetically linked to the M1 protein.* Proc Natl Acad Sci U S A, 1989. **86**(3): p. 1061-5.

15. Zebedee, S.L. and R.A. Lamb, *Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions.* J Virol, 1988. **62**(8): p. 2762-72.

16. Yewdell, J.W. and C.J. Hackett, *Specificity and funciton of T lymphocytes induced by influenza A viruses*, in *The influenza viruses*, R.M. Krug, Editor. 1989, Plenum: New York. p. 361-429.

17. Horisberger, M.A., *The large P proteins of influenza A viruses are composed of one acidic and two basic polypeptides.* Virology, 1980. **107**(1): p. 302-5.

18. Compans, R.W., *Influenza virus proteins. II. Association with components of the cytoplasm.* Virology, 1973. **51**(1): p. 56-70.

19. Krug, R.M. and P.R. Etkind, *Cytoplasmic and nuclear virus-specific proteins in influenza virus-infected MDCK cells.* Virology, 1973. **56**(1): p. 334-48.

20. Krug, R.M. and R. Soeiro, *Studies on the intranuclear localization of influenza virus-specific proteins.* Virology, 1975. **64**(2): p. 378-87.

21. Lazarowitz, S.G., R.W. Compans, and P.W. Choppin, *Influenza virus structural and nonstructural proteins in infected cells and their plasma membranes.* Virology, 1971. **46**(3): p. 830-43.

22. Skehel, J.J., *Polypeptide synthesis in influenza virus-infected cells.* Virology, 1972. **49**(1): p. 23-36.

23. Greenspan, D., et al., *Expression of influenza virus NS2 nonstructural protein in bacteria and localization of NS2 in infected eucaryotic cells.* J Virol, 1985. **54**(3): p. 833-43.

24. Smith, G.L., et al., *Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses.* Virology, 1987. **160**(2): p. 336-45.

25. Gubareva, L.V., L. Kaiser, and F.G. Hayden, *Influenza virus neuraminidase inhibitors.* Lancet, 2000. **355**(9206): p. 827-35.

26. Ito, T., et al., *Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.* J Virol, 1998. **72**(9): p. 7367-73.

27. Gottschalk, A., *Neuraminidase: the specific enzyme of influenza virus and Vibrio cholerae.* Biochim Biophys Acta, 1957. **23**(3): p. 645-6.

28. Claas, E.C., *Pandemic influenza is a zoonosis, as it requires introduction of avian-like gene segments in the human population.* Vet Microbiol, 2000. **74**(1-2): p. 133-9.

29. Klimov, A., et al., *Surveillance and impact of influenza in the United States.* Vaccine, 1999. **17 Suppl 1**: p. S42-6.

30. Sprenger, M.J., et al., *Impact of influenza on mortality in relation to age and underlying disease, 1967-1989.* Int J Epidemiol, 1993. **22**(2): p. 334-40.

31. Julkunen, I., et al., *Inflammatory responses in influenza A virus infection.* Vaccine, 2000. **19 Suppl 1**: p. S32-7.

32. Barker, W.H. and J.P. Mullooly, *Pneumonia and influenza deaths during epidemics: implications for prevention.* Arch Intern Med, 1982. **142**(1): p. 85-9.

33. Ronni, T., et al., *Activation of IFN-alpha, IFN-gamma, MxA, and IFN regulatory factor 1 genes in influenza A virus-infected human peripheral blood mononuclear cells.* J Immunol, 1995. **154**(6): p. 2764-74.

34. Kuszewski, K. and L. Brydak, *The epidemiology and history of influenza.* Biomed Pharmacother, 2000. **54**(4): p. 188-95.

35. Simonsen, L., et al., *The impact of influenza epidemics on mortality: introducing a severity index.* Am J Public Health, 1997. **87**(12): p. 1944-50.

36. Stark, G.R., et al., *How cells respond to interferons.* Annu Rev Biochem, 1998. **67**: p. 227-64.

37. Lu, Y., et al., *Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor.* Virology, 1995. **214**(1): p. 222-8.

38. Lee, T.G., et al., *The 58,000-dalton cellular inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (PKR) is a member of the tetratricopeptide repeat family of proteins.* Mol Cell Biol, 1994. **14**(4): p. 2331-42.

39. Tan, S.L., M.J. Gale, Jr., and M.G. Katze, *Double-stranded RNA-independent dimerization of interferon-induced protein kinase PKR and inhibition of*

dimerization by the cellular P58IPK inhibitor. Mol Cell Biol, 1998. **18**(5): p. 2431-43.

40. Sprenger, H., et al., *Selective induction of monocyte and not neutrophil-attracting chemokines after influenza A virus infection.* J Exp Med, 1996. **184**(3): p. 1191-6.

41. Bussfeld, D., et al., *Differential mononuclear leukocyte attracting chemokine production after stimulation with active and inactivated influenza A virus.* Cell Immunol, 1998. **186**(1): p. 1-7.

42. Matikainen, S., et al., *Influenza A and sendai viruses induce differential chemokine gene expression and transcription factor activation in human macrophages.* Virology, 2000. **276**(1): p. 138-47.

43. Adachi, M., et al., *Expression of cytokines on human bronchial epithelial cells induced by influenza virus A.* Int Arch Allergy Immunol, 1997. **113**(1-3): p. 307-11.

44. Matsukura, S., et al., *Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A.* J Allergy Clin Immunol, 1996. **98**(6 Pt 1): p. 1080-7.

45. Ronni, T., et al., *Regulation of IFN-alpha/beta, MxA, 2',5'-oligoadenylate synthetase, and HLA gene expression in influenza A-infected human lung epithelial cells.* J Immunol, 1997. **158**(5): p. 2363-74.

46. Sareneva, T., et al., *Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells.* J Immunol, 1998. **160**(12): p. 6032-8.

47. Durbin, J.E., et al., *Type I IFN modulates innate and specific antiviral immunity.* J Immunol, 2000. **164**(8): p. 4220-8.

48. Biron, C.A., *Initial and innate responses to viral infections--pattern setting in immunity or disease.* Curr Opin Microbiol, 1999. **2**(4): p. 374-81.

49. Nain, M., et al., *Tumor necrosis factor-alpha production of influenza A virus-infected macrophages and potentiating effect of lipopolysaccharides.* J Immunol, 1990. **145**(6): p. 1921-8.

50. Gong, J.H., et al., *Influenza A virus infection of macrophages. Enhanced tumor necrosis factor-alpha (TNF-alpha) gene expression and lipopolysaccharide-triggered TNF-alpha release.* J Immunol, 1991. **147**(10): p. 3507-13.

51. Bender, A., et al., *Effect of granulocyte/macrophage colony-stimulating factor on human monocytes infected with influenza A virus. Enhancement of virus*

replication, cytokine release, and cytotoxicity. J Immunol, 1993. **151**(10): p. 5416-24.

52. Pirhonen, J., et al., *Virus infection activates IL-1 beta and IL-18 production in human macrophages by a caspase-1-dependent pathway.* J Immunol, 1999. **162**(12): p. 7322-9.

53. Choi, A.M., et al., *Oxidant stress responses in influenza virus pneumonia: gene expression and transcription factor activation.* Am J Physiol, 1996. **271**(3 Pt 1): p. L383-91.

54. Hofmann, P., et al., *Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response.* J Leukoc Biol, 1997. **61**(4): p. 408-14.

55. Pahl, H.L. and P.A. Baeuerle, *Expression of influenza virus hemagglutinin activates transcription factor NF-kappa B.* J Virol, 1995. **69**(3): p. 1480-4.

56. Flory, E., et al., *Influenza virus-induced NF-kappaB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IkappaB kinase.* J Biol Chem, 2000. **275**(12): p. 8307-14.

57. Simonsen, L., et al., *The impact of influenza on mortality in the USA*, in *Options for the control of influenza*, A.W. Hampston and R.G. Webster, Editors. 1996, Elsevier: New York. p. 26-33.

58. Kobasa, D., et al., *Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus.* Nature, 2004. **431**(7009): p. 703-7.

59. Matrosovich, M.N., et al., *Human and avian influenza viruses target different cell types in cultures of human airway epithelium.* Proc Natl Acad Sci U S A, 2004. **101**(13): p. 4620-4.

60. Katz, J.M., et al., *Antibody response in individuals infected with avian influenza A (H5N1) viruses and detection of anti-H5 antibody among household and social contacts.* J Infect Dis, 1999. **180**(6): p. 1763-70.

61. Buxton Bridges, C., et al., *Risk of influenza A (H5N1) infection among health care workers exposed to patients with influenza A (H5N1), Hong Kong.* J Infect Dis, 2000. **181**(1): p. 344-8.

62. Gomolin, I.H., et al., *Control of influenza outbreaks in the nursing home: guidelines for diagnosis and management.* J Am Geriatr Soc, 1995. **43**(1): p. 71-4.

63. Hament, J.M., et al., *Respiratory viral infection predisposing for bacterial disease: a concise review.* FEMS Immunol Med Microbiol, 1999. **26**(3-4): p. 189-95.

64. Anderson, R.N. and B.L. Smith, *Deaths: Leading causes for 2002.* National Vital Statistics Reports, 2005. **53**(17): p. 1-92.

65. Cundell, D., H.R. Masure, and E.I. Tuomanen, *The molecular basis of pneumococcal infection: a hypothesis.* Clin Infect Dis, 1995. **21 Suppl 3**: p. S204-11.

66. Talbot, U.M., A.W. Paton, and J.C. Paton, *Uptake of Streptococcus pneumoniae by respiratory epithelial cells.* Infect Immun, 1996. **64**(9): p. 3772-7.

67. Sternberg, G.M., *A fatal form of septicaemia in the rabbit, produced by the subcutaneous injection of human saliva.* National Board of Health Bulletin, 1881. **2**: p. 781-3.

68. Henrichsen, J., *Six newly recognized types of Streptococcus pneumoniae.* J Clin Microbiol, 1995. **33**(10): p. 2759-62.

69. van Dam, J.E., A. Fleer, and H. Snippe, *Immunogenicity and immunochemistry of Streptococcus pneumoniae capsular polysaccharides.* Antonie Van Leeuwenhoek, 1990. **58**(1): p. 1-47.

70. Casal, J. and D. Tarrago, *Immunity to Streptococcus pneumoniae: Factors affecting production and efficacy.* Curr Opin Infect Dis, 2003. **16**(3): p. 219-24.

71. Ghaffar, F., I.R. Friedland, and G.H. McCracken, Jr., *Dynamics of nasopharyngeal colonization by Streptococcus pneumoniae.* Pediatr Infect Dis J, 1999. **18**(7): p. 638-46.

72. Musher, D.M., *Streptococcus pneumoniae*, in *Mandell, Douglas, and Bennett's principles and practice of infectious diseases*, G.L. Mandell, Bennet, J. E., Dolin, R., Editor. 2000, Churchill Livingstone: Philadelphia, PA. p. 2128-44.

73. Peter, G., Klein, J. O., *Streptococcus pneumoniae*, in *Principles and practice of pediatric infectious diseases*, S.S. Long, Pickering, L. K., Prober, C. G., Editor. 2002, Churchill Livingstone: Philadelphia, PA. p. 739-46.

74. Kalin, M., *Pneumococcal serotypes and their clinical relevance.* Thorax, 1998. **53**(3): p. 159-62.

75. Chantler, T., et al., *Parental views on the introduction of an infant pneumococcal vaccine.* Community Pract, 2006. **79**(7): p. 213-6.

76. Jedrzejas, M.J., *Pneumococcal virulence factors: structure and function.* Microbiol Mol Biol Rev, 2001. **65**(2): p. 187-207 ; first page, table of contents.

77. Henderson, B. and M. Wilson, *Cytokine induction by bacteria: beyond lipopolysaccharide.* Cytokine, 1996. **8**(4): p. 269-82.

78. McCullers, J.A. and E.I. Tuomanen, *Molecular pathogenesis of pneumococcal pneumonia.* Front Biosci, 2001. **6**: p. D877-89.

79. Mitchell, T.J., et al., *Molecular analysis of virulence factors of Streptococcus pneumoniae.* Soc Appl Bacteriol Symp Ser, 1997. **26**: p. 62S-71S.

80. Bridy-Pappas, A.E., et al., *Streptococcus pneumoniae: description of the pathogen, disease epidemiology, treatment, and prevention.* Pharmacotherapy, 2005. **25**(9): p. 1193-212.

81. Austrian, R., *The pneumococcus at the millennium: not down, not out.* J Infect Dis, 1999. **179 Suppl 2**: p. S338-41.

82. Austrian, R., *Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention.* Rev Infect Dis, 1981. **3 Suppl**: p. S1-17.

83. Klein, D.L., *Pneumococcal disease and the role of conjugate vaccines.* Microb Drug Resist, 1999. **5**(2): p. 147-57.

84. Austrian, R., *Some aspects of the pneumococcal carrier state.* J Antimicrob Chemother, 1986. **18 Suppl A**: p. 35-45.

85. Gray, B.M. and H.C. Dillon, Jr., *Clinical and epidemiologic studies of pneumococcal infection in children.* Pediatr Infect Dis, 1986. **5**(2): p. 201-7.

86. Rake, G., *Pathology of pneumococcus infection in mice following intranasal instillation.* J Exp Med, 1936. **63**: p. 17-31.

87. Huxley, E.J., et al., *Pharyngeal aspiration in normal adults and patients with depressed consciousness.* Am J Med, 1978. **64**(4): p. 564-8.

88. Mason, C.M. and S. Nelson, *Normal host defenses and impairments associated with the delayed resolution of pneumonia.* Semin Respir Infect, 1992. **7**(4): p. 243-55.

89. Johnston, R.B., Jr., *Pathogenesis of pneumococcal pneumonia.* Rev Infect Dis, 1991. **13 Suppl 6**: p. S509-17.

90. Spencer, H., *The bacterial pneumonias*, in *Pathology of the lung*. 1977, Pergamon Press: Oxford.

91. Austrian, R., *Pneumococcal infections*, in *Bacterial Vaccines*, R. Germanier, Editor. 1984, Academic Press: London. p. 257-288.

92. Cundell, D.R., et al., *Relationship between colonial morphology and adherence of Streptococcus pneumoniae.* Infect Immun, 1995. **63**(3): p. 757-61.

93. Heffron, R., *Pneumonia: with special reference to pneumococcus lobar pneumonia*. 1939, Cambridge MA: The Commonwealth Fund.

94. Marrie, T.J., *Pneumococcal pneumonia: epidemiology and clinical features.* Semin Respir Infect, 1999. **14**(3): p. 227-36.

95. Ortqvist, A., et al., *Diagnostic and prognostic value of interleukin-6 and C-reactive protein in community-acquired pneumonia.* Scand J Infect Dis, 1995. **27**(5): p. 457-62.

96. Torres, J.M., et al., *Streptococcus pneumoniae bacteremia in a community hospital.* Chest, 1998. **113**(2): p. 387-90.

97. Musher, D.M., *Infections caused by Streptococcus pneumoniae: clinical spectrum, pathogenesis, immunity, and treatment.* Clin Infect Dis, 1992. **14**(4): p. 801-7.

98. Ortqvist, A., et al., *Bacteremic pneumococcal pneumonia in Sweden: clinical course and outcome and comparison with non-bacteremic pneumococcal and mycoplasmal pneumonias.* Scand J Infect Dis, 1988. **20**(2): p. 163-71.

99. Ort, S., et al., *Pneumococcal pneumonia in hospitalized patients. Clinical and radiological presentations.* Jama, 1983. **249**(2): p. 214-8.

100. Ortqvist, A., J. Hedlund, and M. Kalin, *Streptococcus pneumoniae: epidemiology, risk factors, and clinical features.* Semin Respir Crit Care Med, 2005. **26**(6): p. 563-74.

101. Watson, D.A., et al., *A brief history of the pneumococcus in biomedical research: a panoply of scientific discovery.* Clin Infect Dis, 1993. **17**(5): p. 913-24.

102. Spika, J.S., et al., *Antimicrobial resistance of Streptococcus pneumoniae in the United States, 1979-1987. The Pneumococcal Surveillance Working Group.* J Infect Dis, 1991. **163**(6): p. 1273-8.

103. Ward, J., *Antibiotic-resistant Streptococcus pneumoniae: clinical and epidemiologic aspects.* Rev Infect Dis, 1981. **3**(2): p. 254-66.

104. Thornsberry, C., et al., *Resistance surveillance of Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis isolated in the United States, 1997-1998.* J Antimicrob Chemother, 1999. **44**(6): p. 749-59.

105. Doern, G.V., et al., *Antimicrobial resistance with Streptococcus pneumoniae in the United States, 1997 98.* Emerg Infect Dis, 1999. **5**(6): p. 757-65.

106. Thornsberry, C., et al., *Surveillance of antimicrobial resistance in Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis in the United States in 1996-1997 respiratory season. The Laboratory Investigator Group.* Diagn Microbiol Infect Dis, 1997. **29**(4): p. 249-57.

107. Tuomanen, E., et al., *Microbiological and clinical significance of a new property of defective lysis in clinical strains of pneumococci.* J Infect Dis, 1988. **158**(1): p. 36-43.

108. Tomasz, A., A. Albino, and E. Zanati, *Multiple antibiotic resistance in a bacterium with suppressed autolytic system.* Nature, 1970. **227**(5254): p. 138-40.

109. Liu, H.H. and A. Tomasz, *Penicillin tolerance in multiply drug-resistant natural isolates of Streptococcus pneumoniae.* J Infect Dis, 1985. **152**(2): p. 365-72.

110. Novak, R., et al., *Emergence of vancomycin tolerance in Streptococcus pneumoniae.* Nature, 1999. **399**(6736): p. 590-3.

111. Henriques Normark, B., et al., *Clinical isolates of Streptococcus pneumoniae that exhibit tolerance of vancomycin.* Clin Infect Dis, 2001. **32**(4): p. 552-8.

112. Lee, C.J., S.D. Banks, and J.P. Li, *Virulence, immunity, and vaccine related to Streptococcus pneumoniae.* Crit Rev Microbiol, 1991. **18**(2): p. 89-114.

113. White, R.T., *Pneumococcal vaccine.* Thorax, 1988. **43**(5): p. 345-8.

114. Tuomanen, E., *Molecular and cellular biology of pneumococcal infection.* Curr Opin Microbiol, 1999. **2**(1): p. 35-9.

115. Lock, R.A., J.C. Paton, and D. Hansman, *Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against Streptococcus pneumoniae.* Microb Pathog, 1988. **5**(6): p. 461-7.

116. McDaniel, L.S., et al., *PspA, a surface protein of Streptococcus pneumoniae, is capable of eliciting protection against pneumococci of more than one capsular type.* Infect Immun, 1991. **59**(1): p. 222-8.

117. Sampson, J.S., et al., *Limited diversity of Streptococcus pneumoniae psaA among pneumococcal vaccine serotypes.* Infect Immun, 1997. **65**(5): p. 1967-71.

118. Briles, D.E., et al., *PspA and PspC: their potential for use as pneumococcal vaccines.* Microb Drug Resist, 1997. **3**(4): p. 401-8.

119. Overweg, K., et al., *The putative proteinase maturation protein A of Streptococcus pneumoniae is a conserved surface protein with potential to elicit protective immune responses.* Infect Immun, 2000. **68**(7): p. 4180-8.

120. Krivan, H.C., D.D. Roberts, and V. Ginsburg, *Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids.* Proc Natl Acad Sci U S A, 1988. **85**(16): p. 6157-61.

121. Cundell, D.R., et al., *Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor.* Nature, 1995. **377**(6548): p. 435-8.

122. Cundell, D.R. and E.I. Tuomanen, *Receptor specificity of adherence of Streptococcus pneumoniae to human type-II pneumocytes and vascular endothelial cells in vitro.* Microb Pathog, 1994. **17**(6): p. 361-74.

123. van der Flier, M., et al., *Adherence of Streptococcus pneumoniae to immobilized fibronectin.* Infect Immun, 1995. **63**(11): p. 4317-22.

124. Plotkowski, M.C., et al., *Adherence of type I Streptococcus pneumoniae to tracheal epithelium of mice infected with influenza A/PR8 virus.* Am Rev Respir Dis, 1986. **134**(5): p. 1040-4.

125. Rayner, C.F., et al., *Interaction of pneumolysin-sufficient and -deficient isogenic variants of Streptococcus pneumoniae with human respiratory mucosa.* Infect Immun, 1995. **63**(2): p. 442-7.

126. Avery, O.T., Heidelberger, M., *Immunological relationships of cell constituents of pneumococcus.* J Exp Med, 1925. **42**: p. 367-376.

127. Avery, O.T., Morgan, H. J., *Immunological reactions of the isolated carbohydrate and protein of pneumococcus.* J Exp Med, 1925. **42**: p. 347-353.

128. Magee, A.D. and J. Yother, *Requirement for capsule in colonization by Streptococcus pneumoniae.* Infect Immun, 2001. **69**(6): p. 3755-61.

129. Tuomanen, E., R. Rich, and O. Zak, *Induction of pulmonary inflammation by components of the pneumococcal cell surface.* Am Rev Respir Dis, 1987. **135**(4): p. 869-74.

130. Tuomanen, E., et al., *The relative role of bacterial cell wall and capsule in the induction of inflammation in pneumococcal meningitis.* J Infect Dis, 1985. **151**(3): p. 535-40.

131. Austrian, R., *Morphologic variation in pneumococcus. I. An analysis of the bases for morphologic variation in pneumococcus and description of a hitherto undefined morphologic variant.* J Exp Med, 1953. **98**(1): p. 21-34.

132. Weiser, J.N., *Phase variation in colony opacity by Streptococcus pneumoniae.* Microb Drug Resist, 1998. **4**(2): p. 129-35.

133. Weiser, J.N., et al., *Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization.* Infect Immun, 1994. **62**(6): p. 2582-9.

134. Kim, J.O. and J.N. Weiser, *Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of Streptococcus pneumoniae.* J Infect Dis, 1998. **177**(2): p. 368-77.

135. Tuomanen, E., et al., *The induction of meningeal inflammation by components of the pneumococcal cell wall.* J Infect Dis, 1985. **151**(5): p. 859-68.

136. Tomasz, A. and K. Saukkonen, *The nature of cell wall-derived inflammatory components of pneumococci.* Pediatr Infect Dis J, 1989. **8**(12): p. 902-3.

137. Hummell, D.S., et al., *Activation of the alternative complement pathway by pneumococcal lipoteichoic acid.* Infect Immun, 1985. **47**(2): p. 384-7.

138. Winkelstein, J.A. and A. Tomasz, *Activation of the alternative complement pathway by pneumococcal cell wall teichoic acid.* J Immunol, 1978. **120**(1): p. 174-8.

139. Gosink, K., Tuomanen, E., *Streptococcus pneumoniae: invasion and inflammation*, in *Gram-positive pathogens*, V.A. Fischetti, Novick, R. P., Ferretti, J. J., Portnoy, D. A., Rood, J. I., Editor. 2000, ASM Press: Washington, D. C.

140. Hoskins, J., et al., *Genome of the bacterium Streptococcus pneumoniae strain R6.* J Bacteriol, 2001. **183**(19): p. 5709-17.

141. Wren, B.W., *A family of clostridial and streptococcal ligand-binding proteins with conserved C-terminal repeat sequences.* Mol Microbiol, 1991. **5**(4): p. 797-803.

142. Rosenow, C., et al., *Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae.* Mol Microbiol, 1997. **25**(5): p. 819-29.

143. Crain, M.J., et al., *Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of Streptococcus pneumoniae.* Infect Immun, 1990. **58**(10): p. 3293-9.

144. Hammerschmidt, S., et al., *Identification of pneumococcal surface protein A as a lactoferrin-binding protein of Streptococcus pneumoniae.* Infect Immun, 1999. **67**(4): p. 1683-7.

145. Tu, A.H., et al., *Pneumococcal surface protein A inhibits complement activation by Streptococcus pneumoniae.* Infect Immun, 1999. **67**(9): p. 4720-4.

146. Briles, D.E., et al., *Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens.* Infect Immun, 1989. **57**(5): p. 1457-64.

147. Briles, D.E., J. Yother, and L.S. McDaniel, *Role of pneumococcal surface protein A in the virulence of Streptococcus pneumoniae.* Rev Infect Dis, 1988. **10 Suppl 2**: p. S372-4.

148. Mitchell, T.J., *Virulence factors and the pathogenesis of disease caused by Streptococcus pneumoniae.* Res Microbiol, 2000. **151**(6): p. 413-9.

149. Hammerschmidt, S., et al., *SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component.* Mol Microbiol, 1997. **25**(6): p. 1113-24.

150. Zhang, J.R., et al., *The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells.* Cell, 2000. **102**(6): p. 827-37.

151. Johnson, M.K., *Cellular location of pneumolysin.* FEMS Microbiol Lett, 1977. **2**: p. 243-245.

152. Canvin, J.R., et al., *The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus.* J Infect Dis, 1995. **172**(1): p. 119-23.

153. Lock, R.A., D. Hansman, and J.C. Paton, *Comparative efficacy of autolysin and pneumolysin as immunogens protecting mice against infection by Streptococcus pneumoniae.* Microb Pathog, 1992. **12**(2): p. 137-43.

154. Yesilkaya, H., et al., *Role of manganese-containing superoxide dismutase in oxidative stress and virulence of Streptococcus pneumoniae.* Infect Immun, 2000. **68**(5): p. 2819-26.

155. Dintilhac, A., et al., *Competence and virulence of Streptococcus pneumoniae: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases.* Mol Microbiol, 1997. **25**(4): p. 727-39.

156. Berry, A.M. and J.C. Paton, *Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of Streptococcus pneumoniae.* Infect Immun, 1996. **64**(12): p. 5255-62.

157. Talkington, D.F., et al., *Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA).* Microb Pathog, 1996. **21**(1): p. 17-22.

158. Scanlon, K.L., W.F. Diven, and R.H. Glew, *Purification and properties of Streptococcus pneumoniae neuraminidase.* Enzyme, 1989. **41**(3): p. 143-50.

159. Linder, T.E., et al., *Effect of intranasal inoculation of Streptococcus pneumoniae on the structure of the surface carbohydrates of the chinchilla eustachian tube and middle ear mucosa.* Microb Pathog, 1994. **16**(6): p. 435-41.

160. Andersson, B., et al., *Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells.* J Exp Med, 1983. **158**(2): p. 559-70.

161. Berry, A.M., R.A. Lock, and J.C. Paton, *Cloning and characterization of nanB, a second Streptococcus pneumoniae neuraminidase gene, and purification of the NanB enzyme from recombinant Escherichia coli.* J Bacteriol, 1996. **178**(16): p. 4854-60.

162. Camara, M., et al., *A neuraminidase from Streptococcus pneumoniae has the features of a surface protein.* Infect Immun, 1994. **62**(9): p. 3688-95.

163. Camara, M., et al., *Streptococcus pneumoniae produces at least two distinct enzymes with neuraminidase activity: cloning and expression of a second neuraminidase gene in Escherichia coli.* Infect Immun, 1991. **59**(8): p. 2856-8.

164. Kostyukova, N.N., et al., *A study of pathogenic factors of Streptococcus pneumoniae strains causing meningitis.* FEMS Immunol Med Microbiol, 1995. **10**(2): p. 133-7.

165. Kanclerski, K. and R. Mollby, *Production and purification of Streptococcus pneumoniae hemolysin (pneumolysin).* J Clin Microbiol, 1987. **25**(2): p. 222-5.

166. Mitchell, T.J., et al., *Comparison of pneumolysin genes and proteins from Streptococcus pneumoniae types 1 and 2.* Nucleic Acids Res, 1990. **18**(13): p. 4010.

167. Paton, J.C. and A. Ferrante, *Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin.* Infect Immun, 1983. **41**(3): p. 1212-6.

168. Houldsworth, S., P.W. Andrew, and T.J. Mitchell, *Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes.* Infect Immun, 1994. **62**(4): p. 1501-3.

169. Braun, J.S., et al., *Pneumolysin, a protein toxin of Streptococcus pneumoniae, induces nitric oxide production from macrophages.* Infect Immun, 1999. **67**(8): p. 3750-6.

170. Paton, J.C., B. Rowan-Kelly, and A. Ferrante, *Activation of human complement by the pneumococcal toxin pneumolysin.* Infect Immun, 1984. **43**(3): p. 1085-7.

171. Rubins, J.B., et al., *Pneumolysin activates phospholipase A in pulmonary artery endothelial cells.* Infect Immun, 1994. **62**(9): p. 3829-36.

172. Steinfort, C., et al., *Effect of Streptococcus pneumoniae on human respiratory epithelium in vitro.* Infect Immun, 1989. **57**(7): p. 2006-13.

173. Mitchell, T.J., et al., *Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein.* Mol Microbiol, 1991. **5**(8): p. 1883-8.

174. Nugent, K.M. and E.L. Pesanti, *Tracheal function during influenza infections.* Infect Immun, 1983. **42**(3): p. 1102-8.

175. Gerone, P.J., T.G. Ward, and W.A. Chappell, *Combined infections in mice with influenza virus and Diplococcus pneumoniae.* Am J Hyg, 1957. **66**(3): p. 331-41.

176. LeVine, A.M., V. Koeningsknecht, and J.M. Stark, *Decreased pulmonary clearance of S. pneumoniae following influenza A infection in mice.* J Virol Methods, 2001. **94**(1-2): p. 173-86.

177. McCullers, J.A. and J.E. Rehg, *Lethal synergism between influenza virus and Streptococcus pneumoniae: characterization of a mouse model and the role of platelet-activating factor receptor.* J Infect Dis, 2002. **186**(3): p. 341-50.

178. O'Brien, K.L., et al., *Severe pneumococcal pneumonia in previously healthy children: the role of preceding influenza infection.* Clin Infect Dis, 2000. **30**(5): p. 784-9.

179. Muir, R., Wilson, G. H., *Influenza and its complications.* Br Med J, 1919. **1**: p. 3-5.

180. Wilson, C.B., Steer, P., *Bacteriological and pathological observations on influenza as seen in France during 1918.* Br Med J, 1919: p. 634-5.

181. Louria, D.B., et al., *Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza.* J Clin Invest, 1959. **38**(1 Part 2): p. 213-65.

182. Sharrar, R.G., *National influenza experience in the USA, 1968-69.* Bull World Health Organ, 1969. **41**(3): p. 361-6.

183. Schwarzmann, S.W., et al., *Bacterial pneumonia during the Hong Kong influenza epidemic of 1968-1969.* Arch Intern Med, 1971. **127**(6): p. 1037-41.

184. Hakansson, A., et al., *Adenovirus infection enhances in vitro adherence of Streptococcus pneumoniae.* Infect Immun, 1994. **62**(7): p. 2707-14.

185. Selinger, D.S., W.P. Reed, and L.C. McLaren, *Model for studying bacterial adherence to epithelial cells infected with viruses.* Infect Immun, 1981. **32**(2): p. 941-4.

186. Avadhanula, V., et al., *Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner.* J Virol, 2006. **80**(4): p. 1629-36.

187. Ramphal, R., et al., *Murine influenzal tracheitis: a model for the study of influenza and tracheal epithelial repair.* Am Rev Respir Dis, 1979. **120**(6): p. 1313-24.

188. Sweet, C. and H. Smith, *Pathogenicity of influenza virus.* Microbiol Rev, 1980. **44**(2): p. 303-30.

189. McCullers, J.A. and K.C. Bartmess, *Role of neuraminidase in lethal synergism between influenza virus and Streptococcus pneumoniae.* J Infect Dis, 2003. **187**(6): p. 1000-9.

190. Peltola, V.T., K.G. Murti, and J.A. McCullers, *Influenza virus neuraminidase contributes to secondary bacterial pneumonia.* J Infect Dis, 2005. **192**(2): p. 249-57.

191. Peltola, V.T. and J.A. McCullers, *Respiratory viruses predisposing to bacterial infections: role of neuraminidase.* Pediatr Infect Dis J, 2004. **23**(1 Suppl): p. S87-97.

192. Plotkowski, M.C., G. Beck, and E. Puchelle, *A new model for studying bacterial adherence to the respiratory epithelium.* Braz J Med Biol Res, 1988. **21**(2): p. 285-8.

193. Takase, H., et al., *Facilitated expansion of pneumococcal colonization from the nose to the lower respiratory tract in mice preinfected with influenza virus.* Microbiol Immunol, 1999. **43**(9): p. 905-7.

194. Abramson, J.S. and J.G. Wheeler, *Virus-induced neutrophil dysfunction: role in the pathogenesis of bacterial infections.* Pediatr Infect Dis J, 1994. **13**(7): p. 643-52.

195. Abramson, J.S., G.S. Giebink, and P.G. Quie, *Influenza A virus-induced polymorphonuclear leukocyte dysfunction in the pathogenesis of experimental pneumococcal otitis media.* Infect Immun, 1982. **36**(1): p. 289-96.

196. Hartshorn, K.L., et al., *Neutrophil deactivation by influenza A virus. Role of hemagglutinin binding to specific sialic acid-bearing cellular proteins.* J Immunol, 1995. **154**(8): p. 3952-60.

197. Abramson, J.S., et al., *Influenza A virus-induced polymorphonuclear leukocyte dysfunction.* Infect Immun, 1982. **37**(2): p. 794-9.

198. Colamussi, M.L., et al., *Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria.* Blood, 1999. **93**(7): p. 2395-403.

199. Hinshaw, V.S., et al., *Apoptosis: a mechanism of cell killing by influenza A and B viruses.* J Virol, 1994. **68**(6): p. 3667-73.

200. Engelich, G., M. White, and K.L. Hartshorn, *Neutrophil survival is markedly reduced by incubation with influenza virus and Streptococcus pneumoniae: role of respiratory burst.* J Leukoc Biol, 2001. **69**(1): p. 50-6.

201. Martin, R.R., et al., *Effects of infection with influenza virus on the function of polymorphonuclear leukocytes.* J Infect Dis, 1981. **144**(3): p. 279-80.

202. Abramson, J.S., et al., *Characterization of the effect of influenza virus on polymorphonuclear leukocyte membrane responses.* Blood, 1984. **64**(1): p. 131-8.

203. Abramson, J.S., et al., *Polymorphonuclear leukocyte dysfunction during influenza virus infection in chinchillas.* J Infect Dis, 1981. **143**(6): p. 836-45.

204. Wheeler, J.G., et al., *Influenza A virus alters structural and biochemical functions of the neutrophil cytoskeleton.* J Leukoc Biol, 1990. **47**(4): p. 332-43.

205. Abramson, J.S., et al., *Inhibition of neutrophil lysosome-phagosome fusion associated with influenza virus infection in vitro. Role in depressed bactericidal activity.* J Clin Invest, 1982. **69**(6): p. 1393-7.

206. Caldwell, S.E., L.F. Cassidy, and J.S. Abramson, *Alterations in cell protein phosphorylation in human neutrophils exposed to influenza A virus. A possible mechanism for depressed cellular end-stage functions.* J Immunol, 1988. **140**(10): p. 3560-7.

207. Nickerson, C.L. and G.J. Jakab, *Pulmonary antibacterial defenses during mild and severe influenza virus infection.* Infect Immun, 1990. **58**(9): p. 2809-14.

208. Kleinerman, E.S., et al., *Effect of virus infection on the inflammatory response. Depression of macrophage accumulation in influenza-infected mice.* Am J Pathol, 1976. **85**(2): p. 373-82.

209. Kleinerman, E.S., R. Snyderman, and C.A. Daniels, *Depressed monocyte chemotaxis during acute influenza infection.* Lancet, 1975. **2**(7944): p. 1063-6.

210. Astry, C.L. and G.J. Jakab, *Influenza virus-induced immune complexes suppress alveolar macrophage phagocytosis.* J Virol, 1984. **50**(2): p. 287-92.

211. Warshauer, D., et al., *Effect of influenza viral infection on the ingestion and killing of bacteria by alveolar macrophages.* Am Rev Respir Dis, 1977. **115**(2): p. 269-77.

212. Knapp, S., et al., *Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia.* Am J Respir Crit Care Med, 2003. **167**(2): p. 171-9.

213. Hennet, T., et al., *A kinetic study of immune mediators in the lungs of mice infected with influenza A virus.* J Immunol, 1992. **149**(3): p. 932-9.

214. van der Sluijs, K.F., et al., *IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection.* J Immunol, 2004. **172**(12): p. 7603-9.

215. Seki, M., et al., *Immunokinetics in severe pneumonia due to influenza virus and bacteria coinfection in mice.* Eur Respir J, 2004. **24**(1): p. 143-9.

216. McCullers, J.A., *Effect of antiviral treatment on the outcome of secondary bacterial pneumonia after influenza.* J Infect Dis, 2004. **190**(3): p. 519-26.

217. Smith, C.B., et al., *Interactions between viruses and bacteria in patients with chronic bronchitis.* J Infect Dis, 1976. **134**(6): p. 552-61.

218. Kim, P.E., et al., *Association of invasive pneumococcal disease with season, atmospheric conditions, air pollution, and the isolation of respiratory viruses.* Clin Infect Dis, 1996. **22**(1): p. 100-6.

219. Simonsen, L., *The global impact of influenza on morbidity and mortality.* Vaccine, 1999. **17 Suppl 1**: p. S3-10.

220. Glezen, W.P., A. A. Payne, D. N. Snyder, T. D. Downs, *Mortality and influenza.* J Infect Dis, 1982. **146**: p. 313-321.

221. Simonsen, L., K. Fukuda, L. B. Schonberger, N. J. Cox, *The impact of influenza epidemics on hospitalizations.* J Infect Dis, 2000. **181**: p. 831-837.

222. Abramson, J.S. and H.R. Hudnor, *Effect of priming polymorphonuclear leukocytes with cytokines (granulocyte-macrophage colony-stimulating factor [GM-CSF] and G-CSF) on the host resistance to Streptococcus pneumoniae in chinchillas infected with influenza A virus.* Blood, 1994. **83**(7): p. 1929-34.

223. Abramson, J.S., et al., *Depression of monocyte and polymorphonuclear leukocyte oxidative metabolism and bactericidal capacity by influenza A virus.* Infect Immun, 1982. **35**(1): p. 350-5.

224. Abramson, J.S. and E.L. Mills, *Depression of neutrophil function induced by viruses and its role in secondary microbial infections.* Rev Infect Dis, 1988. **10**(2): p. 326-41.

225. Garvy, B.A. and A.G. Harmsen, *The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice.* Inflammation, 1996. **20**(5): p. 499-512.

226. Seki, M., et al., *Acute infection with influenza virus enhances susceptibility to fatal pneumonia following Streptococcus pneumoniae infection in mice with chronic pulmonary colonization with Pseudomonas aeruginosa.* Clin Exp Immunol, 2004. **137**(1): p. 35-40.

227. Cundell, D.R., et al., *PAf receptor anchors Streptococcus pneumoniae to activated human endothelial cells.* Adv Exp Med Biol, 1996. **416**: p. 89-94.

228. Tong, H.H., et al., *Effect of lacto-N-neotetraose, asialoganglioside-GM1 and neuraminidase on adherence of otitis media-associated serotypes of Streptococcus pneumoniae to chinchilla tracheal epithelium.* Microb Pathog, 1999. **26**(2): p. 111-9.

229. Tong, H.H., et al., *Comparison of alteration of cell surface carbohydrates of the chinchilla tubotympanum and colonial opacity phenotype of Streptococcus pneumoniae during experimental pneumococcal otitis media with or without an antecedent influenza A virus infection.* Infect Immun, 2002. **70**(8): p. 4292-301.

230. Hestdal, K., et al., *Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells.* J Immunol, 1991. **147**(1): p. 22-8.

231. Fleming, T.J., M.L. Fleming, and T.R. Malek, *Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family.* J Immunol, 1993. **151**(5): p. 2399-408.

232. Swain, S.D., et al., *Neither neutrophils nor reactive oxygen species contribute to tissue damage during Pneumocystis pneumonia in mice.* Infect Immun, 2004. **72**(10): p. 5722-32.

233. Herigstad, B., M. Hamilton, and J. Heersink, *How to optimize the drop plate method for enumerating bacteria.* J Microbiol Methods, 2001. **44**(2): p. 121-9.

234. Wiley, J.A., et al., *Antigen-specific CD8(+) T cells persist in the upper respiratory tract following influenza virus infection.* J Immunol, 2001. **167**(6): p. 3293-9.

235. Cotter, M.J., et al., *A novel method for isolation of neutrophils from murine blood using negative immunomagnetic separation.* Am J Pathol, 2001. **159**(2): p. 473-81.

236. Voyich, J.M. and F.R. DeLeo, *Host-pathogen interactions: leukocyte phagocytosis and associated sequelae.* Methods Cell Sci, 2002. **24**(1-3): p. 79-90.

237. Cate, T.R., *Impact of influenza and other community-acquired viruses.* Semin Respir Infect, 1998. **13**(1): p. 17-23.

238. Tumpey, T.M., et al., *Neutrophil-mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea.* J Virol, 1996. **70**(2): p. 898-904.

239. de Oca, R.M., et al., *Polymorphonuclear neutrophils are necessary for the recruitment of CD8(+) T cells in the liver in a pregnant mouse model of Chlamydophila abortus (Chlamydia psittaci serotype 1) infection.* Infect Immun, 2000. **68**(3): p. 1746-51.

240. Sayles, P.C. and L.L. Johnson, *Exacerbation of toxoplasmosis in neutrophil-depleted mice.* Nat Immun, 1996. **15**(5): p. 249-58.

241. Czuprynski, C.J., et al., *Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to Listeria monocytogenes infection.* J Immunol, 1994. **152**(4): p. 1836-46.

242. Czuprynski, C.J., C. Theisen, and J.F. Brown, *Treatment with the antigranulocyte monoclonal antibody RB6-8C5 impairs resistance of mice to gastrointestinal infection with Listeria monocytogenes.* Infect Immun, 1996. **64**(9): p. 3946-9.

243. Matsuzaki, J., et al., *Successful elimination of memory-type CD8+ T cell subsets by the administration of anti-Gr-1 monoclonal antibody in vivo.* Cell Immunol, 2003. **224**(2): p. 98-105.

244. Nakano, H., M. Yanagita, and M.D. Gunn, *CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells.* J Exp Med, 2001. **194**(8): p. 1171-8.

245. Colonna, M., G. Trinchieri, and Y.J. Liu, *Plasmacytoid dendritic cells in immunity.* Nat Immunol, 2004. **5**(12): p. 1219-26.

246. Johnston, R.B., Jr., *The host response to invasion by Streptococcus pneumoniae: protection and the pathogenesis to tissue damage.* Rev Infect Dis, 1981. **3**(2): p. 282-8.

247. Boxio, R., et al., *Mouse bone marrow contains large numbers of functionally competent neutrophils.* J Leukoc Biol, 2004. **75**(4): p. 604-11.

248. Zhang, W.J., et al., *Lethal synergism between influenza infection and staphylococcal enterotoxin B in mice.* J Immunol, 1996. **157**(11): p. 5049-60.

249. Hirano, T., et al., *Effects of influenza A virus on lectin-binding patterns in murine nasopharyngeal mucosa and on bacterial colonization.* Otolaryngol Head Neck Surg, 1999. **121**(5): p. 616-21.

250. Haslett, C., *Granulocyte apoptosis and its role in the resolution and control of lung inflammation.* Am J Respir Crit Care Med, 1999. **160**(5 Pt 2): p. S5-11.

251. Hers, J.F., N. Masurel, and J. Mulder, *Bacteriology and histopathology of the respiratory tract and lungs in fatal Asian influenza.* Lancet, 1958. **2**(7057): p. 1141-3.

252. Plotkowski, M.C., O. Bajolet-Laudinat, and E. Puchelle, *Cellular and molecular mechanisms of bacterial adhesion to respiratory mucosa.* Eur Respir J, 1993. **6**(6): p. 903-16.

253. Abdul-Wahab, O.M., G. Ross, and J.M. Bradbury, *Pathogenicity and cytadherence of Mycoplasma imitans in chicken and duck embryo tracheal organ cultures.* Infect Immun, 1996. **64**(2): p. 563-8.

254. Agee, C.C., J.A. Engelhardt, and M.G. Gabridge, *Antimycoplasmal activity of dimethylphenols in a tracheal explant culture system.* Antimicrob Agents Chemother, 1980. **18**(2): p. 243-8.

255. Bakaletz, L.O. and M.S. Rheins, *A whole-organ perfusion model of Bordetella pertussis adherence to mouse tracheal epithelium.* In Vitro Cell Dev Biol, 1985. **21**(6): p. 314-20.

256. Churg, A., B. Gilks, and J. Dai, *Induction of fibrogenic mediators by fine and ultrafine titanium dioxide in rat tracheal explants.* Am J Physiol, 1999. **277**(5 Pt 1): p. L975-82.

257. Cogliano, R.C. and P.A. Small, Jr., *Specific immunity to influenza virus in ferret organ cultures.* Br J Exp Pathol, 1978. **59**(1): p. 21-31.

258. Collier, A.M., L.P. Peterson, and J.B. Baseman, *Pathogenesis of infection with Bordetella pertussis in hamster tracheal organ culture.* J Infect Dis, 1977. **136 Suppl**: p. S196-203.

259. Dai, J., et al., *Mineral dusts directly induce epithelial and interstitial fibrogenic mediators and matrix components in the airway wall.* Am J Respir Crit Care Med, 1998. **158**(6): p. 1907-13.

260. Dai, J., et al., *Air pollution particles produce airway wall remodeling in rat tracheal explants.* Am J Respir Cell Mol Biol, 2003. **29**(3 Pt 1): p. 352-8.

261. DeBey, M.C. and R.F. Ross, *Ciliostasis and loss of cilia induced by Mycoplasma hyopneumoniae in porcine tracheal organ cultures.* Infect Immun, 1994. **62**(12): p. 5312-8.

262. Dugal, F., C. Girard, and M. Jacques, *Adherence of Bordetella bronchiseptica 276 to porcine trachea maintained in organ culture.* Appl Environ Microbiol, 1990. **56**(6): p. 1523-9.

263. Engelhardt, J.A. and M.G. Gabridge, *Effect of squamous metaplasia on infection of hamster trachea organ cultures with Mycoplasma pneumoniae.* Infect Immun, 1977. **15**(2): p. 647-55.

264. Funnell, S.G. and A. Robinson, *A novel adherence assay for Bordetella pertussis using tracheal organ cultures.* FEMS Microbiol Lett, 1993. **110**(2): p. 197-203.

265. Gabridge, M.G., *Respiratory tract organ cultures to assay attachment and pathogenicity of mycoplasmas.* Ann Microbiol (Paris), 1984. **135A**(1): p. 33-8.

266. Gabridge, M.G. and L.E. Hoglund, *Mycoplasma pneumoniae infection of intact guinea pig tracheas cultured in a unique matrix-embed/perfusion system.* In Vitro, 1981. **17**(10): p. 847-58.

267. Gabridge, M.G. and R.B. Polisky, *Quantitative reduction of 2,3,4-triphenyl tetrazolium chloride by hamster trachea organ cultures: effects of Mycoplasma pneumoniae cells and membranes.* Infect Immun, 1976. **13**(1): p. 84-91.

268. Hu, P.C., A.M. Collier, and J.B. Baseman, *Interaction of virulent Mycoplasma pneumoniae with hamster tracheal organ cultures.* Infect Immun, 1976. **14**(1): p. 217-24.

269. Lane, B.P. and S.L. Miller, *Preparation of large numbers of uniform tracheal organ cultures for long term studies. I. Effects of serum on establishment in culture.* In Vitro, 1976. **12**(2): p. 147-54.

270. Nedrud, J.G., A.M. Collier, and J.S. Pagano, *Cellular basis for susceptibility to mouse cytomegalovirus: evidence from tracheal organ culture.* J Gen Virol, 1979. **45**(3): p. 737-44.

271. Nedrud, J.G., J.S. Pagano, and A.M. Collier, *Long-term mouse cytomegalovirus infection of tracheal organ culture: relation to host cell replication.* J Gen Virol, 1982. **60**(Pt 2): p. 247-59.

272. Pavelka, M., *Organ culture of adult rat and mouse tracheal epithelium: I. Ultrastructure following various culture periods.* Cell Tissue Res, 1976. **165**(3): p. 371-82.

273. Pijoan, C., D.H. Roberts, and J.D. Harding, *The effect of porcine mycoplasmas on pig tracheal organ cultures.* J Appl Bacteriol, 1972. **35**(3): p. 361-5.

274. Tong, H.H., et al., *Effect of neuraminidase on receptor-mediated adherence of Streptococcus pneumoniae to chinchilla tracheal epithelium.* Acta Otolaryngol, 2002. **122**(4): p. 413-9.

275. Wang, R.D., et al., *Cigarette smoke produces airway wall remodeling in rat tracheal explants.* Am J Respir Crit Care Med, 2003. **168**(10): p. 1232-6.

276. Williams, P.P. and J.E. Gallagher, *Preparation and long-term cultivation of porcine tracheal and lung organ cultures by alternate exposure to gaseous and liquid medium phases.* In Vitro, 1978. **14**(8): p. 686-96.

277. Williams, P.P. and J.E. Gallagher, *Cytopathogenicity of Mycoplasma hyopneumoniae in porcine tracheal ring and lung explant organ cultures alone and in combination with monolayer cultures of fetal lung fibroblasts.* Infect Immun, 1978. **20**(2): p. 495-502.

278. Azoulay-Dupuis, E., et al., *Lung alterations in guinea-pigs infected with influenza virus.* J Comp Pathol, 1984. **94**(2): p. 273-83.

279. Vishniakova, L.A. and V. Reztsova Iu, *[Streptococcus pneumoniae adhesion to mouse tracheal explants and its inhibition by carbohydrate preparations].* Zh Mikrobiol Epidemiol Immunobiol, 1999(2): p. 26-8.

280. Feldman, C., et al., *The interaction of Streptococcus pneumoniae with intact human respiratory mucosa in vitro.* Eur Respir J, 1992. **5**(5): p. 576-83.

281. Frankova, V., *Inhalatory infection of mice with influenza A0/PR8 virus. I. The site of primary virus replication and its spread in the respiratory tract.* Acta Virol, 1975. **19**(1): p. 29-34.