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Cross-kingdom effects of live attenuated influenza vaccination on dynamics of and disease due to respiratory bacterial pathogens within divergent ecological domains: Introducing the *Generalized Herd Effect*

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Cross-kingdom effects of a live attenuated viral vaccine on dynamics of and disease due to respiratory pathogens within divergent ecological domains: Introducing the *Generalized Herd Effect*

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ABSTRACT

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By

Michael J. Mina

Community interactions between pathogens modulate both health and disease. These interactions might be expected to be most prevalent within gut, respiratory and other mucosal surfaces that harbor complex populations of commensal and, occasionally, pathogenic microbes. In the respiratory tract, multi-species interactions are ubiquitous. A well-known example is the often lethal synergy between influenza virus and *Streptococcus pneumoniae* - believed to be responsible for much of the mortality during the 1918 influenza pandemic. Though precise mechanisms underlying this interaction remain to be fully elucidated, evidence suggests that influenza virus infection enhances virulence of bacterial pathogens through a combination of virus mediated denudation of the epithelial barriers and by inhibiting proper antibacterial innate immune responses.

Although effects of influenza virus on bacterial colonization of the upper respiratory tract and invasion in the lower respiratory tract have been well established, what has not been considered is the natural extension of this relationship to the live-attenuated influenza vaccine (LAIV).

Here I detail direct within-host effects of a live-attenuated influenza vaccine (LAIV) and it's wild type parent strain on multiple serotypes of important bacterial pathogens (*Streptococcus pneumoniae* and *Staphylococcus aureus*) at the mucosal surfaces of the respiratory tract in mice. I find that LAIV, like WT influenza virus significantly enhances density and duration of bacterial carriage and incidence and severity of bacterial acute otitis media (AOM). In a departure from wild-type influenza infection, LAIV vaccination confers significant cross-kingdom protection against lethal pneumonia by the same pathogens that are exacerbated in the upper respiratory tract. Using mathematical modeling approaches, we demonstrate that individual level effects of LAIV on density in the nasopharynx can have profound impacts on bacterial pathogen dynamics across the population. We term these effects of a vaccine "generalized herd-effects" for their ability to confer herd-effects, both detrimental and beneficial, on pathogens outside of the vaccine target species. The results herein are the first to explore the direct effects of an established vaccine on the within- or between-host dynamics of pathogens within divergent ecological domain from the vaccine target species.

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CHAPTER 1. INFLUENZA AND BACTERIAL COINFECTIONS

A REVIEW OF THE HISTORY, IMPACT AND RECENT DISCOVERIES

1.1 INTRODUCTION

Respiratory infections are widespread and common, distributed across all social and economic strata and encompass both pneumonia, the single most important disease state resulting in mortality of children under five years of age globally and otitis media, the primary cause for childhood physician visits and prescription of antibiotic therapy in middle- and high-income countries.[1-3] In 2011, 120.4 million pneumonia cases in children included 14.11 million severe episodes (11.7%) and 1.26 million childhood deaths (18% of all cause mortality), with a case fatality rate of 0.01.[1] Pneumonia is not limited to children. Incidence of community-acquired pneumonia (CAP) in adults across the European continent is estimated at 1.07-1.2 per 1000 person-years (1.54-1.57 per 1000 population) and 14 per 1000 person-years in the elderly.[4] In adults and children, pneumonia is the fourth leading cause of death overall and the number one infectious cause.[5] In the upper

respiratory tract, otitis media effects 80% of all children within the first three years of life and 40% of children go on to have greater than 6 recurrences by seven years of age.[2] Children spend an average of 42 days of their first year and 49 of their second year of life with otitis media, which has consequences on development and spread of antibiotic resistance across bacterial pathogens, exemplified by the 80% of otitis media cases in the United States treated with antibiotics, irrespective of viral vs. bacterial etiology.[2, 6]

A major contributor to both pneumonia and otitis media, influenza viruses rank among the most important pathogens to afflict human health and cause disease and mortality.[1] With relatively low case fatality rates, influenza takes its toll through annual epidemic waves that infect hundreds of millions, causing severe infections in three to five million and 250,000-500,000 deaths annually, 99% of which in low-income countries.[7] In 2008, the last year before pH1N1 disrupted the decades-long foothold that seasonal H3N2 and non-pandemic H1N1 influenza strains retained across the human population, 90 million influenza episodes occurred in children under five years of age, with 20 million acute lower respiratory tract infections, 982,000 cases of severe pneumonia and 137,000 childhood fatalities.[1, 8] Although pneumonia deaths are primarily of bacterial etiology, particularly owing to *Streptococcus pneumoniae* and *Haemophilus influenzae* (contributing 32.7% and 15.7% of all pneumonia deaths, respectively), influenza viruses add considerably, contributing 7.0% of all severe pneumonia episodes and 10.9% of pneumonia deaths.[1]

Despite the annual toll imposed on humans by seasonal influenza, gene segment reassortment, often with avian or swine influenza species, have caused antigenic shifts in influenza hemagglutinin (HA) and neuraminidase (NA) surface proteins important, from the host perspective, for antigenic recognition by immune memory, which along with human adaptation of whole avian influenza viruses, create pandemic strains capable of rapid transmission and translocation owing to a complete, or near complete, lack of immune memory or cross-protection against the novel virus.[9-12] Between the late 19th and early 20th centuries, at least four major influenza pandemics were recorded.[13-17] Three of these: the Russian pandemic of 1889[17], the H2N2 "Asian Flu" of 1957 and the H3N2 "Hong Kong" pandemic of 1968 were of a considerably more mild nature relative to the much more devastating H1N1 "Spanish Influenza" pandemic of 1918-1919.[14, 16] Infecting one-third of the global population in 1918, and estimates of 20-50 million deaths, the 1918 influenza pandemic is the most deadly pandemic in this history of humankind.[9, 18] To put the case fatality differences in perspective, the percent of fatal cases in the pandemics of 1889, 1957 and 1968 were each approximately 0.1%, compared to a conservative estimate of 1.0% (though often cited as >2.5%) in 1918, at least 50-fold greater than the case fatality rate of the 2009 H1N1 pandemic.[18-20] Some areas had vastly higher rates like the Chamorran's of Saipan, a commonwealth island of the United States in the Philippine Sea, and the Kimberly Diamond miners in South Africa with 14% and 22% of infections leading to death.[21-23]

1.2 EVIDENCE FROM THE 19TH CENTURY - PRESENT

Although the 1918 influenza virus was extraordinary in transmissibility and virulence, only seldom did acute respiratory distress and death follow viral infection alone. Current evidence demonstrates that mortality during the 1918 pandemic was primarily a result of an extraordinary capacity of the virus to enhance susceptibility to bacterial infections.[22] A recent analysis of over 8000 autopsy reports found evidence of bacterial invasion in 92% of fatal 1918 influenza cases, evidenced by necrosis and desquamation of the respiratory epithelium, alveolar duct dilation, hyaline membrane formation and exposure, lobar consolidation with high concentrations of infiltrating neutrophils, edema, pleural effusions, high bacterial CFU's and necrosis surrounding bronchiolar damage.[13, 24, 25] *Streptococcus pneumoniae* (the pneumococcus) was the predominant bacterial culprit, while beta-hemolytic *streptococcus*, *Staphylcoccus aureus* and *Haemophilus influenzae* were also detected.[13, 26-28] Similarly, specimens from the asian influenza pandemic of 1956-1957 and more recently the 2009 H1N1 pandemic (pH1N1), show conclusive evidence of bacterial invasion in nearly 30% of fatal influenza cases in the United States, and as high as 50% reported in Japan.[13, 29-31] Influenza also predisposed to bacterial invasion of the bloodstream, evidenced by positive blood cultures in 40% of fatal 1918 cases.[32] Influenza induced susceptibility to invasive disease was further supported by a shift in etiology of pneumococcal bacteremia from predominantly highly invasive capsular serotypes in the absence of influenza,

towards inclusion of less invasive strains more often associated with asymptomatic carriage than disease.[32]

Death from severe influenza alone usually occurs between 2-4 days after onset of flu-like symptoms[9, 33]. In 1918 however, only 5% of deaths took place within this time frame, while a majority occurred at least seven days after symptom-onset, much like the time-course of fatal pneumococcal pneumonia, prior to the advent of antibiotic therapies.[22, 26, 33-35] An analysis of time-to-death curves between fatal cases in 1918 and untreated bacterial pneumonia in the pre-antimicrobial drug era of the 1920's and '30's demonstrated striking similarities between the two disparate disease processes, further supporting a role for bacterial pneumonia as the major cause of death in the 1918 pandemic.[32] These data fit with a very recent report that bridged a within host mechanistic math model to epidemiologic data to detect a strong, but short-lived interaction whereby infection with an influenza virus increases susceptibility to pneumococcal infection more than 100-fold during the week following infection with influenza virus.[36]

Amidst growing concerns of a future pandemic, the past decade has seen an explosion of research aimed at elucidating the etiology of disease during 1918, proving conclusively the role for bacterial coinfections and understanding the mechanisms underlying the apparent synergistic relationship between the two phylogenetically distinct pathogens.[37] Although pending influenza pandemics have recently increased interest in influenza-bacterial coinfections, the combination of influenza and bacteria as a major contributor to acute respiratory disease has been well appreciated for nearly a century.[38-40] Indeed, the idea that a virus alone could

even cause pneumonia in humans was not solidified until the late 1950's when the introduction of antibiotics was found to significantly reduce severe disease and mortality during the Asian flu of 1957.[41-43] This is demonstrated clearly when, in 1931, Richard Shope, referring to the filterable influenza virus, concluded that *"the disease induced by this filtrable infectious agent…was definitely not typical swine influenza and will be referred to hereafter as 'filtrate disease'".*[44] Medical reports by physicians and bacteriologists (most notably Richard Friedrich Johannes Pfeiffer) from as early as 1892 suggested that *Bacillus influenzae* (now *Haemophilus influenzae*) was responsible for much of the mortality associated with pandemic influenza circulating the globe. Unable to culture the same bacteria as Pfeiffer, others believed influenza was the result of a pathogen with low virulence working synergistically with a pneumonia causing bacterial agent to elicit severe and often fatal pathology.[14] In 1918, by demonstrating the ability of influenza causing pathogens to pass though filters fine enough to exclude *B. influenzae*, it was agreed that other pathogens must be at work.[14, 45]

The first conclusive reports date back to 1931, two years before Smith and Laidlaw's discovery of influenza A virus in humans, when a then young Richard Shope at the Rockefeller institute, and his mentor, Paul Lewis demonstrated that sequential infection of pigs with swine influenza virus and *Haemophilus influenzae* induced disease far greater than either pathogen in isolation.[44, 46] These experiments reconciled, at least in part, Pfeiffer's *Haemophilus influenzae* theory and the postulation by Olitsky and Gates in 1921 that the pathogen responsible for influenza was of viral etiology.[47] In light of what is known today, and the subject of

this review, Pfeiffer is probably more deserving than he is given credit for with his discovery that a bacterial pathogen is in fact associated with severe pneumonitis and death during influenza infections.

A number of animal studies throughout the early to mid 20th century have also long supported the idea of influenza-bacterial 'synergy', a term often used to describe the relationship between these two phylogenetically distinct pathogens that was perhaps first used in this context by Frederick Bang in 1943 in an investigation of the interactions between *Haemophilus influenzae suis* and swine influenza in the chick embryo.[48] In 1935 Brightman observed fatal responses in ferrets following sequential infection with sublethal doses of both influenza and pneumococcus.[13, 49] Six years later, Andrewes and Glover demonstrated in 1941 that Group C Streptococcus depends on influenza inoculation for successful transmission between ferrets.[13, 50] Using a mixture of A/Puerto Rico 8/1934 H1N1 (PR8; still the primary influenza strain used throughout a majority of laboratories today) and group C Streptococci, Woolpert noted that "mixtures of the two agents killed a larger proportion of the mice, and brought about death earlier than either agent alone" and that "Streptococci were recovered from the lungs and heart's blood of all mice that had died of the mixed infection."[13, 51, 52] In 1946, Harford, Smith and Wood demonstrated that sulfonamide therapy helped prevent disease by decreasing secondary bacterial infections during influenza epidemics and, later, that pathogenicity of infection with influenza A virus in rhesus macaques was limited in the absence of pathogenic bacteria.[52] [53]

With the exception of the recent emergence of H5N1, H7N9 and now H10N8 in human populations, which kill predominantly via hypervirulence and development of viral acute respiratory distress rather than bacterial coinfections, evidence from pandemics and interpandemics of the previous century have left little room to debate the importance of bacterial secondary infections as a major cause of severe pneumonia and mortality following influenza virus infection. Thus, modern research has emphasized efforts to elucidate the structural and immunologic mechanisms and resulting dynamics underlying enhanced bacterial infections. The remainder of this review will discuss the most important recent advances in our understanding of the influence of influenza virus infection on bacterial pathogen dynamics and the underlying mechanisms modulating these effects. While much of the discussion focuses on the interactions between influenza virus and *Streptococcus pneumoniae*, owing to the clinical importance of this particular pairing of respiratory pathogens, and, it follows, the preponderance of clinical and laboratory studies investigating these two pathogens, where possible we also discuss advances in our understandings of influenza viruses and non-pneumococcal bacterial coinfections including *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, *Pseudomonas aureuginosa* and others. Further, given its known clinical importance, we focus on the unidirectional effects that influenza virus infections have on bacterial disease. However, evidence suggests that the relationship is bidirectional, whereby bacterial infection modulates virus dynamics and disease, an important issue recently reviewed by Short and colleagues.[54]

1.3 INFLUENZA AND BACTERIAL DYNAMICS

Infections with respiratory bacterial pathogens often begin as asymptomatic infections designated as carriage.[55, 56] Replication and migration is maintained at subclinical levels through a combination of host epithelial and mucosal defenses and innate and adaptive immune processes. Under normal conditions for example, pneumococcal colonization begins with bacterial entry into the lumen of the nasal cavity and within hours traverse the mucosal layers to establish colonization at the epithelial surface of the nasopharynx. Contact with epithelial cells induces a primarily TLR-2-dependent signaling cascade that initiates an acute neutrophilic inflammatory response that lasts for a few days. Often insufficient to fully clear the colonizing bacteria, the innate inflammatory response is gradually shifted towards Th17-dependent monocytes/macrophage recruitment over the ensuing days, weeks and even months. This long duration may be beneficial to both the pathogen – providing ample opportunity for transmission, and for the host, providing time for development of robust adaptive immunity against a diversity of pneumococcal antigens, including antibody (Ab) against immunodominant capsular polysaccharides required for efficient opsonophagocytic killing during sequential exposure.[57] Through these processes, the vast majority of cases resolve with few if any clinical symptoms.[55] Occasionally, bacteria replicate and disseminate or invade surrounding tissue causing a spectrum of diseases from sinusitis and otitis media to pneumonia, bacteremia, sepsis and meningitis. Though progression from asymptomatic carriage to disease remains a topic of investigation, it is clear that

prior infection with an influenza virus may undermine normal immunologic processes and provoke or enhance disease.

Due to the complex nature of the interactions between influenza and bacterial infections, the mechanisms underlying disease can be described at different phenotypic and cellular, molecular or immunologic levels. For example, mechanisms of disease underlying post-influenza bacterial infection in the lower respiratory tract may be due, on the one hand, to gross phenotypic changes in bacterial replication in the upper respiratory tract (URT) or to alterations at the cellular and molecular level following influenza mediated disruptions of antibacterial immune defenses. In addition, alterations in immune function resulting in excess bacterial replication and disease may be tissue and compartment specific such that mechanisms underlying increased bacterial colonization may not generalize to those responsible for increased bacterial pneumonia in the post-influenza state. Thus, as best as possible, we aim to review what is known in sequential order, moving from the macroscopic to the microscopic; from influenza-induced: phenotypic changes in bacterial transmission, colonizing dynamics and susceptibility to disease, to structural mechanisms of enhanced colonization and invasion, including alterations in respiratory mucosal and epithelial cell structure and function and finally to influenza mediated disruptions in antibacterial inflammatory processes and immune defenses including cell-cell and within-cell signaling and function within the upper and lower airways.

1.3.1 INFLUENZA ENHANCES BACTERIAL TRANSMISSION

Influenza infections first impact bacterial pathogens in the URT, the natural reservoir of respiratory bacteria. Despite this, only recently are effects of influenza on transmission, acquisition, colonization and URT disease (ie: sinusitis and otitis media) becoming rediscovered - likely driven by concerns of future influenza pandemics and the burgeoning crisis of increasing antibacterial resistance, for which bacterial URT infections are intimately intertwined.[22, 57-61] As discussed previously, influenza epidemics are often associated with increased incidence of bacterial disease primarily from *S. pneumoniae*, S*. aureus* and *H. influenzae*.[62, 63] These associations may be driven by at least one, but likely a combination of three (or more) plausible hypotheses, including influenza induced: increased rates of invasion of already colonizing or newly but normally acquired bacteria; increased rates of bacterial acquisition or increased rates of transmission during influenza infection. While the former of the three has been well established and is the focus of the latter half of this review, the remaining two are much more difficult to test in both humans and animals and remain elusive. One recent analysis that combined epidemiologic data and a within-host mathematical model found evidence for only a mild contribution from increased transmission, attributing most of the association to the first hypothesis suggested above.[36] However, this finding is somewhat inconsistent with data demonstrating that bacterial invasion most often follows recent acquisition events and that most deaths from bacterial secondary infections in 1918 occurred in individuals with normally low bacterial carriage rates – implying acquisition may have been increased.[22, 55]

One particularly well-designed study in ferrets demonstrated a significant role for influenza in driving bacterial transmission and acquisition, in particular the latter.[64] When 'donor' ferrets were colonized with pneumococcus and three days later placed in cages at one meter distance from pneumococcal naïve recipients, initial influenza inoculation of donors (3 days before bacterial colonization) or of recipients (3 days before contact with donors) increased transmission from 50% in influenza naïve controls to 83% and 100% in influenza exposed donors or recipients, respectively. At three meters distance, transmission was 0% for both controls and influenza infected donors, but increased to 100% acquisition in influenza-exposed recipients, irrespective of the influenza status of the donors.

While the mode of transmission in the setting of influenza-exposed donors was not evaluated, significantly elevated nasopharyngeal bacterial titers following influenza infection may, at least in part, underlying this process. In a separate transmission study in infant mice, bacterial titers were increased to the same extent as that seen during influenza infection, but via neutrophil depletion rather than influenza infection to prevent confounding by influenza symptoms.[65] Increased titers alone were sufficient to enhance transmission in a manner similar to that following viral infection. Interestingly, infection with influenza virus or non-microbial induction of inflammation in recipient mice was required for bacterial acquisition. These latter findings support those of the ferret model, and reports from 1918 that suggest a role for influenza as a potent mediator of bacterial acquisition.[22, 64] Both of these findings are in accord with data from numerous studies demonstrating a significant priming effect on nasopharyngeal tissue,

whereby previous influenza virus infection enhances immediate bacterial colonizing density between 100 and 100,000-fold within minutes and hours of initial colonization, relative to influenza naïve controls.[64, 66]

1.3.2 Influenza Enhances Bacterial Colonization

Infection with influenza virus has potent effects on both the density and duration of bacterial colonization.[67-71] In children, influenza virus infection is associated with at least 15-fold increases in pneumococcal nasopharyngeal titers.[71] Although bacterial colonization is largely asymptomatic, it is considered a prerequisite for more severe disease, either through invasion of the epithelial and endothelial barriers leading to blood stream infections or dissemination via microaspiration into the lower airways causing pneumonia.[58] Importantly, new acquisition and increased bacterial colonizing titers have been associated with increased risk of dissemination into sterile sites, thus influenza mediated increases in either of these processes may increase disease, irrespective of other underlying immunologic mechanisms that may contribute further to enhance disease.[55, 60, 67]

Bacterial colonization at select time points following influenza infection have been described in numerous animal experiments.[64-67, 70]. Recently, using bioluminescent in-vivo imaging we described the most complete picture to date of fine scale colonizing dynamics of multiple pneumococcal and *S. aureus* strains in both the presence and absence of influenza virus (Fig. 1.1 and see chapter 2 and referencees[66, 70]) When bacterial inoculation followed seven days after influenza infection, densities of *S. pneumoniae* (serotyeps 19F and 7F) and *S. aureus* (strains

Newman and Wright) were increased up to 100-fold (or up to 100,000-fold for the type II pneumococcal serotype D39; unpublished data) within hours of bacterial inoculation.[66] Infection with influenza virus only 1 day prior to, or during a period of stable bacterial colonization demonstrated that a 3-4 day incubation period (roughly the time to maximal viral titers) was required before excess bacterial replication ensued, although only one day was required before normal bacterial clearance was inhibited. Whether excess bacterial replication followed from physical changes in epithelial tissue or immunologic changes was not determined. However, increased bacterial titers were detected even when bacterial inoculation was given 28 days after viral infection, long after viral clearance was complete.

As discussed in greater detail later (see vaccines under strategies to prevent or treat secondary bacterial infections), these studies also demonstrated that vaccination with a live attenuated influenza virus (LAIV) vaccine had the exact same effects on bacterial colonizing dynamics in the nasopharynx as the wild-type influenza virus. However, unlike WT influenza, LAIV did not increase bacterial pneumonia, invasive disease or mortality[66]. Further, early LAIV vaccination given one month before influenza infection successfully inhibited post-influenza secondary bacterial infection and completely prevented excess influenza-mediated bacterial colonization.[70]

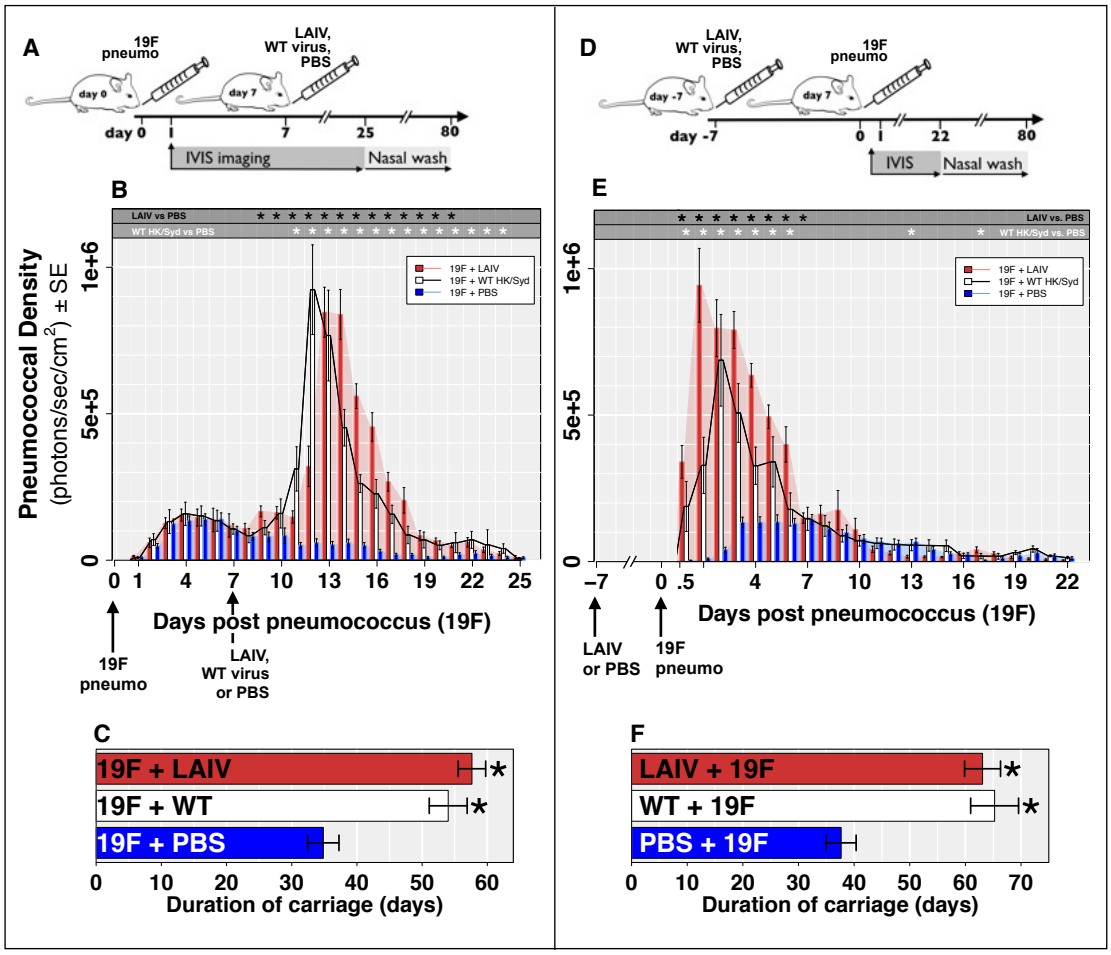

Figure 1.1 LAIV vaccine and WT influenza infection similarly enhance 19F pneumococcal carriage density and duration of colonization.

Adapted from Mina MJ et al. mBio 2014. 5(1):e01040-13. Groups of 12-14 mice were vaccinated with LAIV, infected with WT influenza virus or PBS vehicle at 7 days following colonization with 19F pneumococcus (a-c) or 7 days prior to colonization with 19F (d-f). Bacterial strains constitutively expressed luciferase and nasopharyngeal carriage density was measured via in-vivo imaging (IVIS) at 12 -hours post-bacterial infection and daily thereafter (b, e). Duration of colonization (c, f) was measured via bacterial plating of nasal washes taken daily after carriage density decreased below the limit of detection for IVIS (~1e4 CFU/ml). Asterisks indicate significant differences between vaccinated (Black asterisks in b, e) or WT influenza virus infected (white asterisks in b, e) vs. control groups (students t-test; p<.05) and error bars represent standard errors around the mean.

1.4 WINDOWS OF SUSCEPTIBILITY

1.4.1 UPPER RESPIRATORY TRACT

Similar to effects of influenza on bacterial colonization, influenza exerts its influence on bacterial upper respiratory tract disease, in particular bacterial otitis media (OM).[72] The window of increased bacterial URT disease follows a similar pattern to increased bacterial colonization in the post-influenza state. When infant mice, lacking in adaptive immunity, were colonized with pneumococcus and nine days later infected with influenza virus, middle ear bacterial titers after six days were 90-fold increased over influenza naïve mice, concurrent with increased inflammation and hearing loss.[73] bacterial OM resolved 11-16 days post viral infection, coincident with a sharp decline in viral titers. In ferrets, when bacterial inoculation followed influenza infection by five days, significant increases in both bacterial sinusitis and otitis media were detected.[69] Though it would seam reasonable to assume, considering similarities in timing of excess colonizing and middle ear titers, increased bacterial OM is not simply a product of bacterial 'spill-over' from increased nasopharyngeal titers as bacterial middle ear titers were significantly increased following H3 vs. H1 encoding viruses, despite similar bacterial colonizing densities.[74] Increased susceptibility to bacterial OM following infection with H3 vs. H1 influenza viruses was first described in ferrets and has since been found to result from increased H3 tropism for middle ear epithelial cells.[69]

While no experiments have focused on describing the precise windows of time during which virus infected hosts are at greatest risk of bacterial OM, the data just described suggest that bacterial OM may occur in excess whenever bacteria are present at the time of influenza infection or if bacterial acquisition occurs prior to viral clearance. Further, using the same in-vivo imaging model described above for measuring fine scale effects of influenza on colonizing density, we have found that influenza induces excess bacterial OM even when acquisition occurs up to four days after viral clearance (unpublished data).

1.4.2 PNEUMONIA AND INVASIVE DISEASE

The window of heightened susceptibility to bacterial invasive disease and pneumonia is often considered most pronounced within the first seven days following infection with influenza. This has been recapitulated in both epidemiologic studies and numerous animal experiments.[32, 36, 75, 76] Depending on the model system however, effects and underlying mechanisms may last weeks or, as one study demonstrates, as long as 6 months.[70, 77, 78] Perhaps the most informative display summarizing the dynamics and timing of lethal influenza-bacterial coinfection comes from a 2002 study by McCullers and Rehg (adapted here in Figure 1.2), demonstrating that survival and mean survival time (for lethal infections) follow U-shaped curves as timing of bacterial inoculation, relative to viral infection, is increased from an antecedent bacterial infection seven days before influenza infection to secondary infection 21 days post-influenza inoculation.[38] Greatest mortality occurred when bacterial inoculation followed influenza infection

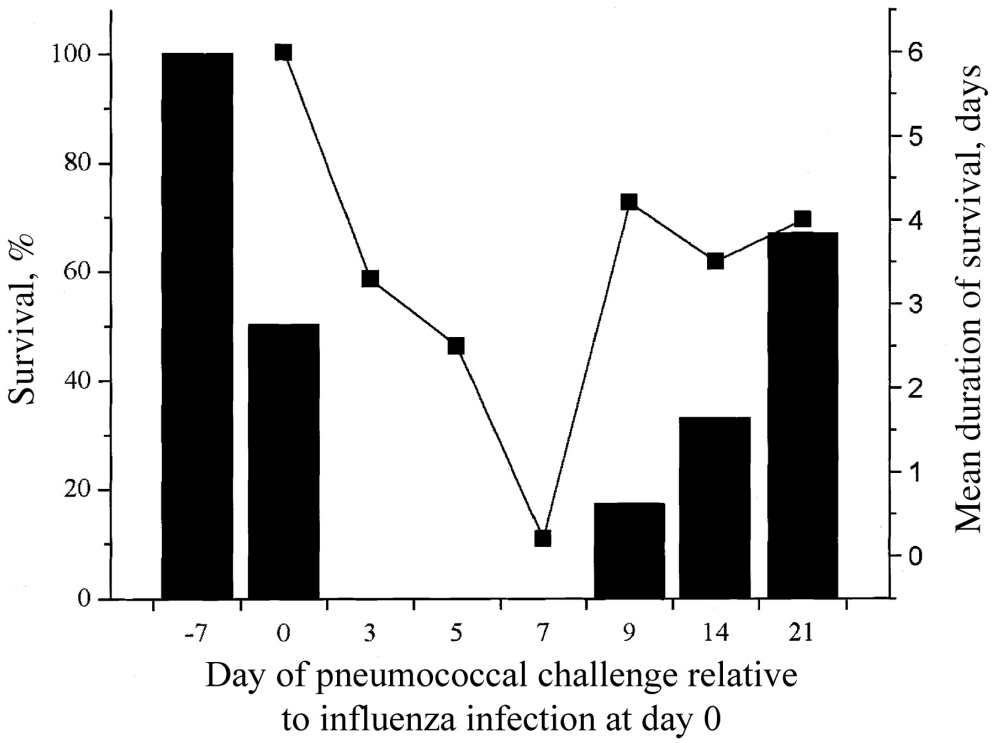

Figure 1.2 Timing of syngergism between influenza and pneumococcus.

Adapted here from McCullers and Rehg. JID 2002; 186:341-350. Groups of mice were challenged with pneumococcus at different times relative to influenza infection at day 0. Percentage survival at d21 post-pneumococcal inoculation is displayed in the bars. The mean duration of survival (only counting those mice that died) is plotted with the line with black squares.

by 3-7 days and mean time-to-death was shortest (< 1 day) when bacterial infection followed influenza by seven days; consistent with the interval between infections that maximizes peak bacterial lung titers and blood.[79-81] Of note, the window of susceptibility to lethal infection is distinct from that of upper respiratory tract colonization and disease as bacterial infection prior to influenza infection has been shown to reduce morbidity and increase survival.[38] While the magnitude of the response (ie: % lethal, measured morbidity, etc…) is dependent upon experimental conditions, the trends displayed in this particular study are generalizable across

nearly every laboratory and epidemiologic investigation describing lethal synergy between influenza and bacteria.[36, 76, 79, 82]

1.5 INFLUENZA AND NON-SPECIFIC BACTERIAL ADHERENCE

For much of the 20th century, dogma suggested that excess bacterial disease following influenza infection followed from increased bacterial adherence and reduced clearance from respiratory epithelial tissue in the post-influenza state. Here we describe the major findings contributing to our understanding of influenza mediated alterations in bacterial adherence and clearance.

1.5.1 ADHERENCE AND EPITHELIAL DESQUAMATION

As early as 1949 pathologists noted virus mediated patches of desquamated epithelium where bacteria adhere and invade with increased vigor.[83] Studies by Hers in 1954 and Parker in 1963 noted desquamation within the week following influenza inoculation led to 'superficial necrosis of the tracheal and bronchial epithelium which spared the basal layer' with development of tracheobronchitis, bronchiolitis and pneumonia [33, 84]. Similar findings in both animals and humans have confirmed their findings. [41, 85-89]. Desquamation exposes basement membrane components ideal for bacterial attachment. [90]. Further, epithelial regeneration yields excess hyalinization and increased production of fibrinogen, fibronectin, and other matrix elements to which bacteria may bind with greater efficiency.[38, 76, 83, 91-95]. Infection of primary and immortalized respiratory epithelial cells with non-

influenza viruses (RSV, human parainfluenza virus type 3 and paramyxovirus) that induce similar epithelial desquamation also increase bacterial adherence.[68]

1.6 INFLUENZA AND SPECIFIC BACTERIAL ADHERENCE

While desquamation may expose non-specific sites of bacterial adherence, specific adhesion molecules expressed in excess during influenza-mediated inflammation or exposed via alterations of epithelial surface proteins also aid bacterial adherence and invasion.

1.6.1 PAFR AND CHOP

The G-protein coupled platelet activating factor receptor (PAFr) is expressed on epithelial and endothelial cells and has numerous ligands, including phosphorylcholine embedded in the cell wall of many respiratory bacterial pathogens (ChoP). Binding of PAFr is thought to aid pathogen docking on PAFr expressing epithelial surfaces. [38, 96-98] Shortly following influenza virus inoculation, a pro-inflammatory cytokine response activates epithelial and endothelial cells which, among other effects, increases expression of PAFr on the cells' surface.[38, 96, 99] By binding ChoP, enhanced expression of PAFr may increase bacterial adherence to the respiratory epithelium in the post-influenza state.

When PAFr was inhibited during secondary pneumococcal infection, neutrophil recruitment and morbidity were reduced at 24 hours post-bacterial infection, however mortality remained unchanged.[38] In contrast another study demonstrated reduced pneumococcal lung titers, bacteremia and mortality during

secondary infection in *Pafr*$^{-/-}$ vs. wild-type mice.[100, 101]. In this latter study, *Pafr*$^{-/-}$ mice had a significantly blunted pro-inflammatory response to secondary infection, including reduced levels of TNF-α, IL-6, and KC (an important murine neutrophil chemoattractant) and consequently reduced macrophage and neutrophil recruitment, a response that has also been repeated in *Pafr*$^{-/-}$ mice following lipopolysaccharide (LPS) inoculation.[102-104]. Yet another report found that mice deficient in PAFr had increased mortality following secondary infection, concomitant with increased bacterial lung titers and inflammatory cytokines and chemokines TNF-α, IL-1β, IL-6, KC, and MIP-1α. This latter finding demonstrated that PAFr is not required for increased susceptibility to bacterial coinfection in the post-influenza state. In agreement with previous studies however, the authors implicated PAFr as necessary for bacteremia, allowing transmigration of bacteria from the respiratory epithelium into the blood stream and across epithelial or endothelial layers. [76, 100, 101, 105] In-vitro, bacteria demonstrated increased adherence to A549 epithelial cells following treatment with influenza virus in both the presence and absence of PAFr antagonists.[106].

Though these reports are contradictory, some of the apparent differences may be explained, at least in part, by the variability of experimental models. When comparing, or attempting to compare data between different models of this nature, a number of elements including strains used, inoculum doses, interval between pathogen inoculations and mouse background must be taken into consideration. For example, although the same influenza strain, PR8, was used in each of the experiments, PAFr was found to have little effect on bacterial binding following

inoculation with 100 $TCID_{50}$ PR8 while an important role for PAFr in increased bacterial adherence was noted following PR8 inoculation with only 10 $TCID_{50}$.[100, 106] Distinct doses effect both the magnitude of inflammation and, in this case, PAFr expression. Further, the choice of bacterial pathogen of clear importance. Indeed, in the studies just mentioned, numerous pneumococcal strains were used including both encapsulated D39, A66.1 and Tigre4 (serotypes 2, 3 and 4, respectively) and the unencapsulted strain R6T. Each of the encapsulated strains will have unique interactions with the respiratory epithelium eliciting variations in immune responses and a range of bacterial adherence, invasion and pathogenicity. PAFr may more readily bind ChoP embedded in the cell surface of unencapsulated vs. encapsulated pneumococcal strains, owing to the closer proximity achievable in the absence of a thick polysaccharide capsule. [105, 107]

The timing of pathogen introduction into the host is also of great importance in these models. PAFr was deemed unnecessary for increased bacterial outgrowth and *Pafr*$^{-/-}$ mice had increased morbidity and mortality when pneumococcal inoculation followed viral infection by 7 days, whereas mice deficient in PAFr showed reduced bacterial replication and mortality when bacterial inoculation followed 14 days after influenza infection.[100] These differences in timing create two very distinct environments into which bacteria are introduced. At seven days post influenza infection, the respiratory epithelium is only beginning to recover, viral particles may still be present and increased sites for bacterial adherence following epithelial desquamation or viral cleavage of sialic acids may blunt the apparent effects of PAFr binding. In contrast, by 14 days following viral infection, viral titers

have fallen to zero, epithelial regeneration has proceeded to sufficiently reduce non-specific binding to basement membrane and viral induced alterations of glycoconjugates will be returning to steady state.[108]

1.6.2 PspA and plgR

The pneumococcal adhesion molecule, pneumococcal surface protein A (PspA), is a choline binding surface protein that aids in immune evasion by inhibiting complement-mediated phagocytosis and prevents killing by host lactoferrin.[109] PspA is also important in pneumococcal adhesion of respiratory epithelial cells by binding epithelial cell polymeric immunoglobulin receptor (plgR), important for epithelial transcytosis of mucosal antibodies, in particular IgA and IgM, and excretion of antigen across mucosal surfaces.[110] By binding plgR, PspA, and other related choline binding proteins like cbpA, enhances pneumococcal adherence to host epithelial cells. Further because plgR is an important host pathway enabling transcytosis of the mucosal and epithelial barriers, binding of plgR may provide an avenue for pneumococcal invasion through these barriers.[58, 111] In an in-vivo mixed competition assay, disruption of PspA (PspA-) led to a 48-fold reduction in pneumococcal colonizing density relative to the WT D39 parent strain. When the same competition assay was performed 7 days following infection with influenza, a nearly 2000-fold reduction was noted in the PspA mutant, suggesting an important synergistic interaction between influenza virus infection and pneumococcal PspA. Similarly PspA immunization reduced post-influenza bacterial lung titers and damage following each of three important pneumococcal serotypes 2, 3 or 4 (D39,

WU2 and TIGR4, respectively).[112] In a different study, plgR expression was increased in an IFN-γ dependent manner, a cytokine expressed during influenza infection.[113-115] Together, influenza induction of IFN-γ may increase expression of plgR, enhancing PspA-plgR mediated bacterial adherence and invasion.

1.6.3 BACTERIAL NANA AND SIALIC ACIDS

In the same study that addressed the role of PspA in post-influenza pneumococcal infections, no effect on primary or secondary colonization was detected for the pneumococcal surface proteins hyaluronidase (Hyl) and neuraminidase (NanA), both important for tissue invasion and colonization through targeting of hyaluronic acid residues on host connective tissue and terminal sialic acid residues on respiratory epithelial glycoconjugates, respectively.[112] [112, 116, 117] The latter finding is however in contrast to previous results that demonstrated attenuated colonization and disease outcomes following NanA- vs. wild-type inoculation, and that prior infection with influenza virus rescued colonization and virulence in the NanA mutants back to wild-type levels.[118]

Variations in experimental models and methods used may help, in part, to explain these conflicting results. NanA was deemed important for colonization using an unencapsulated R6T pneumococcal while no effect of NanA was detected using the encapsulated D39 parent strain. Though R6T is descended from the D39 strain, suggesting similar NanA activities, the capsule is integral for efficient colonization of the respiratory epithelium.[58, 105] Thus effects on colonization due to reduced NanA activity may be masked by the presence of a capsule. Further, using a 50 : 50 NanA- :

wild-type competition model may mask effects of NanA if the wild-type half of the mixture cleaves any excess sialic acids to rescue NanA binding. Indeed, excess cleavage of sialic acids by nearby micrbobial pathogens enabling increased adherence of bacterial is not without precedent.

1.6.4 INFLUENZA NA AND SIALIC ACIDS

Numerous studies have implicated the influenza neuraminidase (NA) in increased bacterial adherence to epithelial tissues. Like bacterial neuraminidase, influenza NA cleaves sialic acid glycoconjugates on the epithelial cell surface and may expose greater numbers of cryptic receptors for bacterial binding than bacterial infection alone.[76, 118, 119]

When numerous recombinant influenza viruses were engineered to differ only in specific NA activity, higher levels of NA activity were associated with increased secondary bacterial infections [116]. In additino, field data from the H3N2 "Asian Flu" pandemic of 1957, caused by a virus with uniquely potent NA activity, demonstrated considerably elevated rates of secondary bacterial colonization and invasion relative to pandemics due to viruses with reduced NA activity.[120].

Through a series of experiments with chinchilla respiratory epithelial tissue, it was demonstrated that NA increases bacterial adherence of pneumococci to tracheal, eustachian tube and middle ear epithelium [121-123]. Further primary infection with influenza restored attenuated NanA mutant pneumococcal adherence and invasion back to wild-type levels.[118] In a different series of experiments treatment with the potent viral NA inhibitor oseltamivir, prior to influenza-bacterial

coinfection improved survival outcomes.[81] Interestingly, treatment was not associated with a decrease in viral titers, but rather reduced bacterial lung titers.

Because bacterial NanA is embedded in the bacterial cell surface, NanA-mediated adherence is generally restricted to regions where bacteria can access sialic acids. Influenza virus however has considerably increased mobility over pneumococci, thus its range for stripping the respiratory epithelium of its sialic acids is greater, complementing the local action of the bacterial NanA and providing increased and more diffusely spaced sites for bacterial adherence [81]. Indeed, oseltamivir treatment in a coinfection model reduced the area to which bacteria adhered back to levels seen in primary infection alone.[81] The portions of lung parenchyma that were colonized during oseltamivir treatment showed similar histopathologic characteristics to those of secondary bacterial infections in the absence of NA inhibition. Thus, viral NA may increase bacterial attachment while bearing no effect on bacterial induced histopathology.[81]

1.7 INFLUENZA AND MUCOCILIARY CLEARANCE

Infection with influenza virus reduces ciliary beat frequency on respiratory epithelial cells. In a chinchilla model of uncomplicated influenza infection, reduced frequency lasted for 14 days post infection and returned to baseline by day 28.[124] Reduced mucociliary clearance of bacteria following influenza virus was first demonstrated in 1983 when clearance of *S. aureus* was significantly diminished in the post-influenza state, despite similar levels of adherence, particularly at day seven post influenza. More recently, using a murine tracheal explant system,

influenza was shown to reduce mucociliary-mediated removal of latex beads.[125] By day six post-influenza, ciliary beating was nearly entirely inhibited despite regeneration of intact tracheal epithelium. In this system, prior influenza infection failed to increase pneumococcal adherence, even when the basement membrane was maximally denuded or during repopulation of basement membrane with undifferentiated epithelial cells. Rather, influenza reduced mucociliary clearance velocity and increased the bacterial density within 2 hours of inoculation. It was suggested that increased adherence found in previous studies is perhaps a relic of using immortalized cell lines and in-vitro work which does not accurately reflect the true nature of the cells and basement membranes.[92, 125]

Reduced ciliary beating in regenerated intact epithelium may follow inhibition of calcium and sodium channels. Indeed, influenza infection inhibits sodium channels through binding of hemagglutinin (HA) to cell surface and activation of phospholipase C and proteinase kinase C.[126] If influenza reduces ciliary beat frequency via inhibition of sodium ion channels, treatment with beta agonists may improve outcomes. However, utility of beta agonists may prove only limited use as influenza has also been shown to downregulate beta receptor function.[126]

1.8 INFLUENZA AND THE INNATE IMMUNE RESPONSE TO COINFECTION

In 1956, Fisher and Ginsberg noted prominent reductions in leukocyte recruitment in the weeks following influenza infection in guinea pigs.[127] Similarly

Walsh and Mogabgab noted a disproportionately low number of cellular infiltrates for the degree of epithelial damage during the 1957 flu pandemic, and Kilbourne and Sellers demonstrated viral attenuation of lymphocytes as a cause of increased bacterial infections following influenza.[41, 128] These early investigations of the innate immune response introduced the idea that secondary bacterial infections are not simply the result of physical alterations in epithelial tissues, but may arise from a system of aberrant and unstable immunologic signaling cascades in the setting of dual influenza and bacterial infections. Indeed, the past two decades have demonstrated overwhelmingly a primary role for dysregulated innate and adaptive antibacterial immunity following viral infection. Although numerous individual cytokines, chemokines and cell-mediated responses have been investigated, a rather small number of key immunologic processes may explain the myriad of studies now represented in the literature. We will consider each of these key processes here.

1.8.1 TYPE I INTERFERON

The type I interferon (IFN) includes multiple IFNalpha proteins and a single IFNbeta protein that signal through a common receptor, IFNAR, resulting in expression of innate immune cytokines important in inhibition of viral replication.[67] Though type I IFNs have traditionally been associated with innate anti-viral responses and polarization of adaptive immunity, they are also increasingly recognized for their role in both beneficial and detrimental antibacterial host defenses.[129-132] Investigations into the effects of type I IFNs on bacterial infections have traditionally focused on intracellular bacteria, where IFNalpha has been shown

to both reduce proliferation of *Chlamydia*, *Listeria monocytogenes* and *Salmonellae* [131, 133-135], and increase proliferation of *Tropheryma whipplei* and *Mycobacterium tuberculosis*.[136-139]. In the setting of post-influenza bacterial infections, excessive production of type I IFN is gaining traction as a key mediator of pneumococcal-bacterial synergy.

Type I IFN and bacterial colonization

During pneumococcal colonization of the nasopharynx, Nod2 recognition of pneumococcal peptidoglycan results in NF-kB activation and CCL2 expression and binding to its receptor, CCR2, important for signaling recruitment of monocytes and macrophage for bacterial clearance. Type I IFN too is expressed in response to the Nod2/CCR2-dependent pathway, and this also requires expression of the pneumococcal pore forming toxin pneumolysin (Ply), presumably to allow Nod2 access to microbial ligands.[67]

Though non-pathologic during single infection with pneumococci, induction of type I IFN by both the pneumococcus and influenza virus may enter a positive feedback loop with subsequent synergistic increase responsible for excess pneumococcal colonization of the URT.[67] Treating mice or macrophage, ex-vivo, with either PR8 influenza or a potent type I IFN inducing TLR3 ligand, poly-ICLC, demonstrated that excess production of IFNbeta in the context of bacterial colonization was alone sufficient to inhibit Nod2-mediated expression of *Ccl2* (via blockade of NF-kB signaling) that prevented macrophage recruitment and pneumococcal clearance. These effects were not seen in the absence of IFNAR signaling and type I IFN had no effect on KC expression or neutrophil recruitment to

the nasopharyngeal tissue. Interestingly, although a type I IFN-mediated blockade of Nod2 signaling was responsible for excess pneumococcal colonization following influenza infection, deletion of *Nod2* or pneumococcal *Ply* (required for Nod2 detection of pneumococcus) abrogated excess IFN production and reduced bacterial colonization to normal levels. This indicates that excess accumulation of type I IFN above a certain threshold may shut down numerous antibacterial immune pathways in an unfortunate game of 'immunologic chicken' whereby the immune system hedges its bets on prioritizing prevention of immunopathology and 'bystander' tissue damage over control of bacterial proliferation; a bet that would explain the significant reduction in deaths from secondary pneumococcal pneumonia in the post- vs. pre-antibiotic era.[140]

Type I IFN and bacterial pneumonia and invasive disease

Although type I IFN had no effect on KC expression or neutrophil recruitment to the upper respiratory tract tissue, excess production of IFNalpha significantly abrogated neutrophil recruitment to and bacterial clearance from the lungs, a process implicated in post-influenza bacterial pneumonia.[130, 141] Indeed, single infection with pneumococcus in combination with inoculation of exogenous IFNalpha into wild-type mice decreased KC expression and neutrophil recruitment, with subsequent development of bacterial pneumonia akin to that seen during coinfection.[130] Administration of KC and MIP-2, both potent neutrophil chemotactic signals, or deletion of IFNAR in mice rescued the neutrophil response during post-influenza bacterial pneumonia while benefit conferred by IFNAR deletion was reversed following neutralization of Cxcr2, the common receptor for KC and MIP-

2.[130] The distinct effects of type I IFN secretion on neutrophil recruitment in the URT (little effect) vs. LRT (potent inhibition) highlights the complexity and heterogeneity of immunity at the tissue specific level that is often disregarded when immune processes are assumed transferable between anatomic compartments.[59]

Effects of type I IFN on neutrophils have also been demonstrated in a non-influenza model utilizing lymphocytic choriomeningitis virus (LCMV).[142] Interestingly LCMV infection led to significant granulocytopenia that was short lived, with reduced neutrophils detected between days 2 and 5 post infection that put mice at increased risk for disease from multiple bacterial pathogens including *Listeria monoctyogenes, Staphylococcus aureus and Salmonella typhimurium*. Interestingly, although LCMV induction of type I IFN was responsible for neutropenia and enhanced bacterial replication (as these effects were not seen following LCMV infection in *IFNAR*$^{-/-}$ mice), neutropenia was not due to reduced chemotaxis, as seen above following influenza virus, but rather type I IFN induction of granulocyte apoptosis in the bone barrow. These data demonstrate the importance of refraining from overgeneralization of immune mechanisms between pathogens even when phenotypes at the organismal (ie: increased susceptibility to infection), cellular (ie: neutropenia) and molecular (ie: excess IFN) levels suggest otherwise.

Type I IFN and Th17 mediated bacterial clearance

Synergistic production of type I IFN has also been implicated in inhibition of IL-17 production, integral for bacterial clearance from both the upper and lower respiratory tracts.[141, 143] Following pneumococcal infection, γδ T-cells produce

>90% of pulmonary IL-17.[141] During secondary pneumooccal infection, IL-17 production was nearly abolished in wild-type mice, coincident with enhanced pneumococcal titers and mortality. This effect was not however seen in *Ifnar*$^{-/-}$ mice and adoptive transfer of γδ T-cells from *Ifnar*$^{-/-}$ mice was sufficient to restore IL-17 production and reduced susceptibility to secondary pneumococcal infections.[141]

Studies of influenza and bacterial coinfections with non-pneumococcal bacteria too have implicated a role for the type I IFN. When mice were coinfected with influenza virus and *Staphylococcus aureus* excess type I IFN led to an attenuated Th17 response following decreased Il-23 expression, and subsequently demonstrated reduced expression of IL-17, IL-22 and reduced MCP-1 mediated recruitment of monocytes/macrophage for *S. aureus* bacterial clearance.[143, 144] Overexpression of IL-23 partially rescued induction of both IL-17 and IL-22 and improved Th17 mediated bacterial clearance. Attenuated IL-1β expression was also demonstrated to play a role.[145] During a primary infection with *S. aurues* early NF-κB activation enhances downstream transcription and activation of pro-IL-1β to IL-1β, also important for Th17 polarization, expression of IL-22 and IL-17 and bacterial clearance. Overexpression of IL-1β rescued IL-17 and IL-22 production and bacterial clearance in wild-type but not IL-17RA deficient mice. IL17RA$^{-/-}$ mice also demonstrated significantly reduced bacterial clearance following *S. aureus* primary infection.

These findings are supported by an interesting observation that patients with hyper-IgE syndrome who present with S. aureus pneumonia often have

mutations in signal transducer and activator of transcription 3 (STAT3), an important transcription factor for Th17 cell polarization.[144]

1.8.2 IFNGAMMA

The type II interferon, IFNg, has been demonstrated to be a major contributor to secondary bacterial pneumonia, implicated in blunting the inflammatory response to post-influenza bacterial lung infections and reducing alveolar macrophage (AM) phagocytosis via downregulation of the AM scavenger receptor MARCO, as well as increasing bacterial adherence to epithelial cells via increased expression of plgR (described above).[79] IFNg is primarily produced by natural killer (NK), CD4+ T-helper and CD8+ cytotoxic-T lymphocytes.[146] In coinfection, IFNg levels peak when bacterial inoculation follows seven day after influenza infection, the time of greatest susceptibility to secondary bacterial pneumonia.[79]

IFNgamma and alveolar macrophage

Monocytes reared in-vitro in the presence of IFNg display reduced phagocytosis associated with depressed pro-inflammatory cytokine secretion, increased levels of oxidative radicals and, in alveolar macrophage (AM), significantly reduced expression of the scavenger receptor MARCO, important for efficient clearance of numerous foreign invaders into the lower respiratory tract.[79, 147]. While IL-12 production is beneficial in the regulation of inflammation, Th1 polarization and development of sterilizing immunity to influenza virus, IL-12 mediated increased IFNg secretion following influenza infection may enhance susceptibility to bacterial outgrowth and invasion in the lower lungs.

In one particularly influential study, excess mortality during secondary vs. primary infection was associated with a synergistic increase in IFNg production detected in the lungs within only four hours of bacterial inoculation, a time point early enough to prevent confounding by excess bacterial replication in influenza infected vs. control mice.[79] In contrast, IFNg levels were undetectable four hours following primary infection.[79] *Ifng*$^{-/-}$ mice coinfected with pneumococcus seven days after influenza infection had reduced mortality, enhanced bacterial clearance and decreased bacterial titers measured in the bronchoalveolar lavage fluid (BALF) relative to wild-type mice.[79] In the absence of influenza infection, IFNg treatment increased bacterial lung titers and pneumonia during primary bacterial infection.[79]. This study however contrasts an earlier report by the same group that showed reduced survival in *Ifng*$^{-/-}$ mice following primary pneumococcal infection that was reversed following administration of IL-12.[148].

A number of other studies demonstrate increased IFNg in the BALF or lungs of mice within 24 hours of secondary vs. primary bacterial infection [77, 129, 149, 150]. Two studies demonstrated increased mortality or reduced bacterial clearance from the lungs following primary pneumococcal infection of *Ifng*$^{-/-}$ mice while another demonstrated a detrimental role for IFNg to reduce AM phagocytic activity in the post-influenza state.[79, 129]. Together, these studies suggest that while IFNg may have a protective effect at physiologic levels following primary bacterial infection, increased levels of IFNg prior to pneumococcal infection induce a synergistic IFNg response that may trigger a reduction of pro-inflamatory cytokines secretion and alveolar macrophage phagocytosis required for efficient bacterial clearance.

IFNgamma and IL-10

In a different series of experiments, increased expression of IL-10 during pneumococcal coinfection, possibly a result of influenza induction of indoleamine 2,3-dioxygenase (IDO), was implicated in increased IFNgamma production. Indeed, inhibition of IL-10 signaling significantly reduced secretion of IFNg, decreased bacterial lung titers and improved survival in mice.[149] These data argue against the dogma that IL-10 acts solely as an anti-inflammatory cytokine and suggests a role for IL-10 expression in the synergistic increase of IFNg and/or subsequent reductions in innate defenses during coinfection.[77] An earlier report however demonstrated that inhibition of IL-10 signaling increased IFNg production during primary bacterial infection.[151]. Although these results seem at odds, it is possible that anti-IL-10 treatment during secondary bacterial infection enabled a relatively unrestricted, robust early pro-inflammatory response that inhibted excess bacterial replication from taking hold. In such a scenario, reduced bacterial titers, not reduced IL-10 signaling, may have underscored the reduced expression of IFNg later on. It is difficult to determine from the data alone whether anti-IL-10 treatment directly improved bacterial clearance, and thereby reduced expression of IFNg or if anti-IL-10 directly inhibited IFNg secretion and thereby enabled proper phagocytic function and bacterial clearance.

IFNgamma and type I IFN

It is interesting to note that an exaggerated type II IFN response during bacterial secondary infection may in fact be just one of the numerous downstream effects of an excessive type I IFN, response discussed above. In human monocyte derived dendritic cells (MDDCs) priming with influenza virus, and subsequent

secretion of type I IFN, or treatment with exogenous IFN alpha alone both led to overexpression of IL-12p70, a major inducer of IFNgamma and Th1 polarization.[152]. Neutralization of IFNalpha on the other hand completely abrogated production of IL-12p70, suggesting that a strong inflammatory Th1 response and excess production of IFNgamma, in the setting of secondary bacterial infections follows from an influenza mediated induction of type I IFN.[153]

1.8.3 INFLUENZA REDUCES TLR SIGNALING

Toll-like receptors (TLRs) are pathogen recognition receptors that exist on and within numerous mucosal sentinel cells and constitute an important family of sensors responsible for detection of pathogens via pathogen associated molecular patterns (PAMPs), including lipopolysaccharide, lipoprotein, flagellin, double stranded RNA and endosomal single stranded RNA, among numerous other epitopes.[154] TLR-PAMP ligation initiates TLR signaling critical for induction of innate immune cascades responsible for cytokine and chemokine secretions that culminate in pathogen clearance.[155-158]

Common dogma posits that following infection, immune memory is relegated to the adaptive arm of the immune response, while innate immunity returns to baseline within an appropriately short duration, often following a period of tightly regulated and dampened innate immunity, likely an evolved trait to reduce innate immune-induced immunopathology as responsibility for pathogen clearance is handed off to adaptive immunity.[155] Indeed this quiescent state is often implicated in heightened susceptibility to secondary pathogens. Contrary to this, influenza

infection may affect TLR functioning (quintessential triggers of innate immunity) for much longer periods of time, with lasting effects on susceptibility to bacterial infections. Four to six weeks following influenza infection, TLR stimulation with flagellin, LPS or lipoteichoic acid (ligands for TLR-5, -4, and -2 respectively) had significantly reduced levels of recruited neutrophils (TLR-5 and -2) and macrophage (TLR-4) relative to influenza naïve mice.[78] Similarly, reduced neutrophil recruitment followed coinfection six weeks after influenza infection with *Pseudomonas aeruginosa,* Group B *Streptococcus* and pneumococcus was detected and led to significant increases in mortality relative to controls. Unlike previous investigations demonstrating a role for increased granulocyte apoptosis as a cause for reduced neutrophil mediated bacterial clearance in the post-influenza state [142] or type I IFN mediated blockade of neutrophil chemotactic signal expression downstream of TLR signaling, reduced neutrophils at six weeks following influenza infection was due to sustained desensitization of TLR to bacterial ligands. In particular, influenza infection reduced flagellin-mediated TLR activation of NF-kB in alveolar macrophage that resulted in decreased expression of the neutrophil chemotactic signals KC and MIP-2 and ultimately reduced recruitment of neutrophils into the lungs. Although six weeks is arguably still within the window of time in which the immune response may be quiescent following infection, airway neutrophils were reduced in influenza infected vs. control mice even 6 months post-influenza infection. Whether reduced neutrophil response due to sustained TLR desensitization is clinically relevant is uncertain, as most laboratory, clinical and epidemiological data point towards a window of increased susceptibility that lasts

on the order of days following onset of influenza symptoms. [36, 76] Further, while infection with influenza virus did reduce neutrophil recruitment at 6 months, the effect was modest and the data is inconclusive as to the impacts on bacterial clearance and disease at this late time point.

Although TLR signaling was desensitized at later points, when mice were inoculated with pneumococcus only two days post-influenza infection, activation of TLR-4 with a TLR-4 mAb agonist, UT_{12}, just prior to infection reduced bacterial load and improved survival.[159] Whereas TLR-5 is critical in induction of neutrophils following bacterial infection, TLR-4 activation is crticial for monocyte/macrophage recruitment. Accordingly, treatment with UT_{12} enhanced early inflammation, including induction of monocyte chemotactic protein 1 (MCP-1; also referred to as CCL2), known to accelerate recruitment of monocytes and macrophage into the respiratory tract. Early induction of inflammation following bacterial coinfection paradoxically suppressed the inflammatory response to post-influenza pneumococcal inoculation and improved survival from otherwise lethal bacterial pneumonia.

Considering the detrimental effects of desensitized TLR signaling and benefit conferred by exogenous stimulation of TLR-4 stimulation on host response to secondary bacterial infection, it is tempting to assume that at least between these two relative extremes (ie: desensitized signaling and overstimulation with UT_{12}) a positive correlation in outcomes and TLR activity would exist. The effects of the negative regulator of TLR signaling, interleukin-1 receptor like 1 (ST2) during secondary bacterial infection may however suggest otherwise.[160] When mice

deficient in ST2 were coinfected with influenza and pneumococcus, $ST2^{-/-}$ mice demonstrated, as expected, an elevated inflammatory cytokine response and increased neutrophil activity that was associated with had higher bacterial lung titers than wild type mice. These results demonstrate the double-edged sword commonly associated with inflammation and immunity. Although pro-inflammatory secretion is largely responsible for severe lung pathology and death during secondary bacterial infections (ie: the cytokine storm), if secretion of inflammatory mediators is sufficiently swift and powerful (ie: via TLR-4 ligation even before bacterial inoculation) to abrogate excess bacterial proliferation, then early increased inflammation may ultimately suppress a more advanced immunopathologic inflammatory responses to high bacterial load later in infection. However, if the magnitude of excess inflammation is insufficient for swift bacterial clearance then the host may be fair better focusing on innate immune regulation to prevent host tissue damage while handing over the task of bacterial clearance to the adaptive immune response.

1.8.4 INFLUENZA INDUCED GLUCOCORTICOIDS REDUCE BACTERIAL CLEARANCE

Post-influenza coinfection with *Listeria monocytogenes,* a bacterium most useful for modeling systemic bacterial infection lends insight into a unique mechanism for influenza-mediated bacterial coinfection. Contrary to coinfection with the more common respiratory bacterial pathogens, influenza coinfection with *L. monocytogenes* was unaffected by type I IFN as *Ifnar*$^{-/-}$ mice retained increased bacterial proliferation following influenza infection.[161] Further, initial infection with

influenza virus markedly reduced IL-6 and IFNgamma production and led to generalized systemic suppression of innate and adaptive immune processes, including production of cytokines, chemokines, immune cell infiltrates and antigen specific T-cells required to clear systemic bacterial infection. Interestingly, immune suppression did not follow an overly abundant anti-inflammatory response, as has been demonstrated previously for influenza and respiratory bacterial coinfections [77] nor a T-cell mediated regulatory response as *Rag2*$^{-/-}$ mice, lacking adaptive immunity, demonstrated a similar phenotype. Rather, severe secondary bacterial infection with *L. monocytogenes* followed an influenza-mediated sustained induction of systemic glucocorticoids.[161]

Glucocorticoids are known for their pleiotropic immunosuppressive effects. When mice were infected with *L. monoctyogenes* five days following influenza infection, secretion of glucocorticoids resulted in generalized suppression of innate immunity that proved both beneficial and detrimental. While influenza induced glucocorticoids suppressed antibacterial innate immunity and significantly increased bacterial titers, sustained levels of glucocorticoids ultimately prevented mortality following secondary infection. Adrenalectomized (ADX) mice, which lack the ability to produce glucocorticoids had a robust pro-inflammatory cytokine response, including elevated levels of IL-6, IL-12p40, TNFalpha and IFNgamma, each implicated in enhancing post-influenza pneumococcal infections,[79, 150, 152] that led to severe lung pathology with high levels of albumin, indicating epithelial tissue damage, and 100% mortality. This was in contrast to 0% mortality in non-ADX mice with elevated bacterial levels. Therefore, although glucocorticoids compromised

innate antibacterial immunity and allowed for increased bacterial replication, glucocorticoid secretion prevented lethal immunopathology during influenza infection and systemic bacterial coinfection.[161]

1.8.5 Alternatively Activated Macrophage and Immune Defenses

While classically activated alveolar macrophage (CAMs) are the first line of defense against pathogens entering into the lower respiratory tract, alternatively activated macrophage (AAM) are increasingly recognized as important contributors to maintenance of tissue homeostasis and remodeling, wound healing and overall suppression of inflammatory immune responses. Unlike their classically activated counterparts, AAMs are poorly bactericidal, differentiate in response to Th2 cytokines IL-4 and IL-13 and produce arginase-1, which competes with iNOS, a product CAM's.

When mice were infected with pneumococcus seven days following influenza infection, 20% of alveolar macrophage demonstrated an alternatively activated phenotype (ie: positive for Arg-1, FIZZ1 and Ym1 AAM markers). By 14 days following influenza infection, although there was not a significantly elevated number of AAMs vs. CAMs, there remained an elevated absolute number of AAMs, which could play a role in numerous studies that have demonstrated sustained anti-inflammatory responses following influenza infection, including desensitized TLR responses and increased regulatory signaling by CD200-CD200R ligation.[162]

1.8.6 NEUTROPHILS AND COINFECTION

The role of neutrophils have been shown to be both beneficial and detriment to the host during post-influenza pneumococcal infection. During secondary infection, numerous studies have demonstrated reduced neutrophil recruitment [78, 100, 129, 130, 142, 163] while others [38, 77, 89, 164, 165] have found increased recruitment.

Neutrophil depletion in mice during primary pneumococcal infection or secondary infection three days post-influenza infection was associated with increased pneumococcal lung titers and mortality in mice. On the other hand, when bacterial inoculation followed six days after influenza infection, neutrophil depletion improved bacterial lung clearance and survival suggesting that at a time when the host is most susceptible to secondary bacterial infections, neutrophils are more detrimental to the host response to infection.[129] Indeed, six days following influenza infection, neutrophil bactericidal function was compromised and intracellular ROS generation reduced in response to *S. pneumoniae*. In these experiments, IL-10, a potent anti-inflammatory cytokine known to reduce neutrophil bactericidal activity was significantly elevated in mice coinfected at six, but not three days post influenza infection. This is in agreement with a previous finding that demonstrated increased neutrophil recruitment that was associated with excess IL-10 production and reduced neutrophil functional response during secondary but not primary pneumococcal infection.[77] In an infant mouse model of post-influenza bacterial acute otitis media, influenza infection resulted in excess neutrophil recruitment into the lumen of the middle ear, however bactericidal capacity was diminished, noted by viable pneumococci localized to the neutrophil

infiltrate.[73] It has also been suggested that excess secretion of KC and MIP-2, both potent neutrophil chemoattractants, during secondary infection in these particular models may recruit a mixed pool of mature and immature neutrophils, the latter unable produce an appropriate anti-bacterial response.[89]

Contrary to the experiments just discussed, reduced neutrophil recruitment has also been detected in response to secondary bacterial infection, associated with reduced expression of KC and MIP-2. As mentioned earlier, reduced expression of these signals may result from an excessive type I IFN response or blunted TLR signaling in the the post-influenza state.[78, 129, 130, 142]

1.8.7 Influenza Induced Neutrophil Extracellular Traps

Influenza virus has been shown in numerous investigations to drive bacterial replication in the middle ear.[69, 73, 74] As well, antibody immune complexes have been associated with otitis media for over two decades, although the underlying mechanisms remained elucisve.[166] Recently, using an infant mouse model, antibodies were shown to increase bacterial replication in the middle ear following the formation of neutrophil extracellular traps (NETs).[167] Using B-cell deficient mice, or local administration of exogenous IgA, NET formation was demonstrated to be an antibody-dependent process. Interestingly, direct injection of DNase into the middle ear significantly reduced bacterial replication, suggesting a potential therapeutic target for post-influenza bacterial otitis media.

In the lower respiratory tract, NETs have also been shown to participate in lung pathogenesis following influenza.[168] NETs were initially identified for their

unique bactericidal activity whereby neutrophils undergo a form of cell death described as NETosis, releasing chromatin bound to neutrophil granules and cytoplasmic proteins, histones and cytotoxic proteases into the immediate extracellular space that develops into an extracellular cytotoxic matrix. While such NET formation can kill bacteria, proteases within NETs can also cause endothelial damage resulting in sepsis and small vessel vasculilits that may also damage alveolar capillary surfaces in the lungs.[168]

During secondary bacterial infection, proteinaceous material and fibrin deposition collects in the lungs. Pneumococcal infection following influenza virus led to both increased development of NETs and increased degredation of NETs, presumably mediated by the production of pneumococcal endonucleases that are known to release pneumococcal bacteria from NET entanglement. Interestingly, NET formation in the pulmonary capillary beds was much more extensive following pneumococcal infection than *S. aureus*, *P. aeruginosa* or *K. pneumoniae*, which could follow from increased DNase activity of *S. pneumoniae* and could suggest an underlying mechanism for the increased prevalence of pneumococcal secondary infections over other common respiratory bacteria.[168]

1.8.8 Influenza Induced Immune Cell Apoptosis

Although leukopenia during secondary infection may follow from reduced chemokine-mediated recruitment, as discussed, leukopenia may also follow from increased apoptosis. When mice were infected with pneumococcus two days after influenza infection, alveolar macrophage (AM) expression of FADD, an activator of

caspase-8 and 3, was significantly increased in the lungs 24 hours after secondary vs. single infection. Earlier and more vigorous activation of apoptosis was associated with more severe inflammation, lung damage and fulminant bacterial pneumonia.[169] In a separate study, over 90% of murine AM were lost to apoptosis during the first week of influenza infection, an effect that lasted for 14 days, at which point AM began to be restored.[82] In this model, early clearance of bacteria 3 hours following inoculation was 50-fold greater during primary vs. secondary bacterial infection and local GM-CSF treatment partially restored bacterial clearance during, and survival from secondary infection.

In a non-influenza model, LCMV induced type I IFN led to bone barrow granulocyte apoptosis that resulted in reduced neutrophil recruitment and coincident increases in secondary infections with numerous bacterial pathogens including *L. monoctogenes*, *salmonella typhimurium*, and *Staphylococcus aurues*. The effect in this model was relative short-lived, lasting between three and five post-viral infection.[142]

1.9 RESISTANCE VS. TOLERANCE TO TISSUE DAMAGE

Understanding the consequences of pathogen infection traditionally places emphasis on pathogen virulence and defects in host resistance to the pathogen. Recently, it was demonstrated however that even when resistance to a secondary invader is fully maintained, inadequate tolerance to host tissue damage may be a primary cause of severe disease and mortality.[170] Utilizing a mouse model of influenza virus and *Legionella pneumophila* coinfection the authors demonstrated

that reduced tolerance to tissue damage during secondary bacterial challenge could account entirely for the excess mortality during secondary vs. primary infection. Indeed, even when bacterial infection was properly controlled and bacterial virulence attenuated and the host made entirely incapable of mounting a cytokine storm via genetic deletions in each of the major innate immune stimulators pathways, mice still succumbed to secondary infection. Interestingly however, excess death was only seen when bacterial inoculation followed influenza by 3-6 days, while all mice survived given prior bacterial or simultaneous infection or when bacteria followed at least 10 days post influenza, presumably providing sufficient time for tissue repair before bacterial inoculation. Utilizing a formal process of elimination, the authors elegantly demonstrated that reduced tolerance to tissue injury may be a major and novel mechanism underlying secondary bacterial infections that are often resistant to antimicrobial and immunomodulatory treatments.[170]

In agreement with this, a study comparing secondary pneumococcal infections following infection with seasonal vs. pandemic H1N1 (pH1N1) found that secondary infection following the pandemic strain had increased mortality that was mediated not by excess inflammation or viral titers, but by significant loss of epithelial cell reproliferation and tissue repair mechanisms.[171] Excess mortality following pH1N1 and pneumococcal coinfection was associated with reduced airway basal epithelial cells and a reduction in the cell reproliferation marker MCM7, which was not reduced during single infections with either virus or bacteria or coinfection with the seasonal H1N1 strain.[171]

1.10 INFLUENZA INDUCED HYPERTHERMIA AND STRESS INCREASE BACTERIAL DISSEMINATION

Bacteria colonization and biofilm formation is aided, in part, to a stringent downregulation of bacterial virulence factors which allows immune evasion and reduces the epithelial pro-inflammatory cytokine to bacteria.[57] During an influenza infection however, hyperthermia, following expression of pyrogenic cytokines, induces expression of bacterial virulence genes and increases release of bacteria from the biofilms that colonize the nasopharynx. Indeed increased temperature alone increased dispersion of bacterial cells that were predominantly opaque, with larger capsules and increased expression of virulence factors than bacteria under normal physiologic temperatures. Further, infection with influenza increases concentrations of glucose, ATP and norepinephrine. While an increase in these products is important for efficient lymphocyte activation and clearance of the primary influenza infection, these signals were each found to induce excess bacterial colonizing titers and increased pneumococcal pneumonia and bacterial otitis media in stably colonized mice.[57, 172, 173]

1.11 INFLUENZA GENOTYPE INFLUENCES BACTERIAL COINFECTION

While the mechanisms demonstrated above are generalizations of the broad interactions between influenza viruses and bacteria, the influenza genotype

significantly interacts with and alters the effects described. A recent analysis of 11 influenza seasons from 1994-'95 through 2004-'05 found that of the 8 seasons that H3N2 predominated 5 had significant epidemiologic association between influenza and invasive pneumococcal pneumonia while there were there no significant associations with bacterial disease when H3N2 was not circulating.[174] This finding is in agreement with numerous animal studies that have described an increased predisposition to bacterial disease following H3N2 vs. H1N1 influenza infections.[74, 116] While the mechanisms underlying this phenomenon remain to be fully elucidated, a recent study demonstrated a significantly stronger tropism for middle ear epithelial tissue from H3 vs. H1 viruses.[74] Using recombinant viruses altered to be isogenic except for the specific HA or NA under investigation, H3 containing viruses grew to higher titers and resulted in greater levels of middle ear inflammation and pathology than H1 viruses. The increased inflammation was further found to be associated with increased bacterial replication in the middle ear, such that infant mice infected with H3 vs. H1 viruses developed higher bacterial counts in their middle ears. Interestingly, while viruses differed in terms of replication and inflammation in the middle ear epithelial tissue, no differences were measured in the nasopharnx where both H1 and H3 viruses grew to equivalent titers. No discrepancies in viral replication or capacity to enhance bacterial proliferation in the middle ear were noted between N2 and N1 viruses.[74]

A different study developed recombinant influenza A viruses to assess changes in neuraminidase activity in seasonal influenza strains for the latter half of the 20th century. In that study it was determined that neuramindase activity

correlated with ability to support secondary bacterial pneumonia. The 1957 and 1997 strains, for example, had highest level of neuraminidase activity and these strains supported increased bacterial adherence and secondary pneumococcal pneumonia.[116] As discussed above, increased NA activity may expose increased binding sites to allow for bacterial adhesion, demonstrated by reduced adhesion to A549 epithelial cells following influenza infection in the presence of neuraminidase inhibitor.[81]

PB1-F2 is a pro-apoptotic influenza A protein that contributes significantly to the virulence and pathogenicity of a given influenza virus.[175] PB1-F2 expression enhances inflammation during primary viral infection, which has ben shown to subsequently increases secondary infections, an effect that was recapitulated via intranasal delivery of synthetic peptide from the C-terminal domain of PB1-F2.[176] Because influenza viruses are subject to significant alterations in their levels virulence via known single amino acid changes in the PB1-F2 protein, these changes have been exploited to better understand the role of PB1-F2 in secondary bacterial infections. In mice, amino acid changes that increased PB1-F2 virulence were associated with increased bacterial titers of *S. pneumoniae*, *S. aureus and S. pyogenes*. Interestingly, the highest bacterial titers and mortality from secondary bacterial infection were seen following infection with the viral strain carrying the same amino acid substitutions as those of the 1918 pandemic.[177] Interestingly, when mice were infected with viruses carrying truncated PB1-F2 proteins with very low virulence, secondary bacterial titers grew to similarly high levels as seen during coinfection with full length PB1-F2 carrying viruses, however nearly 100% survival

was noted, reinforcing the role of host tissue damage and inflammation as an equal or more important factor in severe disease during secondary bacterial infection than bacterial titers alone.[177]

1.12 PREVENTION AND TREATMENT STRATEGIES

Numerous strategies have been suggested to curtail the prevalence of secondary bacterial infections during influenza epidemics and pandemics. Indeed, many of the mechanisms detailed above were discovered through the treatment of cell lines or animals with a chemical that worked to prevent secondary bacterial infection. Here, we will limit out discussion to a few select investigations designed to study a potential therapeutic strategy.

1.12.1 VACCINATION

Influenza and pneumococcal infections are both largely preventable through vaccination. When herd-immunity is considered, vaccination is the number one strategy for prevention of secondary bacterial infections. In a double-blind randomized placebo-controlled trial of the 9-valent pneumococcal conjugate vaccine (PCV) in an urban city in South Africa, vaccination with PCV prevented 41% of influenza virus-associated pneumonias.[178] In an unrelated study, PCV reduced influenza associated hospitalizations by 48% in in children aged 6 months to 5 years.[179].

In mice, FluMist vaccination protected mice from lethal secondary bacterial infection via acquired immunity against the primary viral infection.[180] We recently compared live attenuated influenza vaccination (LAIV) to PCV in their abilities to reduce post-influenza pneumococcal colonizing density. Influenza vaccination significantly reduced secondary bacterial titers measured in the nasopharynx, while PCV demonstrated no significant overall effect as titers were no different than unvaccinated mice following infection with influenza virus.[70] Interestingly however, in the days prior to influenza infection PCV immunized mice had reduced bacterial titers relative to both PBS treated and, especially, LAIV vaccinated mice but this protective effect of PCV to reduce pneumococcal colonization was abolished following influenza infection. Despite increased bacterial colonizing titers however, PCV and LAIV both reduced bacterial invasive disease and pneumonia (data not shown).

In a study looking at effects of LAIV and inactivated influenza vaccine (IIV) on post-influenza infection with *Streptococcus pyogenes*, both vaccines reduced influenza lung titers and pro-inflammatory cytokines following influenza infection. Interestingly although bacterial lung titers were not significantly reduced following vaccination, mortality was. Thus, while mortality from secondary infections was reduced, vaccination was had reduced ability to protect against morbidity.[181]

Although influenza vaccinations are undoubtedly beneficial to reduce influenza and secondary bacterial infections in the long-term, we recently reported an unexpected and transient effect of LAIV to increase upper respiratory tract bacterial colonization for up to a month post-vaccination in a manner highly

analogous to that seen following wild-type influenza infection. While LAIV had no detrimental effect on lower respiratory tract infections, owing to its temperature sensitive and attenuated phenotype, in the upper respiratory tract where it replicates well, LAIV reversed bacterial clearance and significantly enhanced both the density and the duration of bacterial nasopharyngeal colonizion.[66] Thus, while LAIV is beneficial to prevent post-influenza bacterial pneumonia in the long-term, it may be important to consider the level of risk of bacterial pathogen colonization during weeks following vaccination when deciding between vaccination with LAIV vs. IIV.

1.12.2 ANTIBIOTICS AND ANTIVIRALS

Although the Spanish influenza virus was an extraordinary virus unlike any other, the 1918 pandemic was well before the advent of antibiotics. It is thought that had antibiotics been available, mortality rates would have been considerably lower.[182] A recent mathematical model demonstrated that a pandemic akin to 1918 would have substantially fewer deaths in high income countries with adequate access to healthcare, vaccines and antibiotics. According to the model, during an influenza pandemic as widespread and virulent as 1918, 100% antibiotic prophylaxis could reduce mortality by greater than 50%.[140]

In a mouse model of influenza and methicicllin-resistant *Staphylcococcus aureus* (MRSA) coinfection, antibiotic treatment with linezolid, vancomycin and clindamycin decreased pulmonary inflammation and cytokine response. Linezolid, however, known to have immunomodulatory properties was the only antibiotic to

also reduce weight loss. No effect on viral titers was seen with following any of the three antibiotic treatments.[183]

In the context of secondary bacterial infections not all antibiotics are necessarily beneficial, and some may inadvertently increase disease. Beta-lactams are first line antibiotics for the treatment of bacterial lung infections. However, in the highly unstable inflammatory environment of the influenza bacteria coinfected lung, a bactericidal antibiotic like ampicillin may have the adverse effect of enhancing the inflammatory processes already underway.

In a murine model of post-influenza bacterial infection, therapeutic treatment with the beta-lactam antibiotic ampicillin induced robust inflammatory lung injury following rapid bacterial cell wall lysis and release of high concentrations of bacterial PAMPs that stimulated an excessive TLR-2 response.[184] On the other hand the bacteriostatic protein-synthesis inhibitors clindamycin and azithromycin improved survival. While TLR-2 signaling following ampicillin treatment increased neutrophil recruitment and lung injury, combination therapy with azithromycin, a known immunomodulatory antibiotic, significantly reduced neutrophil recruitment and dampened the inflammatory response. This effect was independent of its antimicrobial activities, demonstrated by similar effects seen even with a macrolide resistant bacterium.[185] In humans a beta-lactam and macrolide combination has been demonstrated to be an effect combination to improve community acquired pneumonia survival in the hospital setting.[186]

Similar to the immunomodulatory effects of azithromycin, combination therapy with ampicillin and the synthetic corticosteroid, dexamethasone, improved

survival from secondary bacterial infection in mice. While treatment with ampicillin was beneficial to improve survival following moderate pneumonia, treatment proved detrimental in severe pneumonia cases. However, combination therapy with both ampicillin and dexamethasone improved survival from severe secondary pneumonia from 0% to 70%.[187] Importantly, dexamethasone treatment alone was not beneficial and led to increased viral titers.

Other less conventional antibiotic approaches have also been investigated. For example, purified bacteriophage cell wall hydrolases or lysins have been demonstrated to eradicate nasal carriage due to gram positive bacteria. Lysins are produced by phage to disrupt bacterial cell wall to enable release of progeny phase. The Cpl-1 lysin is specific for *S. pneumoniae*. When tested in mice, Cpl-1 effectively cleared bacterial colonization and prevented bacterial otitis media during post-influenza bacterial infection. [188]

Antiviral agents too have been considered for prevention of secondary infections. When A549 cells were infected with influenza virus, treatment with the neuraminidase inhibitor oseltamivir prior to addition of pneumococcus completely abrogated increased bacterial adherence seen following influenza treatment in the absence of oseltamivir, relative to influenza uninfected cells.[189] When mice received oseltamivir treatment following influenza infection, treatment significantly reduced mortality from secondary bacterial infections, even when treatment was initiated as late as 5 days post influenza infection. This was not a response to reduced influenza infection as delayed treatment with oseltamivir at 3 and 5 days post influenza infection failed to reduce viral titers and morbidity due to the primary infection, but

remained to improve survival and increased survival time following secondary infection.[189]

1.13 CONCLUSION

In a time of unprecedented opportunity for influenza reassortment and global transmission, increasing resistance to antibiotics, and exponential growth in data to understand the interactions between host and pathogens, the importance of and capacity to gain a firm grasp on the mechanisms underlying influenza and bacterial coinfections has never been greater. The 1918 influenza pandemic demonstrated for us nearly 100 years ago the devastating toll the correct combination influenza genes and bacterial pathogens can take. Improving upon available influenza, pneumococcal, Hib and other bacterial vaccines and developing new vaccines for pathogens not yet covered is integral as a first line of defense. However, with ever-changing viral and bacterial genomes and shifting distributions of bacterial sub-types, vaccines may be far from a full-proof plan. In-depth understanding of the mechanisms underlying post-influenza bacterial infections is fundamental towards development of improved therapeutics to care for the patient with a combination of pathogens that has thus far proved difficult, and in some cases impossible to treat. It is clear that a primary cause for severe disease and death during post-influenza bacterial infection is an over-zealous immune response and an inability to balance pathogen clearance with prevention of host-tissue damage. Thus, development of immunomodulatory therapies are likely to prove as or more beneficial than conventional antimicrobial agents to treat complicated coinfection

states. Increasing understanding of the utility of combination therapies of antimicrobials and immunomodulatory agents will be critical to improve treatment outcomes and preventing excess mortality during future influenza seasons and global pandemics.

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CHAPTER 2. LIVE-ATTENUATED INFLUENZA VACCINE ENHANCES COLONIZATION OF STREPTOCOCCUS PNEUMONIAE AND STAPHYLOCOCCUS AUREUS IN MICE

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2.1 ABSTRACT

Community interactions at mucosal surfaces between viruses, like influenza and respiratory bacterial pathogens are important contributors towards pathogenesis of bacterial disease. What has not been considered however is the natural extension of these interactions to live attenuated immunizations, and in particular, live-attenuated influenza vaccines (LAIVs). Using a mouse adapted LAIV against influenza A (H3N2) carrying the same mutations as the human FluMist® vaccine, we find that LAIV vaccination reverses normal bacterial clearance from the nasopharynx and significantly increases bacterial carriage densities of the clinically important bacterial pathogens: *Streptococcus pneumoniae* (serotypes 19F and 7F) and two strains of *Staphylococcus aureus* within the upper respiratory tract of mice. Vaccination with LAIV also resulted in up to 2- to 5-fold increases in mean durations of bacterial carriage. Further, we show that the increases in carriage density and duration were nearly identical in all aspects to changes in bacterial colonizing dynamics following infection with wild-type influenza virus. Importantly, LAIV, unlike WT influenza viruses, had no effect on severe bacterial disease or mortality within the lower respiratory tract. Our findings are, to the best of our knowledge, the first to demonstrate that vaccination with a live-attenuated viral vaccine can directly modulate colonizing dynamics of important and unrelated human bacterial pathogens, and does so in a manner highly analogous to that seen following wild-type virus infection.

2.2 IMPORTANCE

Following infection with an influenza virus, infected or recently recovered individuals become transiently susceptible to excess bacterial infections, particularly *Streptococcus pneumoniae* and *Staphylococcus aureus*. Indeed, in the absence of preexisting comorbidities, bacterial infections are a leading cause of severe disease during influenza epidemics. While this synergy has been known and is well studied, what has not been explored is the natural extension of these interactions to live attenuated influenza vaccines (LAIV). Here we show, in mice, that vaccination with LAIV primes the upper respiratory tract for increased bacterial growth and persistence of bacterial carriage, in a manner nearly identical to that seen following wild-type influenza virus infections. Importantly, LAIV, unlike wild-type virus, did not increase severe bacterial disease of the lower respiratory tract. These findings may have consequences on individual bacterial disease processes within the upper respiratory tract as well as on bacterial transmission dynamics within LAIV vaccinated populations

2.3 INTRODUCTION

The conventional view of pathogen dynamics posits that pathogen species act independently of one another. More recently however, community interactions between pathogens have been recognized as necessary to modulate both health and disease [1-7]. These interactions might be expected to be most prevalent within gut, respiratory and other mucosal surfaces that harbor complex populations of

commensal and, occasionally, pathogenic microbes. In the respiratory tract for example, viral infections are known to predispose to secondary bacterial invasive disease and pneumonia from pathogens that are most commonly benign, but occasionally become virulent, particularly following a viral infection [8-10]. A well-known example is the often lethal synergy between influenza virus and pneumococcal or staphylococcal bacterial secondary infections.

Infection with influenza viruses increase susceptibility to severe lower and upper respiratory tract bacterial infections resulting in complications such as pneumonia, bacteremia, sinusitis and acute otitis media [11]. Bacterial infections may be a primary cause of mortality associated with influenza infection in the absence of pre-existing comorbidity [12, 13]. Primary influenza infection increases acquisition, colonization and transmission of bacterial pathogens [14], most notably *Streptococcus pneumoniae*, the pneumococcus, and *Staphylococcus aureus* [11, 15].

Though the underlying mechanisms, while well studied, are not entirely defined, they likely include a combination of influenza mediated cytotoxic breakdown of mucosal and epithelial barriers [16-18] and aberrant innate immune responses to bacterial invaders in the immediate post-influenza state, characterized by uncontrolled pro- and anti-inflammatory cytokine production, excessive leukocyte recruitment and extensive immunopathology [11, 19-22]. When coupled with diminished epithelial and mucosal defenses, such an environment becomes increasingly hospitable for bacterial pathogens to flourish and invade in the days and first few weeks following influenza infection.

Increasingly, evidence is linking the early innate immune response

triggered by infection or vaccination to sustained adaptive immunity [23]. Thus, a broad goal of vaccination is to elicit an immune response analogous to that of the pathogen itself, without subsequent disease [24]. The intranasally administered live attenuated Influenza vaccine (LAIV) contains temperature sensitive and attenuated virus designed to replicate efficiently in the cooler temperatures of the upper respiratory tract (URT) but fails to do so in the warmer temperatures of the lower respiratory tract (LRT) [25, 26]. Through selective replication in the URT, LAIV proteins are exposed to the host immune system in their native conformation, eliciting highly robust (IgA), serum (IgG) and cellular immune responses mimicking those of the pathogenic virus itself [27].

Although an innate immune response to vaccination is beneficial for long-term protection from influenza [28] and influenza-bacterial coinfections [29], the direct consequences of such a response to a viral vaccine, with respect to secondary colonization and disease due to entirely unrelated bacterial pathogen species are unknown. As increased susceptibility to and transmission of bacterial pathogens following influenza are due in large part to the innate immune response and breakdowns of the epithelial barriers of the URT, it is important to understand whether similar effects, elicited by live attenuated virus replication, may also predispose to bacterial infection. We sought here to determine the effects of a live attenuated influenza vaccine on URT and LRT bacterial infections. In particular, we ask whether LAIV vaccination alters bacterial colonization dynamics of the upper or disease in the lower respiratory tract of mice.

2.4 MATERIALS AND METHODS

2.4.1 INFECTIOUS AGENTS AND VACCINES

Viral infections were carried out with an H3N2 1:1:6 reassortant virus developed as described previously [30], containing the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) from A/Hong Kong/1/68 (HK68) and A/Sydney/5/97 (Syd97) isolates, respectively, and the six internal protein gene segments from A/Puerto Rico/8/34 or PR8 (referred to as WT influenza virus). LAIV vaccinations consisted of a temperature sensitive (*ts*) attenuated variant of HK/Syd, HK/Syd$_{att/ts}$ (LAIV) that contains site-specific mutations in the PB1 and PB2 RNA segments of the genome (see Fig. S1 in appendix A) as described previously [30]. These are the same mutations found in the attenuated A/Ann Arbor/6/60 master donor strain used to produce the influenza A virus strains found in the commercial product FluMist® [30]. WT and LAIV viruses were propagated in 10-day-old embryonated chicken eggs at 37°C and 33°C, respectively) and characterized in Madine-Darby canine kidney cells to determine the dose infectious for 50% of tissue culture wells ($TCID_{50}$). The pneumococcal carrier isolates ST425 (serotype 19F) and ST191 (serotype 7F), chosen based on their colonizing potential as previously described [14] were used for colonization experiments. The highly invasive type-2 and type-3 pneumococcal isolates D39 and A66.1, respectively were used for pneumonia, and survival studies. The 19F and 7F strains were engineered to express luciferase, as described previously[14]. *Staphylococcus aureus* strains Wright (ATCC 49525) and Newman(ATCC 25905) were engineered to express luciferase by

Caliper Life Sciences (Alameda, CA).

2.4.2 Animal and Infection Models

8-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments with the exception of mice treated with early vaccination to demonstrate vaccine efficacy and effectiveness. In these cases, 4-week–old BALB/c mice were vaccinated or administered PBS and monitored for four weeks before further inoculation. All inoculations and vaccinations were via the intranasal route under general anesthesia with inhaled isoflurane 2.5% (Baxter Healthcare, Deerfield, IL). LAIV vaccination consisted of 2e6 $TCID_{50}$ HK/$Syd_{ts,att}$ LAIV in 40ul PBS. Lethal and sub-lethal doses of WT HK/Syd used 5e7 and 1e5 $TCID_{50}$ in 50ul PBS, respectively. Pneumococcal infections with 19F and 7F were as described previously [14], except inoculation was in 40uls PBS. Infection with *S. aureus* strains Wright and Newman contained 1e7 colony forming units (CFU) in 40uls PBS. Mortality studies were performed as previously [31] with sub-lethal doses of the invasive type-2 and type-3 pneumococcal serotypes D39 and A66.1 isolates, consisting of 1e5 and 1e3 CFU in 100uls PBS (to ensure bacterial entry into the lower lungs), respectively. Animals were monitored for body weight and mortality at least once per day for all survival studies. Mice were sacrificed if body weight fell below 70% initial weight.

2.4.3 Bacterial CFU titers for duration studies

Bacterial CFU titers were measured in nasal washes using 12uls of PBS administered and retrieved from each nare, and quantitated by serial dilution

plating on blood agar plates. Washes were performed daily only after pneumococcal density fell below the limit of detection for IVIS imaging (~1e4 CFU/ml).

2.4.4 MEASURING BACTERIAL AND VIRAL TITERS IN LUNGS AND NP HOMOGENATES

Viral and bacterial titers were measured in whole lung and nasopharyngeal (NP) homogenates. Whole lungs were harvested and homogenized using a gentleMACS™ system (Miltenyi Biotech), as per manufacturers protocol. NP was isolated via careful dissection dorsally across the frontal bones, laterally via removal of the zygomatic, posterially by dislocation of the upper jaw from the mandible and inferiorly just posterior to the soft palate. Isolated NP was homogenized via plunging in 1.5 mL PBS through a 40 micron mesh strainer. Bacterial titers were measured via plating of serial dilutions and viral titers measured by determining the $TCID_{50}$ as previously described [30].

2.4.5 CYTOKINE LEVELS IN NP AND BAL BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Nasopharyngeal isolates and BAL were collected as above and cytokines were measured using commercially available kits from R&D systems (MIP-1β, TGF-β, and IFN-β) or eBiosciences (IL-4, IL-6, IL-10, IL-17, IL-23 and IFN- ɣ).

2.4.6 BIOLUMINESCENT IMAGING

Mice were imaged using an IVIS CCD camera (Xenogen) as described [14, 32]. Nasopharyngeal bacterial density was measured as total photons/sec/cm^2 in pre-specified regions covering the NP and background (calculated for each mouse on a

region of equal area over the hind limb) was subtracted. Each NP measurement represents an average of two pictures, one for each side of the mouse head. Quantitation was performed using LivingImage software (v. 3.0; Caliper Life Sciences) as described [14].

2.4.7 STATISTICAL ANALYSES

All statistical analyses were performed within the R statistical computing environment (R version 2.14, R foundation for statistical computing, R Development Core Team, Vienna, Austria). Specific statistical tests used are as indicated in each figure. The R package 'survival' was used for all survival analyses, Kaplan Meier plots and KM log-rank tests. All other statistical tests were performed using R base functions.

2.4.8 ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee (protocol #353) at SJCRH under relevant institutional and American Veterinary Medical Association guidelines and were performed in a Biosafety level 2 facility that is accredited by AALAAS.

2.5 RESULTS

Using a live-attenuated influenza A vaccine, HK/Syd 6:1:1 (LAIV), containing many of the same mutations and demonstrating similar growth dynamics to those found in the commercially available human FluMist® vaccine (MedImmune,

Gaithersburg, MD; see ref. [30] and Fig. S1 in Appendix A for vaccine details), we evaluated the effects of LAIV, and its WT HK/Syd parent strain (referred to as WT virus) on *Streptococcus pneumoniae* (the pneumococcus) and *Staphylococcus aureus* replication and disease.

2.5.1 LAIV IS RESTRICTED IN GROWTH AT 37°C BUT NOT AT 33°C.

To determine whether LAIV grows efficiently at temperatures seen within the nasopharynx (NP) while remaining restricted in growth at warmer temperatures of the LRT, WT influenza virus and its LAIV derivative were grown in MDCK cells at 37°C. As expected [30], a >3-log decrease in viral titers was measured for LAIV, relative to the WT parent strain ($p<.001$; Figure 1a). When LAIV was propagated at 33°C however, a temperature often associated with the nasopharyngeal environment [33], viral replication was no different than WT virus titers measured at 37°C.

2.5.2 HK/SYD 1:1:6 LAIV VACCINATION IS SAFE AND EFFECTIVE

Although LAIV is attenuated, inoculation with very high doses may cause morbidity and weight loss. Via a series of dosing experiments (data not shown), a vaccinating dose of 2e6 $TCID_{50}$ LAIV in 40ul PBS vehicle was determined safe, with no weight loss or other detectable signs of morbidity in mice (Fig. 1b). This dose is in agreement with previous studies [28, 30]. Inoculation with the same dose of the WT parent virus led to significant morbidity and mortality (5/12 mice succumbed by day 7 post infection; Fig. 1b), demonstrating the attenuated nature of the LAIV.

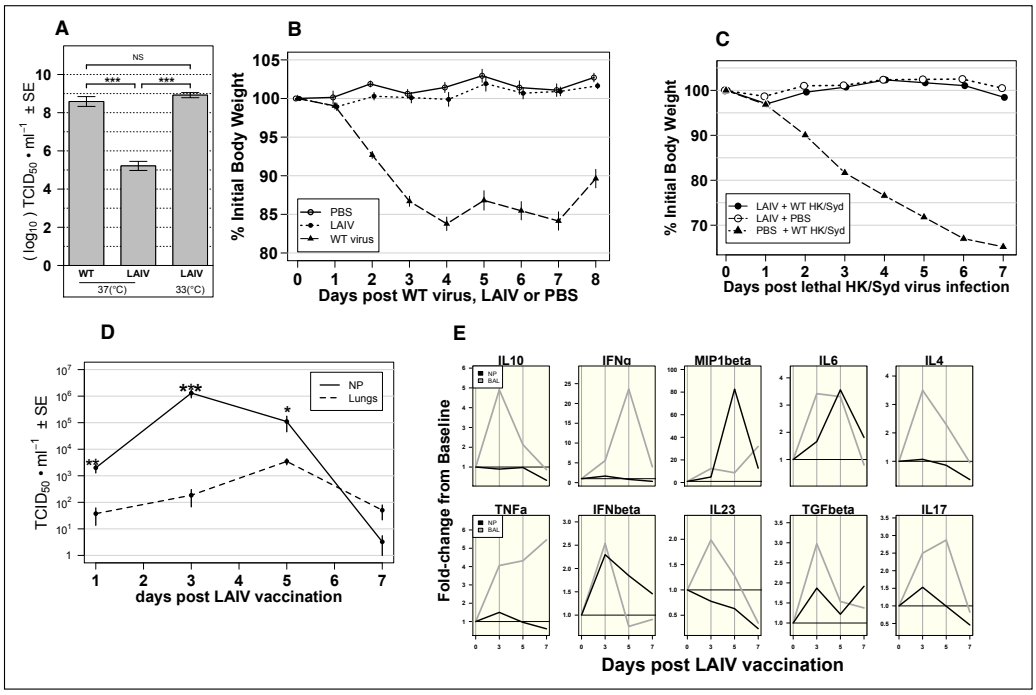

Figure 2.1 LAIV is safe, effective, replicates well within the URT and elicits a robust cytokine response.

*(A) WT and LAIV HK/Syd viruses were grown in MDCK cells at 37°C, and LAIV at 33°C and viral titers measured via median $TCID_{50}$ (n=3 per group). (B) Groups of 12-14 8-week-old BALB/c mice were inoculated with 2e6 $TCID_{50}$ LAIV, WT HK/Syd virus or PBS and monitored for weight loss. 3/12 mice and 2/12 mice died at 4 and 7 days post infection with WT HK/Syd virus while no mice died following LAIV or PBS. (C) Groups of 8 4-week-old BALB/c mice were inoculated with 2e6 $TCID_{50}$ LAIV (2 of the 3 groups) or PBS and 4 weeks later infected with a lethal dose (5e7 $TCID_{50}$) of WT HK/Syd virus or PBS control. Infection was considered lethal if body weight (BW) fell below 70% initial BW. (D) Four groups of 5 mice each were vaccinated with LAIV and whole lung and NP viral titers measured at 1, 3, 5 and 7 days post vaccination. (E) Four groups of 5 mice were vaccinated with LAIV and NP and BAL cytokines were measured at days 0 (unvaccinated mice) and days 3, 5 and 7 following vaccination. Error bars represent standard errors of the mean. Asterisks indicate statistically significant differences from controls; two-sided students t-test. *= p<.05, **=p<.001, ***=p<.0001 and NS indicates no difference between groups.*

Vaccine efficacy and antibody response using this LAIV strain have been described previously.[30] To phenotypically confirm efficacy here, groups of 8 4-week old mice were inoculated with LAIV or PBS control and four weeks later with a lethal dose of the WT virus. Early vaccination with LAIV conferred complete

protection from any detectable morbidity or weight loss due to infection with the WT strain, vs. 100% mortality in unvaccinated control mice (Fig. 1c).

2.5.3 LAIV IS RESTRICTED IN GROWTH IN THE LOWER BUT NOT THE UPPER RESPIRATORY TRACT.

To determine whether the differences in replication seen in-vitro also occur in-vivo in the upper (~33°C) vs. lower (~37°C) respiratory tracts, groups of 5 mice were vaccinated with LAIV and viral titers measured in whole lung and whole NP homogenates (Figure 1d). By 3 days post vaccination, NP titers were 10,000-fold greater than in the lungs (1.3e6 vs. 1.2e2 $TCID_{50}$, p<.001). In contrast, the WT virus grew to high viral titers in both the NP and lungs (>5e5 $TCID_{50}$; data not shown), in agreement with previous reports [34], which led to significant morbidity and mortality, as demonstrated in the controls in Fig. 1b. Overall, maximal NP titers occurred earlier and were nearly 400-fold greater than maximum lung titers (1.3e6 vs. 3.4e3 $TCID_{50}$; p<.001). Importantly, these NP viral dynamics are in agreement with viral shedding in NP aspirates from human subjects following vaccination with the FluMist® vaccine [35].

2.5.4 LAIV CYTOKINE RESPONSE IN THE NASOPHARYNX AND LUNGS.

While LAIV replication in the NP induces robust systemic inflammatory response [36, 37], the cytokine response in the NP has, to our knowledge, not been observed. Nasopharyngeal homogenates and BAL cytokines were measured in groups of 5 mice each at days 0, 3, 5 and 7-post vaccination (Fig. 1e). Of particular interest, the type I interferon (IFN-beta) was significantly increased in the NP and BAL following

LAIV vaccination and this cytokine has been demonstrated to play a pivotal role in excess bacterial colonization of the nasopharynx following WT influenza virus infection [38]. As well, MIP-1 beta was also significantly upregulated following LAIV similar to that seen following influenza-pneumococcal coinfections of human middle ear epithelial cells [39]. In general, the responses measured here in the NP are similar to those measured from nasopharyngeal washes in humans infected naturally with seasonal influenza A viruses [40].

2.5.5 LAIV ENHANCES PNEUMOCOCCAL BACTERIAL DYNAMICS IN THE URT IN A MANNER HIGHLY ANALOGOUS TO WT INFLUENZA VIRUS.

Numerous previous investigations have demonstrated that replication of WT influenza virus within the URT predisposes to excess bacterial replication and colonization within the NP, particularly by *Streptococcus pneumoniae* [32, 38, 41]. Because, as demonstrated above, LAIV replicates to near WT levels when in the cooler temperatures of the URT, we sought to study effects of LAIV on bacterial carriage density within the NP of mice, and compared them to the changes in bacterial carriage following WT virus infection. LAIV vaccination or sublethal

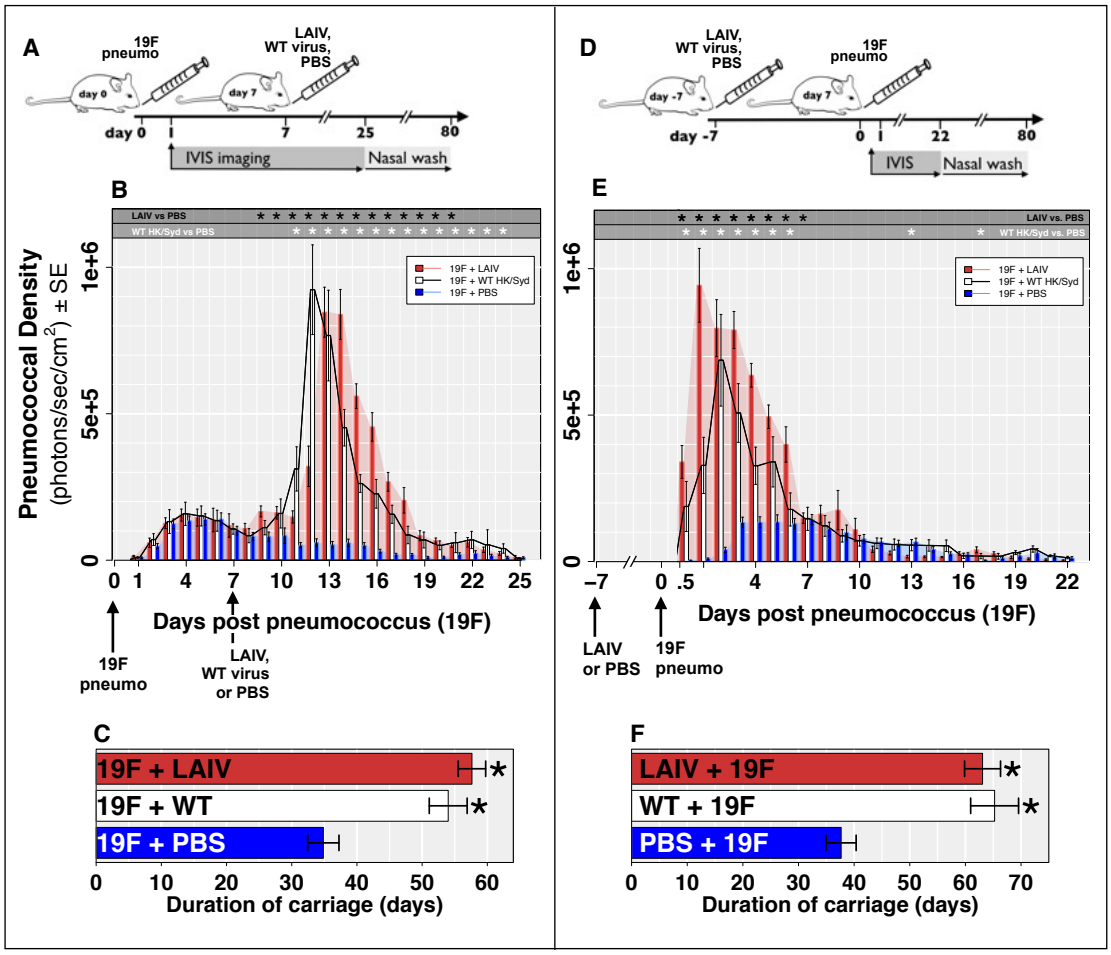

Figure 2.2 LAIV vaccine and WT influenza infection similarly enhance 19F pneumococcal carriage density and duration of colonization.

Groups of 12-14 mice were vaccinated with LAIV, infected with WT influenza virus or PBS vehicle at 7 days following colonization with 19F pneumococcus (a-c) or 7 days prior to colonization with 19F (d-f). Bacterial strains constitutively expressed luciferase and nasopharyngeal carriage density was measured via in-vivo imaging (IVIS) at 12 -hours post-bacterial infection and daily thereafter (b, e). Duration of colonization (c, f) was measured via bacterial plating of nasal washes taken daily after carriage density decreased below the limit of detection for IVIS (~1e4 CFU/ml). Asterisks indicate significant differences between vaccinated (Black asterisks in b, e) or WT influenza virus infected (white asterisks in b, e) vs. control groups (students t-test; p<.05) and error bars represent standard errors around the mean.

infection with the WT parent strain were delivered seven days following inoculation with a common nasopharyngeal colonizing strain of pneumococcus type 19F (Fig. 2a-c), included in the current pneumococcal conjugate vaccine [42]. Following vaccination, normal bacterial clearance from the NP was halted, and

reverted to exponential growth within 3 days post-vaccination (Fig. 2b). Receipt of LAIV significantly increased density of bacterial carriage and extended mean duration of colonization from 35 to 57 days (Fig. 2c). Of particular importance, these effects were nearly identical in all aspects to the effects of the WT influenza virus on bacterial carriage density and duration (Figs. 2b and c). Though no detectable morbidity was associated with vaccination alone (Fig 1b above), vaccination in the presence of bacterial colonization resulted in very mild, though sustained weight loss (~3-5%, $p<.05$) relative to colonized, unvaccinated controls (Fig. S2 in Appendix A) that corresponded with the time of greatest excess in bacterial proliferation.

To test whether order and timing of vaccination relative to bacterial acquisition is important, LAIV or WT virus was administered 7 days before (rather than after) 19F colonization (Fig. 2d-f). Early vaccination or infection with WT virus each led to immediate excess bacterial outgrowth following pneumococcal inoculation, relative to mice pretreated with PBS vehicle (Fig. 2e). This increase was generally more pronounced following LAIV vaccination relative to WT virus infection, but the difference only reached statistical significance at day 1 post bacterial infection. Increases in mean durations of carriage were also demonstrated and were similar between the two groups, with duration extending from 38 days following treatment with PBS to 63 days or 65 days following LAIV or WT virus, respectively (Fig. 2f).

To further define the temporal nature of these interactions and simultaneously test whether this response is strain specific, vaccination was given

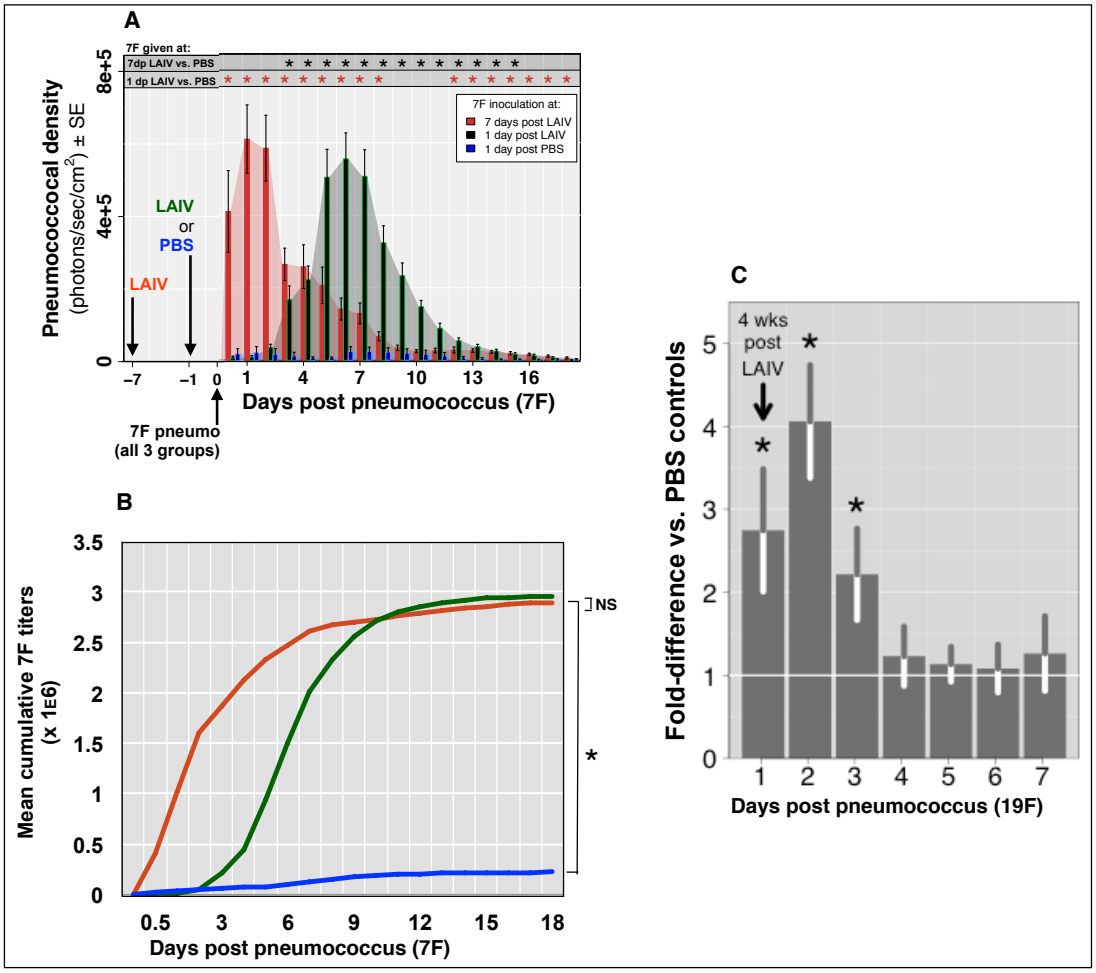

Figure 2.3 LAIV enhancement of pneumococcal density is time-dependent and long-lasting.

Groups of 12-14 mice were vaccinated with LAIV or PBS vehicle at 1 or 7 days prior to colonization with pneumococcal serotype 7F. Bacteria strains constitutively expressed luciferase and bacterial NP density was measured via IVIS in-vivo imaging (A-B). Mean cumulative bacterial titers in B were calculated by first calculating the cumulative bacterial titers per individual mouse NP at each time point, and then calculating the average and SE across the individual cumulative titers per time point, rather than simply averaging the areas under the mean density curves shown in A. Asterisks indicate significant differences in bacterial densities between vaccinated and PBS control groups (dark green = LAIV given 7 days prior and red = LAIV given 1 day prior to 7F inoculation; two-tailed students t-test; p<.05). (C) Groups of mice were vaccinated with LAIV (n=20) or PBS vehicle control (n=30), respectively, at 28 days prior to colonization with 19F pneumococcus. Fold-differences per day between mean bacterial densities measured in mice treated 28 days prior with LAIV vs. PBS are reported. Error bars indicate standard errors of the mean and asterisks indicate significant differences (p<.05) from PBS controls. (ie: PBS controls; two-tailed single sample t-test).

at either 1 or 7 days prior to infection with a slightly more invasive type 7F pneumococcus (Fig. 3a). Maximum bacterial density in both groups of vaccinated mice reached a near 100-fold increase vs. PBS controls. When inoculation with bacteria followed only 1 day (vs. 7) post-vaccination, similar but delayed dynamics (Fig. 3a) and cumulative bacterial titers (Fig. 3b) were measured. Interestingly, the delay was consistent with the difference in time from vaccination to bacterial inoculation between the two groups.

We sought to understand whether these effects of LAIV vaccination on bacterial proliferation would continue over a longer duration of time. Mice were infected with pneumococcus a full 28 days following LAIV vaccination; well after viral clearance from the NP was complete (~7 days post vaccination). Despite the 28 day lag between LAIV and pneumococcal infection, LAIV continued to yield immediate excess bacterial proliferation, relative to PBS controls (Fig. 3c), however the effect was modest and short-lived with only a 2 to 4-fold increases over PBS controls measured between days 1-3 post infection, respectively. By day 4, bacterial density in the NP returned to control levels and duration of colonization was not increased.

2.5.6 LAIV ENHANCES *STAPHYLOCOCCUS AUREUS* DYNAMICS IN THE URT.

We next sought to test the effects of LAIV on carriage of an entirely distinct, but important Gram-positive bacterium, *Staphylococcus aureus.* LAIV was administered 7 days prior to infection with *S. aureus* strains Wright (Fig. 4a-c) or Newman (Fig. 4d-e). Similar to the previous experiments using two strains of

pneumococcus, density of these two strains of *S. aureus* following vaccination was increased at all measured time points for both Wright and Newman strains (Fig. 4d, g) and duration of colonization was significantly extended 3-5 fold over PBS controls (Fig. 4e, h).

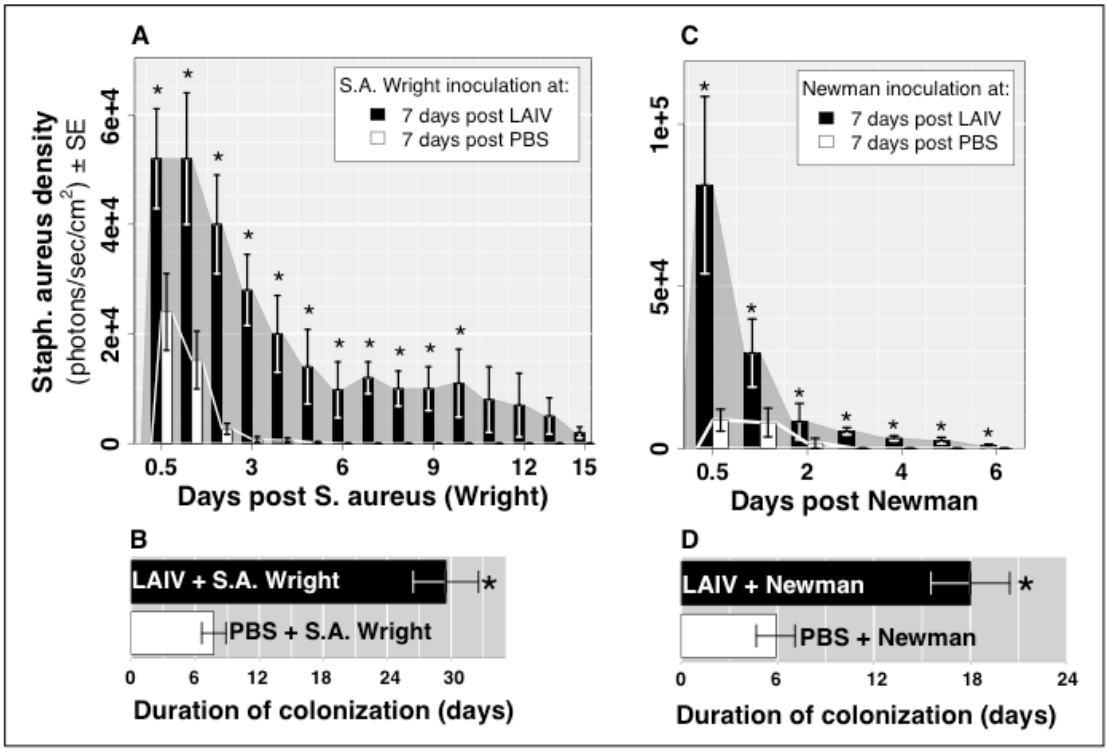

Figure 2.4 LAIV vaccine enhances bacterial load and duration of staphylococcal carriage.

Groups of 12-14 mice were vaccinated with LAIV or PBS vehicle 7 days prior to colonization with S. aureus strain Wright (a, b) or Newman (c, d). S. aureus constitutively expressed luciferase and bacterial density was measured via IVIS in-vivo imaging. Duration of colonization (b, d) was measured via bacterial plating of nasal washes taken daily after carriage density decreased below the limit of detection for IVIS imaging. Asterisks indicate significant differences between vaccinated and control groups (two-sided students t-test; p<.05) and error bars represent standard errors around the mean.

2.5.7 LAIV DOES NOT INCREASE MORBIDITY OR MORTALITY FROM BACTERIAL LRT INFECTIONS.

Given the severe and often lethal interaction seen between circulating influenza virus strains and bacterial lower respiratory tract infections [11, 43], we assessed the effects of LAIV on bacterial LRIs and mortality and compared these effects to those seen following WT influenza-bacterial coinfection and single

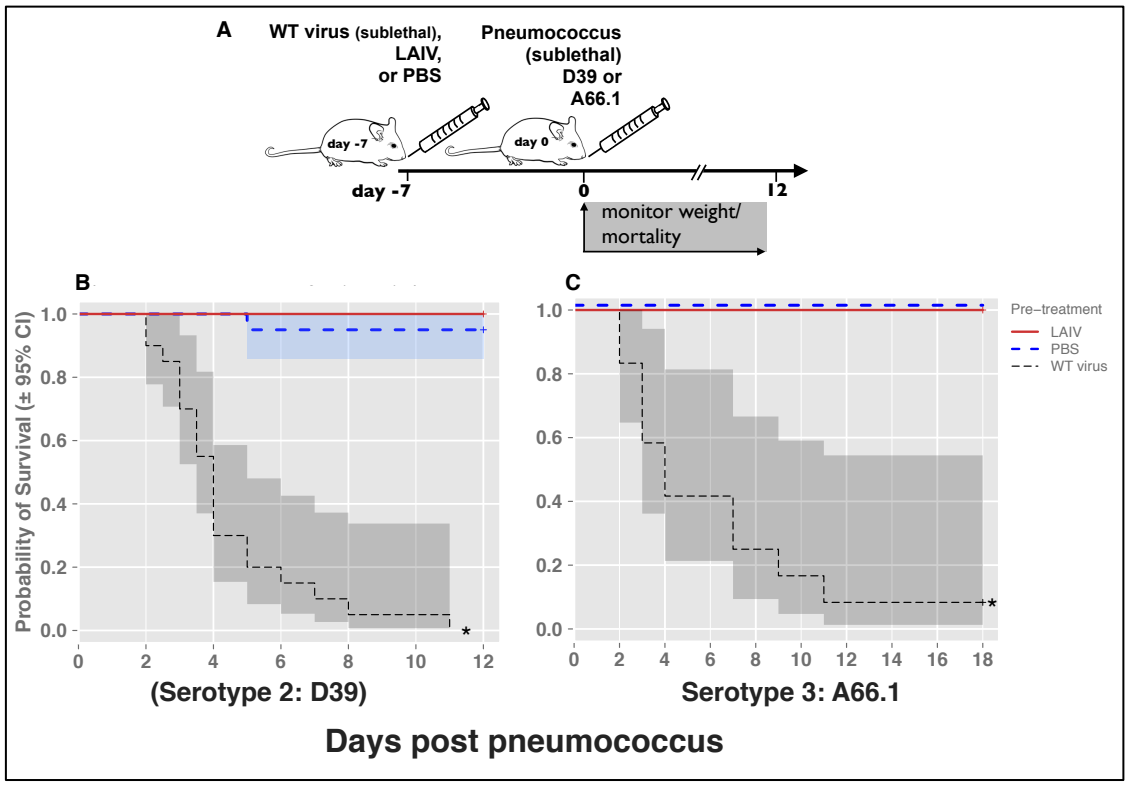

Figure 2.5 LAIV vaccine does not increase bacterial pneumonia or severe disease.

Groups of mice received intranasal LAIV vaccination (solid red curves), sublethal infection with WT influenza virus (broken black curves) or PBS (broken blue curves) seven days prior to inoculation with a sublethal dose of Streptococcus pneumoniae type 2 (1e5 CFU D39; n=20 per group; figure b) or type 3 (1e3 CFU A66.1; n=12-15 per group; figure c) and body weight and mortality were observed at least every 12 hours for the first 4 days post pneumococcal inoculation and daily thereafter. Kaplan-Meier survival curves with 95% confidence intervals were constructed and asterisks indicate statistically significant differences (log-rank test; p<.05) between LAIV or WT virus infected groups vs. PBS controls.

infections with bacteria. Mice received LAIV, WT influenza virus or PBS control and seven days later (a time known to maximize the lethal effects of influenza-bacterial coinfections 31) were inoculated with a sublethal dose of either of the highly invasive type 2 or 3 pneumococcal serotypes D39 or A66.1, respectively (Figs 5a-c). In contrast to 100% mortality observed when sublethal inoculation with D39 or A66.1 followed pretreatment with wild-type influenza virus, bacterial inoculation following pretreatment with LAIV demonstrated no increases in morbidity (ie: weight loss; data not shown) or mortality (Fig. 5b, c) relative to bacterial infection alone.

2.6 DISCUSSION

The potent and often lethal effects of an antecedent viral influenza infection on secondary bacterial disease have been reported previously [11, 21, 44-46]. Viral replication induced epithelial and mucosal degradation and the ensuing innate immune response yield diminished capacity to avert secondary bacterial infections. Recent clinical and experimental data suggest that influenza virus infection may exert its influence beginning in the URT by enhancing susceptibility to bacterial colonization [14, 47, 48] and increasing NP carriage density [38].

Though vaccination with LAIV, in the longer-term, thwarts secondary bacterial infections by inhibiting primary infections with influenza virus [32, 49], the immediate effects of LAIV vaccines on bacterial replication and disease have never before been described. Indeed, although vaccines are among our greatest achievements in the constant battle against microbial pathogens, the effects of vaccination on distinct

pathogens species unrelated to vaccine-targeted pathogens have, until now, remained entirely unexplored. LAIVs selectively replicate in the URT, partially denude the epithelium [50] and induce robust innate immune responses that ultimately contribute to long-term protective immunity [28]. In so doing, LAIVs may, like WT influenza viruses, condition the site of replication for enhanced secondary bacterial colonization.

Here, we demonstrated that vaccination with LAIV, like a WT influenza virus, induces swift increases in bacterial density within the URT with no discernable differences in effects on bacterial dynamics in the NP between the two virus strains. A lag between viral inoculation and excess bacterial replication of at least 3-5 days was consistently measured, no matter the bacterial strain. Of particular interest, the type I interferon, IFN-beta, known to play a pivotal role in excess pneumococcal colonization following WT influenza virus infections [38] was maximally upregulated at 3 days post LAIV, coincident with commencement of excess bacterial proliferation. After the 3-5 day threshold following vaccination was met, the murine NP remained conditioned for excess pneumococcal replication for at least 28 days (our furthest time point out) post vaccination. However, as the delay between vaccination and bacterial infection was increased, the magnitude of the effects of vaccination on bacterial dynamics became considerably more modest, though still statistically significantly excess growth measured when acquisition followed 28 days post vaccination.

While the studies described here are limited in scope to murine models, enhanced bacterial load in the URT following LAIV may agree with human

data [51] where LAIV has been associated with increases in adverse upper respiratory tract symptoms. Although adverse URT symptoms following FluMist® are considered to be of viral etiology, they are most evident in children <5 years of age, where rates of bacterial carriage are greatest [52]. Potentially corroborating this, are data from a large prospective double-blinded trial of Flumist®, (trial MI-CP111, [53]) that assessed reactogenicity and adverse URT events within the first 28 days following vaccination in ~3000 children between the ages of 6-59 month. This trial demonstrated a bimodal increase in URT symptoms following FluMist®, the first between day 2 and 4 post vaccination and the second between days 5-10 post vaccination [53]. While these increased URT events (relative to TIV controls) were considered normal reactions to the live-vaccine, the bi-modal nature of the increased symptoms suggests that two distinct mechanisms may be in place. In the context of the current findings, the first peak may correspond with viral replication while the second, more sustained peak may, at least in part, be driven by symptoms due to excess bacterial carriage.

Perhaps the most important finding from our study, with regard to the health of the public and potential concerns regarding vaccination, is that LAIV did not enhance lower respiratory tract infections, morbidity or mortality following bacterial infections which are, by most accounts, the most significant issues to be concerned with in terms of respiratory tract bacterial disease. Indeed, this finding is consistent with numerous epidemiologic reports all failing to detect any serious adverse sequelae of LAIV vaccination in humans [51, 54]. Further, this finding is consistent with significantly diminished LAIV virus replication within the lower

respiratory tract, suggesting perhaps that viral replication is a requirement for the synergistic response seen between WT influenza viruses and bacterial LRT infections.

While care should be taken to not overgeneralize the data described here to all vaccines, the broad implications suggest that live-attenuated viral vaccines may have unintended consequences on important human bacterial pathogens unrelated to the vaccine target species. Further, our findings suggest a role for laboratory models of multispecies interactions with vaccine strains to inform future vaccine monitoring and evaluation programs aimed at identifying, thus far entirely unrealized 'unconventional' effects, both beneficial or detrimental, of live-attenuated viral vaccines and cross-species microbial dynamics.

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CHAPTER 3. LIVE ATTENUATED INFLUENZA VACCINATION PREDISPOSES TO AND INCREASES DURATION OF BACTERIAL ACUTE OTITIS MEDIA IN MICE

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3.1 ABSTRACT

Background: Infection with Influenza A virus (IAV) increases host susceptibility to lower and upper respiratory tract bacterial infections, resulting in increased bacterial colonization and complications such as pneumonia, bacteremia and acute otitis media (AOM). Recently, vaccination with live attenuated influenza virus (LAIV) was reported to enhance subclinical bacterial colonization within the nasopharynx, akin to that following WT IAV. Although LAIV did not predispose to bacterial pneumonia or bacteremia, whether LAIV predisposes to clinically relevant bacterial AOM has not been investigated. **Methods**: Eight week old Balb/c mice received LAIV or PBS either during stable pneumococcal colonization or one or seven days prior to pneumococcal inoculation with either of two clinical isolates, 19F or 7F, each engineered to express luciferase. AOM was monitored daily via in-vivo imaging. **Results:** LAIV significantly increased incidence and duration of bacterial AOM episodes irrespective of whether LAIV was antecedent or subsequent to pneumococcal infection or pneumococcal serotype. Onset of excess bacterial AOM was strongly dependent on time since LAIV vaccination, with a minimum of approximately four days post LAIV required before excess bacterial AOM was detected. **Conclusions**: Although LAIV vaccination is safe and highly effective at reducing influenza virus infections, our results indicate that interactions between LAIV and bacterial pathogens may have clinical implications, predisposing vaccinees to excess pneumococcal AOM. These data warrant further investigations into interactions between live attenuated viral vaccines and the human microbiome, particularly at mucosal surfaces lining the gut and respiratory tracts.

3.2 INTRODUCTION

Infection with influenza A virus (IAV) increases susceptibility to severe lower and upper respiratory tract bacterial infections resulting in complications such as pneumonia, bacteremia, sinusitis and bacterial acute otitis media (AOM);[1] the latter being a major contributor to the global burden of pediatric disease and remains one of the most common diagnoses leading to the prescription of antibacterial agents in the United States.[2] While bacterial AOM often occurs in isolation, increasing evidence suggests that primary or concurrent viral respiratory infections of the upper respiratory tract (URT) may play uniquely important roles in enhancing bacterial acquisition, colonization and, ultimately, progression from asymptomatic bacterial carriage to AOM,[3] notably from *Streptococcus pneumonia* (the pneumococcus), and *Staphylococcus aureus.*[1, 4]

Although the mechanisms underlying influenza mediated susceptibility to bacterial AOM are not entirely defined, they likely include a combination of IAV mediated cytotoxic breakdown of mucosal and epithelial barriers of the URT[5-8] and aberrant innate immune responses to bacterial invaders in the immediate post-influenza state, characterized by uncontrolled pro- and anti-inflammatory cytokine production, excessive leukocyte recruitment and immunopathology.[1, 9-13] When coupled with diminished mucosal defenses, such an environment becomes increasingly hospitable for bacterial pathogens to flourish and cause clinical disease in the days and weeks following influenza infection.

Increasing evidence links the early innate immune response triggered by vaccination to long-term vaccine efficacy.[14] Thus, a goal of vaccination is to elicit an immune response as close to that elicited by the pathogen itself, without subsequent disease. The intranasally administered live-attenuated Influenza vaccine (LAIV) is comprised of 1:1:6 reassortant viruses containing the hemagglutinin (HA) and neuraminidase (NA) surface proteins from wild-type viruses on a temperature sensitive and attenuated 'backbone' designed to enable efficient viral replication in the cooler temperatures of the (URT) but not the warmer temperatures of the lower respiratory tract (LRT).[15, 16] Through selective replication in the URT, LAIV proteins are exposed to the host immune system in their native conformation, eliciting highly robust (IgA), serum (IgG) and cellular immune responses mimicking those of the pathogenic virus itself. [17] without subsequent virus mediated disease in the LRT.[18, 19]

Recently our lab reported that LAIV, while safely providing long-term immunity against influenza and significantly reducing post-influenza secondary bacterial infections,[20] inadvertently enhances duration and density of bacterial carriage in the URT of mice.[21] Importantly, in contrast to wild-type IAV infections, LAIV did not increase bacterial outgrowth in the LRT, having no effect on bacterial pneumonia or bacteremia. What is not known is whether LAIV replication in the URT may inadvertently catalyze the transition from asymptomatic bacterial carriage to clinically important bacterial AOM - akin to what has been shown following WT IAV infections.[13, 22, 23]

3.3 MATERIALS AND METHODS

3.3.1 VACCINATIONS AND INFECTIOUS AGENTS

Live-attenuated influenza vaccinations were developed from a parent H3N2 1:1:6 reassortant virus developed as described previously,[24] containing the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) from A/Hong Kong/1/68 (HK68) and A/Sydney/5/97 (Syd97) isolates, respectively, and the six internal protein gene segments from A/Puerto Rico/8/34 or PR8 (referred to hereafter as HK/Syd or WT virus). LAIV consisted of a temperature sensitive (*ts*) attenuated variant of HK/Syd (HK/Syd_{ts} or LAIV) that contains site-specific mutations in the PB1 and PB2 RNA segments of the genome as described previously. These are the same mutations found in the attenuated A/Ann Arbor/6/60 master donor strain used to produce the commercial product known as FluMist®.[16] LAIV viruses were propagated in 10-day-old embryonated chicken eggs at 33°C and characterized in Madine-Darby canine kidney cells ($TCID_{50}$). In-vitro and in-vivo growth dynamics have been reported.[21] The pneumococcal carrier isolates ST425 (serotype 19F) and ST191 (serotype 7F) have been previously described.[3] These strains were engineered to express luciferase, as described.[3, 25]

3.3.2 ANIMAL AND INFECTION MODELS

8-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. All inoculations were via the intranasal route. LAIV vaccination consisted of 2e6 $TCID_{50}$ HK/$Syd_{ts,att}$ LAIV in 40ul PBS. Pneumococcal infections with

19F and 7F *Streptococcus pneumoniae* were as described previously.[3] Briefly, bacterial cultures were grown in Todd Hewitt broth (Difco Laboratories, Detroit, MI) containing 0.5% yeast (THY) until mid- to late-log phase (O.D. ~0.3) and aliquots stored at -80° C in 10% glycerol and quantified via serial dilution on blood agar plates. Inoculations were prepared from frozen aliquots and consisted of $1x10^6$ and $1x10^5$ colony forming units of 19F and 7F pneumococci, respectively, in 25 µls PBS. Infections were initialized via careful administration of 12.5 µls of bacteria to each nare under general anesthesia with 2.5% inhaled isoflurane (Baxter Healthcare Corporation, USA). All experiments were conducted in biosafety level 2 facilities, in a manner in accordance with the guidelines of the committee on care and use of laboratory animals.

3.3.3 BIOLUMINESCENT IMAGING

Mice were imaged using an IVIS CCD camera (Xenogen) as described.[3] Middle ear bacterial density was measured as total photons/sec/cm^2 in pre-specified regions covering the middle ear canal and background (calculated for each mouse on a region of equal area over the hind limb) was subtracted. AOM was defined as a bacterial density > 40,000 photons/sec/cm^2. This threshold has been previously described for this particular infection model with the same CCD camera within the same laboratory environment.[26] as the lowest threshold that was consistently exceeded only when bacteria were present in the middle ear while remaining high enough to eliminate false positives. Quantitation was performed using Living Image software (v. 3.0; Caliper Life Sciences) as described.[3]

A single episode of AOM was defined as any continuous detection of AOM that was not interrupted by greater than 2 days. This two-day 'interruption allowance' was necessary to account for normal fluctuations in bacterial density that enter sub-threshold levels before rebounding back to full AOM, as defined above. Additionally episodes were categorized as early or late onset. Early onset was defined as an initial episode of AOM in a given mouse that occurs within 5 days of bacterial inoculation. Late onset was defined as any episode that commenced at least 2.5 days after clearance of a previous episode or at least five days post pneumococcal infection.

3.3.4 Statistical Analyses

All statistical analyses were performed within the R statistical computing environment (R version 2.14, R foundation for statistical computing, R Development Core Team, Vienna, Austria). Kaplan Meier curves were constructed for freedom from acute otitis media for each mouse per group and the log-rank test was used to calculate statistically significant differences between groups. Frequencies of AOM were plotted using Loess smoothing (span 0.2) and differences in daily frequencies of AOM in each vaccinated group, relative to PBS controls were calculated using Fisher's Exact test for differences in proportions. Differences in mean duration of acute otitis media were calculated using two-tailed two-sample students t-tests. The false detection rate was used to adjust for multiple comparisons where appropriate,

and statistical significance was considered when the calculated probability is less than an alpha of 0.05.

3.4 RESULTS

3.4.1 LAIV INCREASES ATTACK RATE OF AOM IN PNEUMOCOCCAL PRE-COLONIZED MICE

Nasopharyngeal carriage of pneumococcus is believed to be a prerequisite for bacterial AOM and elevated bacterial density has been associated with transition from asymptomatic carriage to middle ear infections.[27] We have previously demonstrated that vaccination with LAIV significantly enhances bacterial titers in the nasopharynx.[21] As such, we sought to determine whether LAIV vaccination of pneumococcal colonized mice may enhance transition to bacterial AOM. Groups of 12-14 mice were colonized with the type 19F pneumococcus, a clinical isolate often found colonizing the nasopharynx of children, and a well established model organism for colonization and AOM in mice.[3] Bacteria were given sufficient time (7 days) to establish stable colonization of the nasopharynx, assessed via IVIS imaging of the nasopharynx and previously reported.[28] before inoculation with either LAIV or PBS vehicle. Within 12 hours of LAIV inoculation, mice demonstrated increased incidence of bacterial acute otitis media (Fig. 1), as determined by in-vivo imaging of the middle ear (see materials and methods). By four days post-LAIV, 85% of mice had at least one episode of AOM, vs. only 25% of PBS controls. The majority of initial onset of AOM episodes in the LAIV group occurred within the first 4 days following

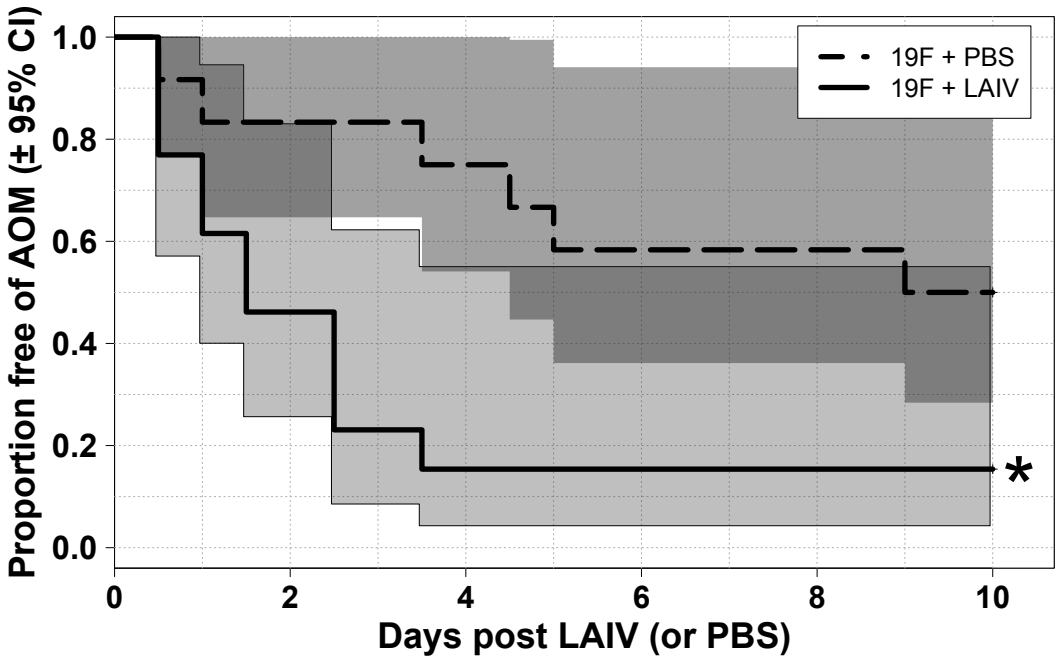

Figure 3.1 LAIV enhances incidence of acute otitis media in pre-colonized mice.

Groups of 12-14 8-week old Balb/c mice were colonized, intranasally, with a type 19F pneumococcal bacteria, engineered to express luciferase, and seven days later inoculated with LAIV or PBS vehicle as a control. Acute otitis media was measured via in-vivo imaging of the middle ear at 12 hour intervals for the first two days following LAIV or PBS and daily thereafter. Initial onset of bacterial AOM was recorded for each mouse and Kaplan-Meier survival curves were constructed. Data is reported as freedom from acute otitis media from time of LAIV or PBS inoculation and the log-rank test was used to determine statistically significant differences between groups. Asterisks (*) indicate a p-value less than 0.05 vs. PBS controls.

vaccination and freedom from AOM stabilized in both groups after day five (with the exception of a single new case in the PBS group at day nine). By day ten following LAIV or PBS inoculation, incidence of AOM in LAIV vaccinated mice remained significantly greater than PBS controls (85% in LAIV vs. 50% in PBS controls, p=.017).

3.4.2 ANTECEDENT LAIV VACCINATION PREDISPOSES TO AOM FOLLOWING BACTERIAL INFECTION

To address the question of whether antecedent inoculation with LAIV predisposes to AOM following bacterial infection, and to ensure the effect of LAIV on bacterial AOM is not a 19F strain specific phenomenon, mice received a colonizing dose of the pneumococcal serotype 7F (a slightly more invasive clinical strain and a well described model organism for pneumococcal AOM in mice[3]) at either seven or one day post LAIV (n=26 for each group) or 1 day post PBS (n=20), referred to hereafter as 7dpV, 1dpV and 1dpPBS, respectively (Fig. 2). Inoculation with LAIV seven days

before pneumococcal infection (7dpV group), led to immediate increases in incidence of AOM with only 30% of 7dpV mice (8/26) remaining free from bacterial AOM 24 hours post-infection. This was in contrast to 81% (21/26) of 1dpV group and 75% (15/20) of the 1dpPBS group that remained free from AOM at 24 hours post-infection. Following initial enhancement of AOM in the 7dpV group, only two new cases (ie: cases in mice previously free from AOM) were seen over the following two weeks, at days five and eight post bacterial infection.

Excess AOM was also eventually detected in the 1dpV group. Interestingly however, onset of excess AOM was distributed in this group over the first 5 days post infection, with the burden of excess AOM commencing between days 3 and 5 post-infection, corresponding to days 4-6 post-LAIV, a time previously demonstrated to maximize bacterial colonization of the nasopharynx.[21]

3.4.3

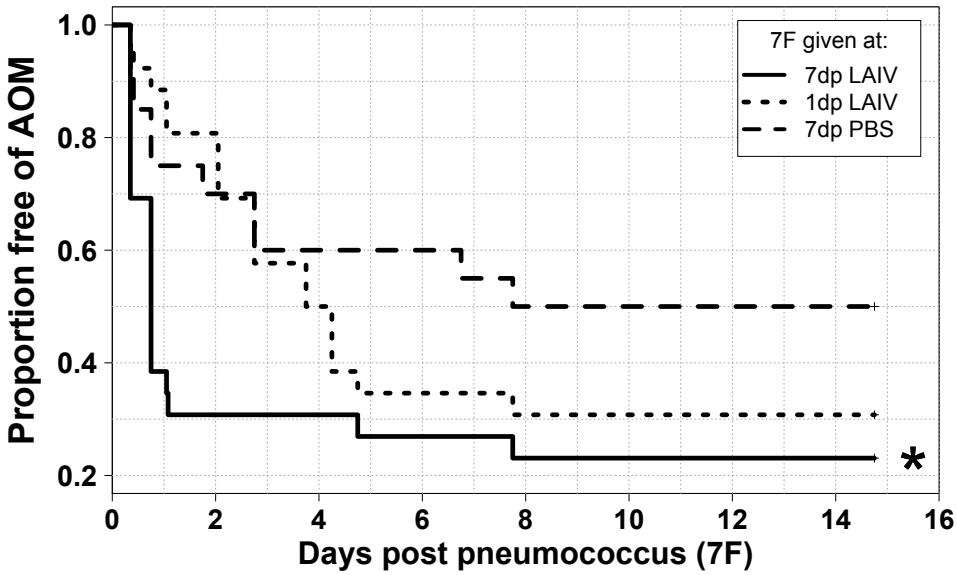

Figure 3.2 Freedom from acute oitis media following bacterial infection in recently vaccinated mice.

Groups of 8-week old Balb/c mice received LAIV at 7 days (n=26) or 1 day (n=26) or PBS (n=20) 1 day before inoculation with the type 7F pneumococcal bacteria, engineered to express luciferase. In-vivo imaging was used to detect bacterial acute otitis media every 12-15 hours for the first two days following pneumococcal infection and at least daily thereafter. Initial onset of pneumococcal acute otitis media was recorded for each mouse and Kaplan Meier Survival curves were constructed to describe freedom from pneumococcal acute otitis media. Asterisks (*) indicated a log-rank test p-value less than 0.05, vs. PBS controls, corrected for multiple comparisons using the false discovery rate.

3.4.4 LAIV MEDIATED ENHANCEMENT OF BACTERIAL AOM IS DELAYED POST VACCINATION

To better understand the dynamics of AOM, we investigated overall frequency per day of AOM for each group (Fig. 3), which differs from our KM analysis above in that KM considers only time of first onset in a given mouse, rather than overall proportion with AOM at any particular time in our experimental groups. Consistent with the KM analysis, mice vaccinated 7 days prior to

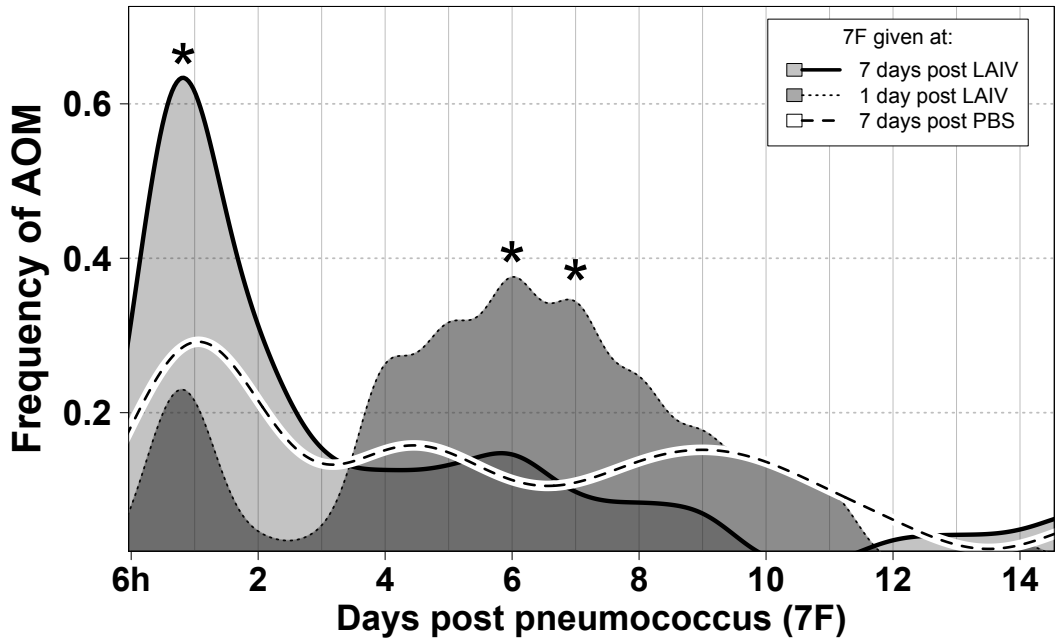

Figure 3.3 Frequency of acute otitis media following bacterial infection of recently vaccinated mice.

Mice were inoculated with LAIV either 7 days or 1 day (n=26 for both) or with PBS at 1 day (n=20) prior to infection with the type 7F pneumococcus, engineered to express luciferase, and in-vivo imaging of the middle ear was performed to measure presence of pneumococcal acute otitis media. Frequency of otitis media is plotted for each group and differences in daily frequency of AOM between groups was tested for statistical significance using fishers exact test for differences in proportions. Asterisks (*) indicate statistically significant differences from PBS based on a p-value < 0.05.

pneumococcal infection had significantly increased frequencies of AOM for the first 24-48 hours post infection relative to PBS controls. Frequency peaked in this group approximately 24 hours post-infection with slightly greater than 60% (16/26) of mice with AOM. In contrast, only 20-30% of mice receiving LAIV or PBS one day prior to bacterial infection had AOM, and these episodes were very short lived with almost no AOM in these groups by day 2. While maximum frequency of AOM was reached 24 hours post infection in the 7dpV group, mice infected only one day following LAIV had a second 'wave' of AOM episodes that began 4 days post vaccination (see dark grey curve in Fig. 3). This second wave of AOM, while lower in

maximum frequency (~40%) than the 7dpv group had a broader and more sustained peak that lasted from days 4-8 post bacterial infection.

3.4.5 LAIV INCREASES DURATION OF BACTERIAL AOM INFECTIONS

Duration of AOM was measured for each AOM episode per mouse, as defined above, and mean durations calculated for each group. Duration of AOM was significantly increased across all vaccinated groups, regardless of pneumococcal strain (19F or 7F) or whether LAIV was given prior to or following pneumococcal infection. When LAIV or PBS was administered to mice with pre-established 19F colonization, LAIV recipients had a mean duration of AOM nearly 2-fold greater than PBS controls (2.3 vs. 1.2 days; $p < 0.05$; Fig 4a). Similarly, when mice received LAIV seven days or one day prior to bacterial infection, mean duration of AOM episodes were nearly 3- and 2-fold increased over PBS controls ($p < 0.05$ for each; Fig 4b). Interestingly, when episodes were classified into early and late onset (see materials and methods for classification criteria) durations of early onset cases in the 7dpv group were nearly identical to durations of late onset cases in the 1dpv group (~3.75 days in each group) and each were greater than 2-fold increased over their respective PBS controls (~1.5 days; $p < 0.05$; Fig. 4c). Alternatively, durations of early onset episodes in the 1dpv group and duration of late onset episodes in the 7dpv group were no different than PBS controls. Taken together with the Kaplan Meier analyses above, these data demonstrate a strong dependence on time since LAIV inoculation, and not time since bacterial infection, with a minimum of four days post vaccination required before enhancement of AOM is detected.

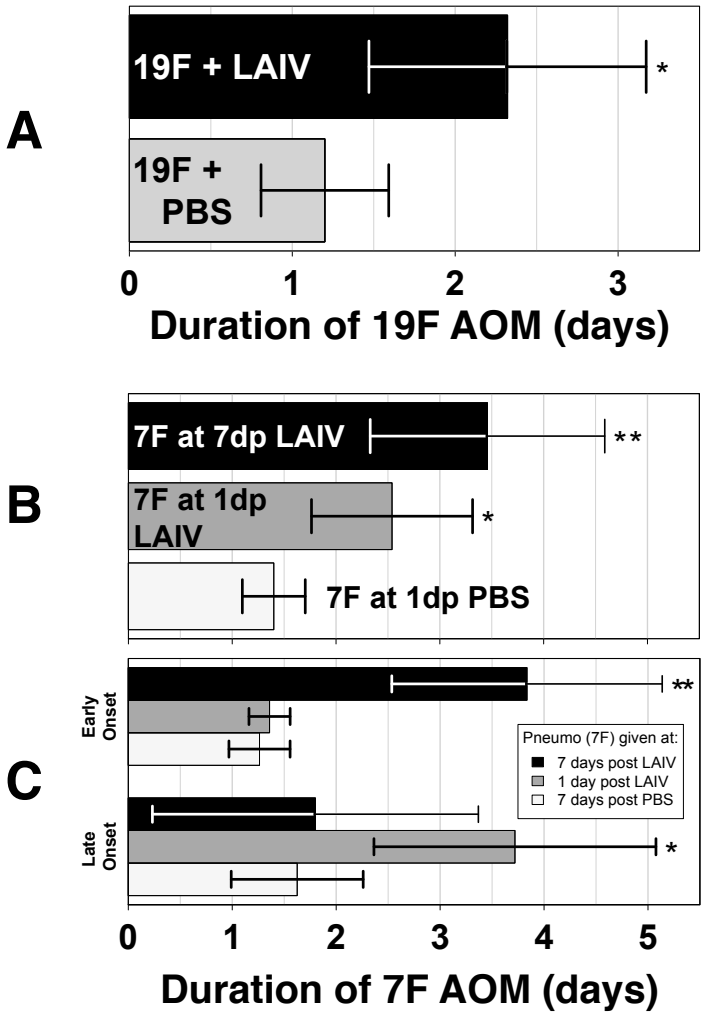

Figure 3.4 LAIV enhances duration of bacterial otitis media.

Groups of Mice were colonized with 19F pneumococcus 7 days prior to inoculation with LAIV (n=14) or PBS (n=12) (A) or received LAIV at 7 or 1 day (n=26 for each) or PBS at 1 day (n=20) prior to infection with type 7F pneumococcus. Duration of acute otitis media episodes were measured and mean durations reported for 19F (A) and 7F (B) AOM where a single episode was defined as any continuous detection of AOM in a given mouse that was not interrupted by greater than 2 days. Episodes of 7F AOM were further classified as early (onset within the first 5 days following infection) or late onset (>2 days following termination of an early episode or >5 days post infection) and mean durations reported for each group (C). Statistical significant differences (vs. PBS controls) were tested using two-tailed two-sample students t-tests with FDR correction for multiple comparisons. Asterisks (*) indicate corrected p-values < 0.05 and error bars represent 95% confidence intervals around the mean.

3.5 DISCUSSION

The potent and often lethal effects of an antecedent viral influenza infection on secondary pneumococcal invasive disease and pneumonia have been reported.[1, 11, 29-31] Viral replication induced epithelial and mucosal degradation and the ensuing innate immune response yield diminished capacity to avert secondary bacterial

infections. Recent clinical and experimental data suggest that influenza viruses may exert their influence beginning in the URT by enhancing susceptibility to bacterial colonization,[3, 32, 33] increasing NP carriage density [22] and enhancing incidence of AOM.[13]

Though vaccination with a live-attenuated influenza virus, in the longer-term, thwarts influenza-bacterial coinfections by inhibiting the viral infection itself, [18, 28] LAIV vaccines have recently been found to transiently enhance density and duration of bacterial colonization within the nasopharynx of mice, with dynamics nearly identical to those following WT IAV.[21] Importantly, unlike WT IAV, LAIV did not result in increased bacterial proliferation or disease in the LRT, presumably due to temperature sensitive nature of LAIV abrogating viral growth within the warmer temperatures of the lungs. Although LAIV did not effect clinical bacterial LRT infections, the effects of LAIV on transition from colonization to bacterial disease within the URT, a region where LAIV replicates efficiently, had not not been studied.

Here, we discovered that vaccination with LAIV significantly increased incidence and duration of pneumococcal AOM, irrespective of bacterial serotype or order of viral vs. bacterial inoculation. Interestingly, a minimum period of time of approximately four days was required before enhancement in AOM was noted when LAIV preceded pneumococcal infection.

The dynamics, with regard to time-since-vaccination of increased AOM closely match pneumococcal colonizing dynamics of the nasopharynx following WT IAV or LAIV,[21, 28] and support the notion that NP colonizing density may be associated with progression to AOM. Interestingly, the delay in increased onset of

AOM in mice vaccinated only one day before bacterial inoculation was approximately the same as the time to peak LAIV viral titers in the URT.[21] Thus, a majority of excess AOM occurs during or soon after viral clearance from the URT. This finding supports numerous reports[1, 10-12, 22] that point towards a complex coupling of poorly coordinated antibacterial innate immune defenses and epithelial damage following influenza infection underlying excess susceptibility to bacterial disease following influenza infections.

On the other hand, the steady increase in onset of AOM measured immediately following LAIV vaccination in 19F pre-colonized mice suggests that introduction of LAIV in the presence of existing bacterial colonization yields enhanced bacterial AOM that is concurrent with viral replication and precedes viral enhanced nasopharyngeal colonizing density, which tend to increase beginning at 4 days post LAIV inoculation. This suggests that the mechanisms of virus induced bacterial AOM may differ according to order of inoculation. Indeed, it may be that even low levels of viral replication in the URT, while not immediately effecting overall bacterial carriage density in the nasopharynx, may rapidly disrupt a delicate balance that naturally exists to prevent asymptomatic carriage from transitioning to bacterial AOM.

While our data suggest that vaccination with LAIV may enhance progression to bacterial AOM when vaccination is administered to pneumococcal colonized individuals or when bacterial acquisition occurs shortly after vaccination, the overall effect of LAIV vaccines measured in humans has been that of significant reductions in viral influenza infections, including otitis media.[34] We hypothesize

that large reductions in virus mediated AOM following LAIV vaccination may well mask LAIV mediated increases in bacterial AOM, which may occur in only a fraction of vaccinated individuals. As well, LAIV mediated protection from primary influenza infections significantly reduces opportunity for secondary bacterial infections,[20] further reducing LRT and URT bacterial disease, including bacterial AOM.

While we are confident that the overall effects of live-attenuated influenza vaccines are beneficial to reduce all-cause AOM across populations, as has been reported,[35] our data suggest a need for future investigations to closely evaluate effects of LAIV vaccination on bacterial respiratory pathogens. Indeed, as medicine becomes increasingly personalized,[36] it may become possible to tailor classes of vaccines and avenues of vaccine delivery to the individual. Indeed, in this particular example, considering the many benefits of LAIV over inactivated injectable influenza vaccines[37] one could imagine that the choice between a killed injectable vaccine and an intranasal live attenuated vaccine might incorporate risk of pneumococcal carriage or acquisition (ie: number of children in the household, age of vaccine recipient) as a potential variable in the decision making process.

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CHAPTER 4. CROSS-KINGDOM PROTECTION AGAINST LETHAL BACTERIAL INFECTION BY LIVE ATTENUATED VACCINES

Michael J. Mina with Keith P. Klugman and Jonathan A. McCullers

4.1 ABSTRACT

Interactions between pathogen species is increasingly understood to modulate both human health and disease. Viruses often predispose to severe secondary infections by pathogens within distinct ecological kingdoms from the primary infecting agent. More recently, benefit of immunomodulation during primary infection to prevent secondary pathogen invasion has been explored. Influenza virus infection reduces disease from respiratory syncytial virus by suppressing the inflammatory response to sequential infection and infection with aerosolized *Haemophilus influenzae primes the innate immune response to confer complete protection from lethal doses of S. pneumoniae or influenza virus. Live attenuated influenza vaccination (LAIV) contains temperature sensitive and attenuated viral particles that replicate well in the upper respiratory tract, but are restricted in growth in the lower respiratory tract, eliciting an innate immune and adaptive immune responses without subsequent pathology in the lungs. We sought here to determine if vaccination with a live attenuated vaccine could prime the innate immune response to more effectively clear bacterial lung infections and protect from lethal bacterial pneumonia, and we compared the effects of priming with LAIV to priming with a sub-lethal infection of wild-type influenza virus or PBS controls. When mice were primed with LAIV, PBS or WT influenza virus 7 days before inoculation with a lethal dose 50 of either of two highly invasive pneumococcal bacteria, A66.1 or D39, priming with live attenuated vaccine reduced mortality by 87% and 62% for the two bacterial strains, respectively, compared to PBS controls. These benefits conferred by live attenuated virus were in stark contrast to 100% mortality when mice were primed with the unattenuated wild-type parent virus. Improved survival was associated with 100-fold fewer bacteria in the lungs of LAIV vs. PBS primed mice and 100,000 fold fewer bacteria in the lungs than WT influenza virus primed mice. Interestingly however, in the upper respiratory tract, where LAIV grows efficiently, bacterial titers in the nasopharynx were nearly 1000-fold increased*

following LAIV relative to PBS controls, and were no different than nasopharyngeal titers in the WT influenza mice. Our results suggest that priming of the innate immune response with a live attenuated viral vaccination may have the novel secondary benefit of conferring significant cross-kingdom protection against pathogens unrelated to the vaccinating strain. Further these data also support a significant role for viral replication in addition to immune modulation during lethal influenza and bacterial coinfections.

4.2 INTRODUCTION

Interactions between multiple pathogen species have been increasingly recognized as important in modulating both human health and disease. Interactions may be mediated either directly by, for example, virulence factors or quorum sensing systems[1] or indirectly through the host environment and immune response to infection.[2] Infection with influenza A virus (IAV), for example, predisposes to increased bacterial respiratory infections,[3-5] mediated through reduced pathogen-tolerance following epithelial tissue damage[6] and poorly controlled antibacterial innate immune defenses.[3] On the other hand, mixed infection with IAV reduces disease from respiratory syncytial virus (RSV) through a dampening of the cytokine responses that contributes to RSV related immunopathology.[7] Infection with aerosolized nontypeable *Haemophilus influenzae* has been shown to trigger a pulmonary inflammatory responses in the lungs that provides near complete protection from lethal infections with either *Streptococcus pneumoniae* (the pneumococcus) or influenza virus.[8] These findings suggest that immune modulation, when in the absence of pathology, may be a useful and viable modality for treatment or prevention of infectious diseases.

Recently, pulmonary delivery of virus-like particles (VLPs), comprised of noninfectious self-assembling protein cage nanoparticles (often of interest for their use as vaccine adjuvants to enhance immunity), have been shown to prevent disease and mortality from infection with multiple pathogens including influenza, severe acute respiratory syndrome virus, mouse adapted pneumovirus and methicillin-resistant *Staphylococcus aureus* (MRSA).[9, 10] In particular VLP's conferred complete protection from lethal MRSA infection and reduced MRSA lung titers by at least six orders of magnitude.[10] Although the exact mechanisms of protection were not entirely defined, protection was broadly triggered by VLP mediated low-grade non-pathologic inflammation in the lungs. Specifically, protection from MRSA was dependent on VLP induction of the anti-inflammatory cytokines interleukin-13 (IL-13) and/or -4, which amongst other actions, reduced excess production of type II interferon, IFN-γ, following MRSA infection and reduced MRSA immunopathology.

Live attenuated influenza virus (LAIV) vaccines are comprised of temperature sensitive and attenuated viruses that grow well in the upper, but not the lower respiratory tract[11] and promote low-grade pulmonary inflammation, required for development of immune memory, without causing clinical pathology.[12] Interestingly, in contrast to WT IAV, which predisposes to lethal bacterial pneumonia from otherwise sublethal doses of *Streptococcus pneumoniae* or *Staphylococcus aureus* inoculated 7 days apart,[3] vaccination with LAIV was recently shown to have no effect on increasing bacterial pneumonia or mortality following sublethal bacterial infection seven days after vaccination.[11]

Considering that protection may be conferred by provocation of non-pathological low-grade inflammation against lethal viral and bacterial infections, we sought here to investigate whether intranasal vaccination with LAIV might too protect from bacterial pneumonia and mortality following infection with lethal doses of highly invasive pneumococcal bacteria.

4.3 MATERIALS AND METHODS

4.3.1 INFECTIOUS AGENTS AND VACCINES

Viral infections were carried out with an H3N2 1:1:6 reassortant virus (HK/Syd) developed as described previously,[11] containing the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) from A/Hong Kong/1/68 (HK68) and A/Sydney/5/97 (Syd97) isolates, respectively, and the six internal protein gene segments from the St. Jude variant of the mouse adapted influenza virus strain A/Puerto Rico/8/34 (PR8). LAIV vaccinations used a temperature sensitive (*ts*) attenuated variant of HK/Syd (HK/Syd$_{ts,att}$ or LAIV) containing the same site-specific mutations in the PB1 and PB2 RNA segments as those in the A/Ann Arbor/6/60 master donor strain used to produce the human FluMist® vaccine, described previously.[11, 13] WT and LAIV viruses were propagated and characterized as described previously.[11] The encapsulated type 2, D39 and type 3, A66.1 pneumococcal isolates were used for bacterial infections. These strains were engineered to express luciferase, as described previously,[14] enabling in-vivo imaging for detection and quantitation of pneumonia or bacterial carriage.

4.3.2 Animal and Infection Models

8-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. Inoculations were via the intranasal route. Sublethal WT IAV infection and LAIV vaccination were as described previously[5, 11] and 7 days later (an interval that causes maximal bacterial proliferation and disease[3, 11, 15]), bacteria were inoculated at doses optimized to cause lethal infections in 50% of mice (LD_{50}). Specifically, LD_{50} bacterial infection was with either 2e6 or 2e4 colony forming units (CFUs) of D39 or A66.1 in 100uls PBS, respectively. Animals were monitored for pneumonia, via in-vivo imaging using an IVIS CCD camera (Xenogen) as described,[14] as well as body weight and mortality at least once per day and mice were sacrificed when body weight fell below 70% initial weight.

4.3.3 Determination of bacterial CFUs in lungs and nasopharyngeal homogenates

Bacterial titers were measured in whole lung and nasopharyngeal (NP) homogenates. Whole lungs were harvested and homogenized using a gentleMACS™ system (Miltenyi Biotech), as per manufacturers protocol. NP was isolated via careful dissection dorsally across the frontal bones, laterally via removal of the zygomatic, posterially by dislocation of the upper jaw from the mandible and inferiorly just posterior to the soft palate. Isolated NP was homogenized via plunging in 1.5 mL PBS through a 40 micron mesh strainer. Bacterial titers were measured via serial dilution plating on blood agar plates.

4.3.4 STATISTICAL ANALYSES

All statistical analyses were performed within the R statistical computing environment (R version 2.14, R foundation for statistical computing, R Development Core Team, Vienna, Austria). Specific statistical tests used are as indicated in each figure. The R package 'survival' was used for all survival analyses including Kaplan Meier plots and KM log-rank tests. All other statistical tests were performed using R base functions.

4.4 RESULTS

4.4.1 LAIV IMPROVES SURVIVAL FOLLOWING LETHAL PNEUMOCOCCAL INFECTIONS

To measure effects of vaccination with LAIV on mortality from *Streptococcus pneumoniae*, mice were vaccinated with LAIV and seven days later received a LD_{50} bacterial infection with either of two invasive pneumococcal capsular serotypes, D39 or A66.1. We used an interval of seven days as this maximizes both survival and mortality following lung inflammation when in the absence[10] or presence[3] of prior lung pathology, respectively. We compared mortality following pretreatment with LAIV vs. WT IAV infection and PBS controls. An LD_{50} dose was chosen to allow detection of either protective or detrimental effects of LAIV. Pretreatment with LAIV conferred significant protection from LD_{50} bacterial inoculation. Following infection with either D39 or A66.1, pretreatment with LAIV reduced mortality by 71% and 75% relative to PBS controls, respectively

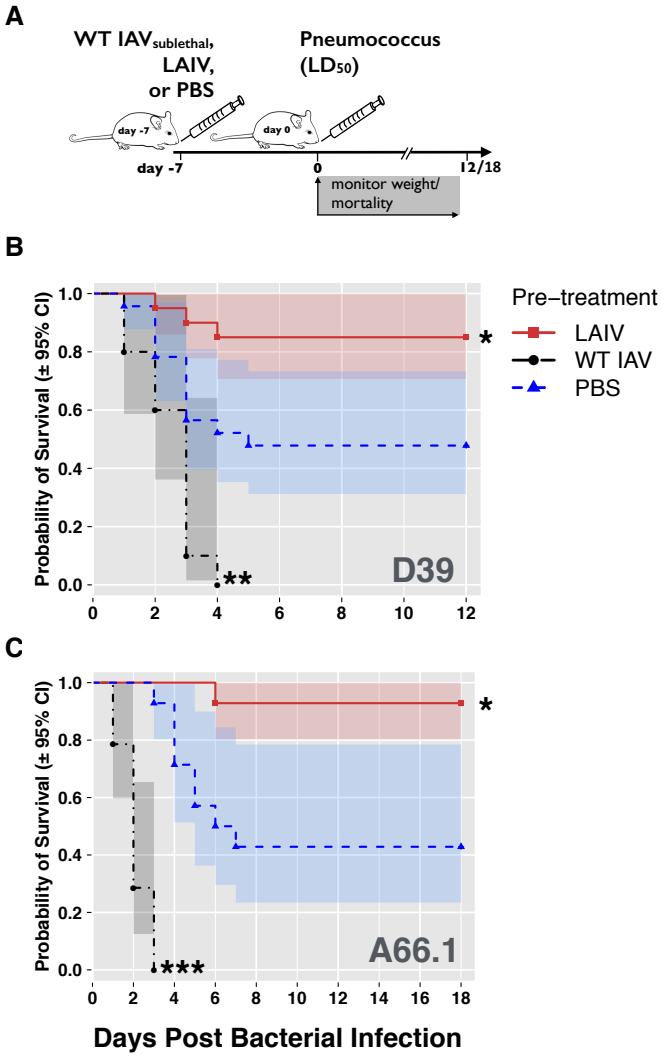

Figure 4.1 LAIV protects from lethal infection with invasive *Streptococcus pneumoniae*.

*Groups of mice were primed intransasally with LAIV, PBS or a sub-lethal dose of WT influenza virus prior to inoculation with a lethal dose 50 of Streptococcus pneumoniae type D39 (n=20, 23 or 10 for LAIV, PBS or WT virus primed mice, respectively) or S. pneumoniae type A66.1 (n=14 for all groups). Groups were monitored daily and probability of survival was plotted for each group with 95% confidence intervals. Survival differences were tested between groups using the Kaplan-Meier log-rank test and significant differences relative to PBS controls are marked with *, ** or *** for p-values < .05, .001 and .0001, respectively.*

(15% vs. 52% following D39; and 11% vs. 44% following A66.1; p<.01 for both comparisions; Fig 1.) In contrast, pretreatment with the wild-type influenza parent strain led to rapid mortality of 100% of mice within 4 days following inoculation with either pneumococcal strain (black curves in Fig. 1b, c).

To determine if protection was associated with changes in bacterial clearance, bacterial titers were measured in the lungs at 24 and 72 hours post-infection in each of the three treatment groups. Twenty-four hours post-infection, pretreatment with LAIV led to a 99.1% reduction in bacterial titers relative to PBS

controls (8.5e5 vs. 9.4e3 CFUs; p<.05). By 72 hours this difference was even greater with lung titers in controls nearly 3000-fold increased over LAIV treated mice (2.0e7 vs. 5.5e4 CFUs; p<.05). In contrast, pretreatment with WT virus increased lung titers by 1,000-fold and ~100,000-fold over PBS and LAIV treated mice, respectively (5.2e8 CFU's post WT virus; p<.05 for each).

4.4.2 VIRAL REPLICATION FACILITATES BACTERIAL REPLICATION

Finally, we sought to determine if opposing effects of LAIV vs. WT influenza virus on bacterial titers was associated with viral replication, and implicitly epithelial tissue damage, as has been suggested [6]. Unlike in the lungs, LAIV replicates efficiently in the nasopharynx (NP), reaching levels akin to WT IAV.[11] Thus we hypothesized that if a lack of viral replication is important to protection conferred by LAIV, then LAIV would fail to protect from bacterial infection within the NP and would instead mirror effects of WT IAV. Indeed, LAIV increased NP titers nearly 1000-fold by 24 hours (1.6e6 vs. 2.4e3 CFUs; p<.05), which persisted at 72 hours (1.4e6 vs. 4.0e3 CFUs; p<.05). Similarly, titers were increased 2000-fold following WT IAV (5.1e6 vs. 2.4e3 CFUs; p<.05). No significant differences were measured in bacterial NP titers in LAIV vs. WT IAV treated mice.

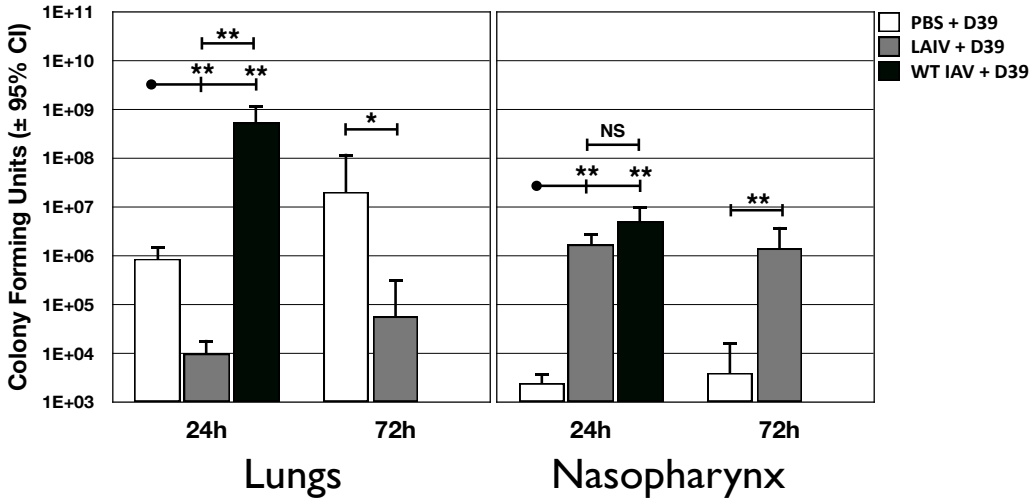

Figure 4.2 LAIV increases bacterial clearance in the lungs but not the nasopharynx..

*Groups of mice were primed with LAIV, WT influenza virus or PBS 7 days prior to infection with a 50% lethal dose of Streptococcus pneumoniae type 2 serotype, D39. At 24 hours (n=4 per group) or 72 hours (n=8 per group) post-pneumococcal infection, whole lungs and nasopharyngeal tissue were harvested and bacterial counts quantified via serial dilution plating on 5% blood agar plates. Students t-test with FDR correction for multiple comparisons was used to determine significant differences between groups. • signifies the reference group being tested against (PBS primed mice) and significant differences are reported as * or ** for p-values <.05 or .001, respectively.*

4.5 DISCUSSION

The potent and often lethal effects of an antecedent viral influenza infection on secondary pneumococcal invasive disease and pneumonia have been reported.[3, 15-18] Viral replication induced epithelial and mucosal degradation and the ensuing innate immune response yield diminished capacity to avert secondary bacterial infections. Recent clinical and experimental data suggest that influenza viruses may exert their influence beginning in the URT by enhancing susceptibility to bacterial

colonization,[14, 19, 20] increasing NP carriage density[21] and enhancing incidence of AOM.[4]

Though vaccination with a live-attenuated influenza virus, in the longer-term, thwarts influenza-bacterial coinfections by inhibiting the viral infection itself, [22, 23] LAIV vaccines have recently been found to transiently enhance density and duration of bacterial colonization within the nasopharynx of mice, with dynamics nearly identical to those following WT IAV.[11] Importantly, unlike WT IAV, LAIV did not result in increased bacterial proliferation or disease in the LRT, presumably due to temperature sensitive nature of LAIV abrogating viral growth within the warmer temperatures of the lungs. Although LAIV did not effect clinical bacterial LRT infections, the effects of LAIV on transition from colonization to bacterial disease within the URT, a region where LAIV replicates efficiently, had not not been studied.

Here, we discovered that vaccination with LAIV significantly increased incidence and duration of pneumococcal AOM, irrespective of bacterial serotype or order of viral vs. bacterial inoculation. Interestingly, a minimum period of time of approximately four days was required before enhancement in AOM was noted when LAIV preceded pneumococcal infection.

The dynamics, with regard to time-since-vaccination of increased AOM closely match pneumococcal colonizing dynamics of the nasopharynx following WT IAV or LAIV,[11, 22] and support the notion that NP colonizing density may be associated with progression to AOM. Interestingly, the delay in increased onset of AOM in mice vaccinated only one day before bacterial inoculation was approximately the same as the time to peak LAIV viral titers in the URT.[11] Thus, a

majority of excess AOM occurs during or soon after viral clearance from the URT. This finding supports numerous reports[3, 15, 21, 24, 25] that point towards a complex coupling of poorly coordinated antibacterial innate immune defenses and epithelial damage following influenza infection underlying excess susceptibility to bacterial disease following influenza infections.

On the other hand, the steady increase in onset of AOM measured immediately following LAIV vaccination in 19F pre-colonized mice suggests that introduction of LAIV in the presence of existing bacterial colonization yields enhanced bacterial AOM that is concurrent with viral replication and precedes viral enhanced nasopharyngeal colonizing density, which tend to increase beginning at 4 days post LAIV inoculation. This suggests that the mechanisms of virus induced bacterial AOM may differ according to order of inoculation. Indeed, it may be that even low levels of viral replication in the URT, while not immediately effecting overall bacterial carriage density in the nasopharynx, may rapidly disrupt a delicate balance that naturally exists to prevent asymptomatic carriage from transitioning to bacterial AOM.

While our data suggest that vaccination with LAIV may enhance progression to bacterial AOM when vaccination is administered to pneumococcal colonized individuals or when bacterial acquisition occurs shortly after vaccination, the overall effect of LAIV vaccines measured in humans has been that of significant reductions in viral influenza infections, including otitis media.[26] We hypothesize that large reductions in virus mediated AOM following LAIV vaccination may well mask LAIV mediated increases in bacterial AOM, which may occur in only a fraction

of vaccinated individuals. As well, LAIV mediated protection from primary influenza infections significantly reduces opportunity for secondary bacterial infections,[5] further reducing LRT and URT bacterial disease, including bacterial AOM.

While we are confident that the overall effects of live-attenuated influenza vaccines are beneficial to reduce all-cause AOM across populations, as has been reported,[27] our data suggest a need for future investigations to closely evaluate effects of LAIV vaccination on bacterial respiratory pathogens. Indeed, as medicine becomes increasingly personalized,[28] it may become possible to tailor classes of vaccines and avenues of vaccine delivery to the individual. Indeed, in this particular example, considering the many benefits of LAIV over inactivated injectable influenza vaccines[29] one could imagine that the choice between a killed injectable vaccine and an intranasal live attenuated vaccine might incorporate risk of pneumococcal carriage or acquisition (ie: number of children in the household, age of vaccine recipient) as a potential variable in the decision making process.

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CHAPTER 5. LAIV BUT NOT PCV PROTECTS AGAINST INCREASED DENSITY AND DURATION OF PNEUMOCOCCAL CARRIAGE FOLLOWING INFLUENZA INFECTION IN PNEUMOCOCCAL COLONIZED MICE

Michael J. Mina with Keith P. Klugman and Jonathan A. McCullers

Adapted from:

Mina MJ, Klugman KP, McCullers JA. Live attenuated influenza vaccine, but not pneumococcal conjugate vaccine, protects against increased density and duration of pneumococcal carriage after influenza infection in pneumococcal colonized mice. Journal of Infectious Diseases. 2013; **208**(8): 1281-5

5.1 ABSTRACT

Secondary bacterial infections due to Streptococcus pneumoniae and Staphylococcus aureus, responsible for excess morbidity and mortality during influenza epidemics, are often preceded by excess bacterial density within the upper respiratory tract. Influenza and pneumococcal vaccines reduce secondary infections within the lungs, however their effects on upper respiratory tract carriage remain unknown. We demonstrate that a live attenuated influenza vaccine significantly reduces pneumococcal growth and duration of carriage during subsequent influenza to levels seen in influenza-naïve controls. No benefit was seen following pneumococcal conjugate vaccine. Our results suggest that live attenuated influenza vaccines may significantly reduce bacterial disease during influenza epidemics.

5.2 INTRODUCTION

Infection with influenza A virus (IAV) increases susceptibility to pneumonia, bacteremia, sinusitis, and acute otitis media (AOM) from several bacterial species including *Streptococcus pneumoniae* and *Staphylococcus aureus*[1, 2] *a*nd bacterial infections are an important cause of mortality associated with IAV infection in the absence of pre-existing comorbidity[3] Previous investigations by our lab and others have demonstrated that early influenza vaccination can significantly reduce or prevent severe IAV mediated pneumococcal lower respiratory tract infections (LRI) and death.[4, 5] Pneumococcal conjugate vaccine (PCV) has also demonstrated significant efficacy to reduce severe post-influenza pneumococcal disease.[6]

Bacterial LRIs and invasive disease (irrespective of influenza status) often are preceded by a primary colonizing event with dissemination of bacteria to the lower respiratory tract. Further, the probability of developing an LRI is believed to be associated with increased pneumococcal colonizing density.[7]

Animal models and human studies suggest that intranasal infection with IAV can enhance carriage density of pneumococci in the nasopharynx (NP)[1] which likely contributes to the excess incidence of severe pneumococcal disease following IAV infection.[8] While the mechanisms underlying this excess colonization within the NP are not entirely defined, they include a combination of IAV mediated cytotoxic breakdown of mucosal and epithelial barriers[9] and aberrant innate immune responses to bacterial invaders in the URT in the post-influenza state.[1]

We sought here to determine the efficacy of the 7-valent pneumococcal conjugate vaccine (PCV) and a live attenuated influenza vaccine (LAIV) to prevent excess pneumococcal colonization density and to decrease duration of pneumococcal carriage following infection with IAV.

5.3 MATERIALS AND METHODS

5.3.1 INFECTIOUS AGENTS

Viral infections were carried out with an H3N2 reassortant virus (HK/Syd) developed as described previously,[10] containing the surface glycoprotein hemagglutinin (HA) from A/Hong Kong/1/68 and the neuraminidase (NA) from A/Sydney/5/97 (Syd97). The six internal protein gene segments are from the St Jude variant of the mouse adapted influenza virus strain A/Puerto Rico/8/34 (PR8). LAIV vaccinations used a temperature sensitive (*ts*) attenuated variant of HK/Syd (HK/Syd_{ts} or LAIV) containing the same site-specific mutations as those in the attenuated A/Ann Arbor/6/60 master donor strain used to produce the influenza A virus strains found in the human FluMist® vaccine, as described previously.[10] The growth dynamics, safety, efficacy and antibody response of our LAIV vaccine have been previously described.[4]

All viruses were propagated in 10-day-old embryonated chicken eggs at 37°C and 33°C for the WT and LAIV strains, respectively. The dose infectious for 50% of tissue culture wells ($TCID_{50}$) was performed using Madine-Darby canine kidney cells. The serotype 19F (ST^{425}) pneumococcal clinical isolate BHN97 was engineered

to express luciferase and used for all experiments due to its colonizing potential as described in.[2] The pneumococcal conjugate vaccine (PCV7; Prevenar, Wyeth Vaccines) consisted of capsular polysaccharides conjugated to CRM197 carrier protein of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F.

5.3.2 Animal and Infection Models

Four week old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used for all experiments and all procedures were approved by the Animal Care and Use Committee at SJCRH. Mice were vaccinated with PCV7, LAIV or sham vaccine (PBS vehicle) and monitored for four weeks. At 8 weeks, PCV7 recipients received a PCV7 booster. Inoculations were via the intranasal route under general anesthesia with inhaled isoflurane (2.5%) except for PCV7 which was given IM in 0.05-ml. LAIV vaccination consisted of 2e6 $TCID_{50}$ HK/Syd$_{ts,att}$ LAIV in 40ul PBS. Sub-lethal doses of WT HK/Syd used 1e5 $TCID_{50}$ in 50ul PBS. Pneumococcal infection consisted of 1e5 colony-forming units in 40uls of PBS administered intranasally evenly between the two nares. For duration of carriage studies, once bacterial density fell below the limit of detection for in vivo imaging (~1e4 CFU) bacterial CFU titers were measured in nasal washes using 12uls of PBS administered and retrieved from each nare and quantitated by serial dilution plating on blood agar plates (lower limit of detection was 100 CFU/ml nasal wash).

5.3.3 BIOLUMINESCENT IMAGING

Mice were imaged using an IVIS CCD camera (PerkinElmer, Waltham, MA) as described.[2] Nasopharyngeal bacterial density was assessed by measuring the flux of light as total photons/sec/cm^2 in pre-specified regions covering the NP, and background (calculated for each mouse on a region of equal area over the hind limb) was subtracted. Each NP measurement represents an average of two pictures, one for each side of the mouse head. Quantitation was performed using LivingImage software (v. 3.0; PerkinElmer) as described.[2]

5.3.4 STATISTICAL ANALYSES:

All statistical Statistical analyses were performed using the R statistical environment (R Foundation for Statistical Computing, Vienna, Austria; URL: http://www.R-project.org). Pairwise comparisons were performed using Students t-tests with Bonferonni correction for multiple comparisons.

5.4 RESULTS

5.4.1 WILD-TYPE INFLUENZA VIRUS ENHANCES DENSITY OF BACTERIAL COLONIZATION IN THE NASOPHARYNX

We sought to measure the effect of an H3N2 influenza virus on density of pneumococcal colonization in mice pre-colonized with 19F pneumococci and without any previous exposure to prophylactic vaccination. Mice were given sham vaccine and 4 weeks later colonized with pneumococci. Seven days post-

colonization, during normal bacterial clearance, mice were infected with either wild-type influenza virus or PBS control - referred to hereafter as 'controls' (see Fig 1a for experimental design). Within 12 hours post viral inoculation, normal pneumococcal clearance ceased and swift, exponential bacterial growth ensued within the NP (Fig. 1b). By 2 days-post infection (dpi) with influenza, bacterial density in the NP was significantly increased over controls and maximal bacterial titers were 15-fold higher than PBS controls (8.4e5 vs. 5.5e4; $p<.001$). Density remained significantly elevated until 15 days following influenza infection. Overall, cumulative bacterial load was increased nearly 500% as a result of influenza infection (Fig 1f; $p=1e-7$). To ensure that the density and dynamics of pneumococcal growth measured by luminescence (Fig. 1b-d, f) accurately represents true bacterial growth dynamics within the nasopharynx, the experiment just described was repeated and nasal washes were performed on select days following influenza infection to quantitate via serial dilution plating, the true bacterial colony forming units within the NP of influenza infected vs. control mice (Fig. 1e). A comparison between Figures 1a and 1e demonstrate agreement between these two methods of bacterial quantitation.

5.4.2 Early prophylactic LAIV but not PCV reduces excess pneumococcal density in the NP following WT influenza.

The effectiveness of early prophylactic LAIV or PCV vaccination to reduce influenza-associated excess bacterial carriage density and duration was assessed. Four-week old mice received either LAIV, PCV or sham vaccine and four weeks later

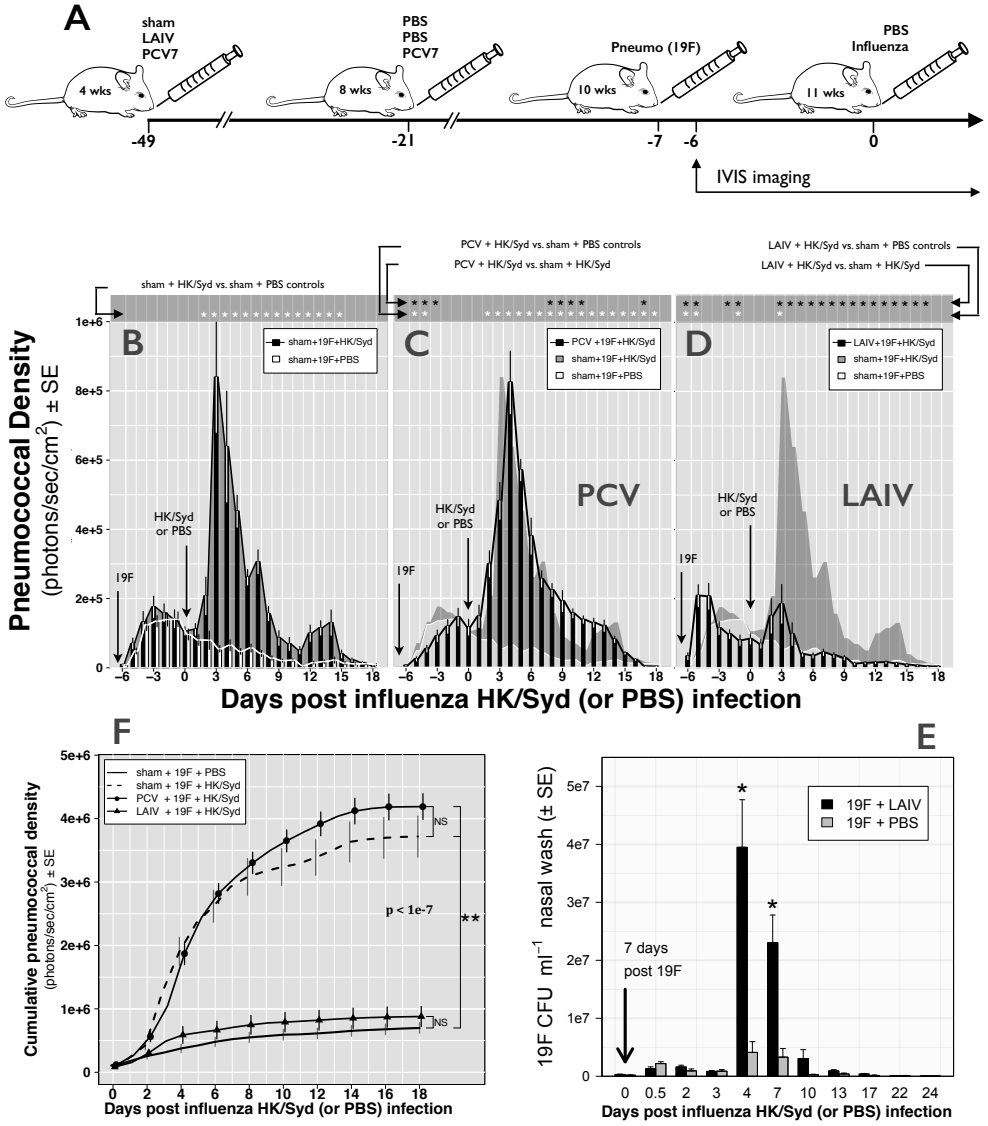

Figure 5.1 Dynamics of pneumococcal carriage following influenza infection with and without prophylactic vaccination

Overall Experimental design is depicted in (A). Groups of 12-15 mice were given either sham (B), pneumococcal conjugate (C) or live attenuated influenza (D) vaccine. Four-weeks later all mice were colonized with a type 19F pneumococcus 7 days prior to intranasal infection with an H3N2 influenza virus (HK/Syd) or PBS vehicle control (white bars/shaded regions). Nasopharyngeal carriage density is plotted for days 1 through 24-post 19F colonization (6 days prior to 18 days following influenza/PBS inoculation). Density of colonization following PCV and LAIV are plotted over grey and white shaded regions representing pneumococcal densities following sham vaccinated and influenza infected or PBS vehicle controls, respectively. To very the utility of IVIS imaging for bacterial quantification, the experimental design for (B) was repeated and nasal washes were serially diluted and plated on blood agar plates for manual quantitation of bacterial colony forming units (E). Cumulative bacterial titers following influenza infection are plotted in (F) for each of the four groups. Asterisks (*) indicate significant differences in bacterial titers

between respective groups as labeled (students t-test with Welch's approximation of degrees of freedom; $p < .05$ unless otherwise indicated).

PCV recipients received PCV boosters, while LAIV and sham vaccinated mice received PBS. Two weeks following PCV boosters, all mice were colonized with pneumococci, followed seven days later by intranasal infection with WT influenza virus. Prophylactic vaccination of colonized mice with PCV demonstrated no efficacy to prevent or reduce excess bacterial titers following influenza infection (Figs 1c, e) relative to controls. In fact, the dynamics of pneumococcal growth following influenza infection were nearly identical between the PCV and sham-vaccinated groups except that peak density in the PCV group occurred one day later than the sham vaccinated group.

It is interesting to note however that in the days just following 19F colonization, prior to infection with influenza, prophylactic PCV vaccination resulted in 2- to 3-fold decreased pneumococcal titers relative to the sham vaccinated groups ($p<.05$; Fig. 1c & d). However, any potential benefit initially conferred by early vaccination with PCV was lost entirely following influenza infection.

In contrast, prophylactic LAIV led to significantly reduced excess bacterial outgrowth following WT influenza virus infection (Fig 1d), with maximum excess pneumococcal density following influenza infection only 2-fold increased over PBS controls (compared to the 15-fold increases seen in the sham- and PCV-vaccinated groups), and this increase was significant at only a single time point (3-dpi with influenza). Early LAIV vaccination also reduced the duration that bacterial titers were elevated over PBS controls 5-fold, to only 3 days of excess titers in the LAIV recipients (vs. 15 days in the PCV and sham vaccine groups). Overall, prophylactic

LAIV led to a 77% reduction in cumulative pneumococcal titers following influenza inoculation (8.8e5 vs. 3.7e6; p=1.3e-7; Fig. 1e), resulting in cumulative titers no different than the non-influenza infected PBS controls (8.8e5 vs. 7.0e5; p=0.327; Fig 1e). In contrast, the PCV treated group had mean cumulative titers ~600% greater than PBS controls (4.2e6 vs. 7.0e5; p=1e-10; Fig. 1e).

5.4.3 EARLY PROPHYLACTIC LAIV BUT NOT PCV REDUCES EXCESS PNEUMOCOCCAL DENSITY IN THE NP FOLLOWING WT INFLUENZA.

The effectiveness of early prophylactic LAIV or PCV vaccination to reduce influenza-associated excess bacterial carriage density and duration was assessed. Four-week old mice received either LAIV, PCV or sham vaccine and four weeks later PCV recipients received PCV boosters, while LAIV and sham vaccinated mice received PBS. Two weeks following PCV boosters, all mice were colonized with pneumococci, followed seven days later by intranasal infection with WT influenza virus. Prophylactic vaccination of colonized mice with PCV demonstrated no efficacy to prevent or reduce excess bacterial titers following influenza infection (Figs 1c, e) relative to controls. In fact, the dynamics of pneumococcal growth following influenza infection were nearly identical between the PCV and sham-vaccinated groups except that peak density in the PCV group occurred one day later than the sham vaccinated group.

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5.4.4 WILD-TYPE INFLUENZA INFECTION AND PROPHYLACTIC VACCINATION SIGNIFICANTLY ALTER DURATION OF PNEUMOCOCCAL CARRIAGE.

Duration of carriage was assessed via daily nasal washes after bacterial NP titers fell below the limit of detection for IVIS. In accordance with human carriage studies, carriage was significantly increased following influenza infection, with bacterial CFU's present in nasal wash for a mean of 52 days post pneumococcal colonization, vs. 31 days in the PBS controls (p = 0.0004; Fig. 2). Prophylactic

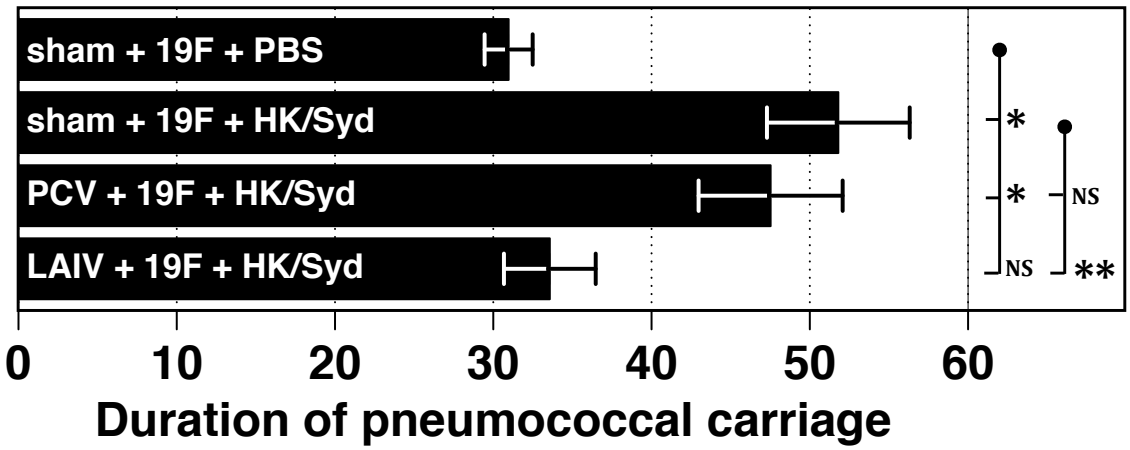

Figure 5.2 Duration of pneumococcal carriage following influenza infection with or without PCV or LAIV vaccination.

Duration of pneumococcal carriage was measured via daily nasal washes and serial dilution plating beginning after nasopharyngeal titers fell below the limit of detection for in-vivo imaging. Interior most asterisks (*) indicate significant differences in duration of carriage relative to sham vaccinated and PBS infected controls. Outer asterisks (**) indicated significant differences relative to sham vaccinated and influenza infected mice (Students t-test with Bonferroni correction; p<0.005 for all asterisks and all NS indicate p-values of greater than 0.40).

vaccination with PCV had no effect on prolonged duration of carriage (47.5 days vs. 52 days; p=0.51; Fig. 2). Early vaccination with LAIV however almost entirely abrogated prolonged duration of carriage due to influenza infection with carriage lasting a modest 33.5 days (vs. 52 days; p = 0.0023; Fig. 2). This duration was no different than the mean duration of carriage detected in the influenza uninfected controls (33.5 days vs. 31 days; p=0.435; Fig. 2).

5.5 DISCUSSION:

While vaccine efficacy is traditionally measured as the ability to directly prevent disease from the vaccine target pathogen, it is important to consider

indirect benefits of vaccination as well. In the context of influenza vaccination, an important indirect benefit is prevention of secondary bacterial disease.

Influenza infections drive pneumococcal transmission and disease. In agreement with studies in humans,[11] we have shown here, in mice, that H3N2 influenza virus significantly enhances pneumococcal density and duration of carriage within the nasopharynx. Excess bacterial density in the URT enhances risk of lower[7] and upper respiratory infections like acute otitis media[12] in humans and has been shown to increase the likelihood of transmission.[13] Extended duration of carriage further increases opportunity for transmission.

Pneumococcal conjugate vaccine has been extremely successful in reducing overall incidence of invasive pneumococcal disease,[14] and has shown efficacy to reduce severe influenza-pneumococcal co-infections of the lower respiratory tract within vaccinated individuals.[6] However, key to efficient and wide-scale prevention of excess bacterial co-infections during influenza epidemics is disruption of influenza induced transmission events across the general population.[15] We have demonstrated here a desirable secondary effect of influenza vaccination to significantly reduce influenza mediated excess pneumococcal carriage density, an important contributor to pneumococcal transmission.[13] That this effect was not achieved through the use of pneumococcal conjugate vaccine is in keeping with the observation that PCV's reduce pneumococcal serotype specific carriage by reduction in acquisition[14] but have not been shown to reduce density of existing carriage or duration of carriage. While PCVs may reduce lower respiratory infections of vaccinated hosts who become co-infected with influenza and pneumococcus, our

results suggest benefit normally conferred by PCV to prevent upper respiratory tract colonization, and subsequent transmission, may be compromised in the setting of an influenza infection. Meanwhile, LAIV demonstrated significant efficacy in reducing post-influenza pneumococcal carriage density and duration to levels near those seen in the absence of influenza infections.

Our data suggest that effective LAIV immunization may have a greater impact on influenza–associated pneumococcal transmission than concurrent PCV administration, emphasizing the importance of prophylactic rather than reactive PCV immunization to prevent pneumococcal transmission during influenza infection.

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CHAPTER 6. DYNAMICS OF EXTENDED IFN-GAMMA EXPOSURE ON MURINE MH-S CELL-LINE ALVEOLAR MACROPHAGE PHAGOCYTOSIS OF *STREPTOCOCCUS PNEUMONIAE*

Michael J. Mina with Lou Ann S. Brown and Keith P. Klugman

6.1 ABSTRACT

Previous investigations have demonstrated that activation with the pro-inflammatory type II interferon, IFN-γ, down-regulates alveolar macrophage (AM) cell surface receptors required for efficient clearance of bacterial infections. While these studies have shown clear effects at discrete time points, the kinetics of the macrophage response to IFN-γ, with respect to bacterial phagocytosis, remain unknown. Here we describe these kinetics in the murine MH-S alveolar macrophage cell-line, a well-established model for investigations of alveolar macrophage function. We measure binding and internalizing rates of *Streptococcus pneumoniae* following exposure to increasing durations of physiologic levels of IFN-γ. When MH-S murine alveolar macrophage (mAM) were exposed to IFN-γ for increasing durations of time, from 0 to 6 days prior to inoculation with the type 2 *Streptococcus pneumoniae*, D39, exposure for only 6 hours led to a rapid reduction in bacterial binding that slowly recovered after three days of exposure before falling again by day six. Bacterial internalizing capacity too was significantly reduced, in an exponential fashion, before falling below 5% that of IFN-γ naïve controls after 6 days. We describe these non-linear dynamics of bacterial phagocytosis, as functions of IFN-γ exposure, and offer insights that may reconcile seemingly contradictory reports from the literature regarding timing between infections and reductions in macrophage function.

6.2 INTRODUCTION

The pro-inflammatory type II interferon, IFN-γ, has historically been known for its role as an immunomodulator while type I interferons were classically associated with

interference of viral replication (a role for which the interferons were named). However, today, both type I and II IFN's are known as important modulators of the innate immune system, aiding in viral as well as bacterial and protozoal clearance [1]. During the early course of an infection, IFN-γ is secreted in response to IL-12 and IL-18, amongst other cytokines [2], by professional antigen presenting cells (APCs), including monocyte/macrophage and dendritic cells, as well as natural killer (NK) cells and later on by CD4+ T helper and CD8+ cytotoxic lymphocytes [1, 3]. Once secreted, the type II IFN aids in leukocyte recruitment, upregulation of antigen presentation by both MHC class I and II, differentiation of multiple cell types and importantly in the context of the experiments described herein, alteration of macrophage function [1]. While IFN-γ is widely known to activate macrophage activity (it was originally termed 'macrophage activating factor'), enhancing phagocytosis of microbial invaders as well as bi-products of inflammation, it has paradoxically been implicated in the down-regulation of alveolar macrophage capacity to phagocytose *Streptococcus pneumonia* (the pneumococcus) [4-6]. Sun et al. have previously demonstrated that this reduction in phagocytic activity occurs within 24 hours post inoculation of mice with IFN-γ and was shown to be a result of IFN-γ mediated reduction in expression of the class A scavenger receptor MARCO (macrophage receptor with collagenous structure) on the surface of alveolar macrophages (AM) [4], a receptor known to be important in pneumococcal surveillance and clearance [7]. As well, Chroneos et. al demonstrated that IFN-γ exposure results in downregulation of C-type lectin receptors on the surface of macrophage, receptors that too are important in the detection and ingestion of pneumococcus [5]. We sought here to better understand the dynamical effects of IFN-γ on AM phagocytosis of pneumococcus. In particular, we

sought to phenotypically describe pneumococcal binding and internalization as a function of duration of exposure to IFN-γ in a model system of murine AM's using the well established MH-S murine AM cell-line [8].

6.3 METHODS:

6.3.1 CELL CULTURE AND IFN-GAMMA EXPOSURE

The mouse alveolar macrophage (mAM) cell line, MH-S (American Type Culture Collection, Manassas, VA), was used as a model system to investigate the effects of IFN-γ on binding and internalization of *Streptococcus pneumoniae*. MH-S cells were harvested and quantified using a Countess™ (Invitrogen) automated cell counter with 0.4% trypan blue and plated at 200,000 cells/ml in RPMI 1640 media containing 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2. Cells were plated at 7 days prior to phagocytosis assays in all conditions. Once plated, cells were allowed to adhere for at least 5 hours. Five hours after plating, and daily thereafter, cells were cultured in media with, or without, 25ng/ml of recombinant mouse IFN-γ (eBioscience), depending on the designated conditions for the particular cells/well. Media was changed daily to ensure consistent levels of IFN-γ during exposure periods and cells were not passaged during any of the experimental conditions (ie: between initial plating and assays 7 days later). Twenty-four hours prior to phagocytosis assays, cells were washed 3 times in 1 ml of sterile phosphate-buffered saline to remove antibiotics, and antibiotic-free media, with or without IFN-γ was added, as per experimental condition.

6.3.2 BACTERIA

All phagocytosis assays were performed using the type 2 encapsulated *Streptococcus pneumoniae* (pneumococcal) strain D39. Bacteria were grown in Todd-Hewitt broth supplemented with 0.5% (w/v) yeast extract (Difco, Detroit, MI) to an optical density (OD_{600}) of 0.3-0.35, corresponding to mid- to late-log phase growth and an approximate bacterial density of 1E8 colony forming units (CFU) per ml. Bacteria were then pelleted at 10,000 g's, and resuspended in 100uls RPMI for use in phagocytosis assays. For all experiments, the number of bacterial CFU's was verified by serial dilution plating on blood agar plates.

For experiments measuring the phagocytic index, a modified D39 pneumococcal strain was developed to express the green-fluorescence protein (GFP), referred to hereafter as D39-GFP. Briefly, WT D39 was transformed with the mobilizable plasmid, pMV158, harboring the gene encoding GFP [9]. The plasmid confers resistance to tetracycline by constitutive expression of the *tetL* gene, encoding an energy dependent tetracycline efflux pump [10] and GFP is under the P_M promotor inducible by inoculating the bacterial culture in the presence of 2% maltose. D39-GFP can be quantitated via fluorescent microscopy and measurement of mean fluorescent units.

6.3.3 PHAGOCYTOSIS ASSAYS: MULTIPLICITY OF INFECTION:

Pneumococci were added for phagocytosis at an MOI of 15 bacteria per MH-S cell. Because IFN-γ may alter replication rates of MH-S cells, to ensure consisiten MOIs, two wells of MH-S cells per experimental condition (where each 'condition' is defined as

a particular duration of IFN-γ exposure) were plated along with each experiment solely for cell counting just prior to addition of bacteria into the experimental wells. Viable cell counts were measured, in duplicate, for each experimental condition using a Countess™ (Invitrogen) automated cell counter with 0.4% trypan blue, and numbers of bacteria added to experimental wells were adjusted for each condition depending on numbers of live MH-S cells accordingly. Numbers of bacterial CFU's were verified by serial dilution plating on 5% sheep-blood agar plates.

6.3.4 TOTAL PHAGOCYTIC ACTIVITY: BINDING AND INTERNALIZATION

Overall phagocytic activity was measured as the ratio of the sum of both bound and internalized bacteria per macrophage using standard phagocytosis assays, as have been previously reported [11]. Briefly, IFN-γ exposed or unexposed MH-S cells were cultured with pneumococci at an MOI of 15 for 50 minutes at 37°C in 5% CO_2. Media was then aspirated off and cells washed twice in 1ml of sterile phosphate-buffered saline to remove any unbound bacteria within the supernatant. Cells were gently lysed by incubation with 1% saponin (Sigma) in RPMI 1640 for 15 minutes at 37°C to release both surface bound and internalized bacteria. Cell lysate was collected and serial dilutions were plated on 5% sheep-blood agar plates for bacterial CFU enumeration. Three wells per experimental condition were analyzed and the whole experiment was repeated three times on three different days. For statistical analysis, the nine data points were pooled.

6.3.5 BACTERIAL INTERNALIZATION

Bacterial internalization was measured using a well established gentamicin protection assay [12] by first incubating pneumococci with MH-S cells for 50 minutes and washing with PBS to remove unbound bacteria as described above. After removal of unbound bacteria, cells were incubated for a further 50 minutes in fresh media containing 120μg/ml gentamicin sulfate to kill extracellular membrane bound bacteria that had not yet been internalized. Cells were then washed twice in ice-cold PBS prior to gentle lysis using 1% saponin as described above. Cell lysate was collected and internalized bacteria were enumerated by serial dilution plating on blood agar plates and are reported per MH-S cell. Three wells per experimental condition were analyzed and the whole experiment was repeated three times on three different days. For statistical analysis, the nine data points were pooled.

6.3.6 BACTERIAL BINDING

Bound bacteria were quantified, indirectly, by subtracting the number of bacterial CFU's measured following gentamicin protection assays (e.g. internalized bacteria) from CFU's enumerated in the absence of gentamicin, described above under 'total phagocytic activity: binding and internalization'. Binding activity is reported as the number of bound bacteria per MH-S cell. Three wells per experimental condition were analyzed and the whole experiment was repeated three times on three different days. For statistical analysis, the nine data points were pooled.

6.3.7 FLUORESCENT MICROSCOPY AND THE PHAGOCYTIC INDEX

The phagocytic index (PI) has been described previously [13]. Briefly, MH-S cells were seeded as described above, except that wells were lined with 12mm glass coverslips (#1 thickness) prior to seeding. As above, cells were exposed to varying durations of IFN-γ prior to inoculation with the GFP expressing pneumococcus, D39-GFP (described above). Fifty minutes following bacterial inoculation (MOI of 15) and incubation at 37°C in 5% CO_2, cells were gently washed 3 times with 1 ml ice-cold PBS to remove unbound bacteria immediately before fixing with 4% paraformaldehyde. Cover slips were mounted using ProLong® Gold anti-fade reagent with DAPI (Invitrogen) and phagocytic index was calculated as the fraction of GFP positive MH-S cells per field (e.g. % MH-S cells associated with at least one bacterium) multiplied by the mean fluorescence intensity (MFI; representing both bound and internalized bacteria) per cell. The use of GFP-expressing pneumococci allowed for MFI quantitation per macrophage at 100x magnification. Data were analyzed as the mean of at least 5 randomly selected fields for each of two replicates per experimental condition, over two entirely distinct experiments. To ensure results were counting bacteria physically associated with the cell surface, or internalized, and not simply residual bacteria remaining in the vicinity of the macrophage, binding was further verified on a subset of samples by staining actin to visualize MH-S cell membrane and high-powered magnification (1000x) was used to ensure pneumococcal-MH-S binding.

6.3.8 STATISTICS

All statistical tests were performed within the R statistical computing environment (R version 2.14, R foundation for statistical computing, R Development Core Team, Vienna, Austria). ANOVA plus Dunnett's test, were used for hypothesis testing for bacterial CFU results when multiple comparisons were made against the control group. Similarly, single-sample t-tests with Bonferroni correction for multiple comparisons were used for hypothesis when ratio of the phagocytic index was the outcome. In this case, single sample t-tests were used because standardization of the control to a value of 1 results in a zero variance in the controls, leaving ANOVA and two-sample T-tests as inappropriate statistical approaches for testing.

6.4 RESULTS:

6.4.1 NON-LINEAR EFFECTS OF IFN-Γ EXPOSURE ON PNEUMOCOCCAL BINDING

We first sought to investigate the dynamics of pneumococcal binding to MH-S mouse alveolar macrophage (mAM) given varying durations of exposure to IFN-γ. mAM were cultured in-vitro with increasing durations (6 hours - 6 days) of exposure to physiologic levels (25ng/ml) of recombinant IFN-γ. Binding was measured as the difference between total bacteria CFU's remaining following lysis with 1% saponin (bound and internalized bacteria) and CFUs remaining following treatment with gentamicin followed by lysis with 1% saponin (internalized only; see methods above). Relative to IFN-γ naïve controls, exposure for only 6 hours significantly reduced pneumococcal binding rates of mAM by ~50% ($p<.05$; Fig 1).

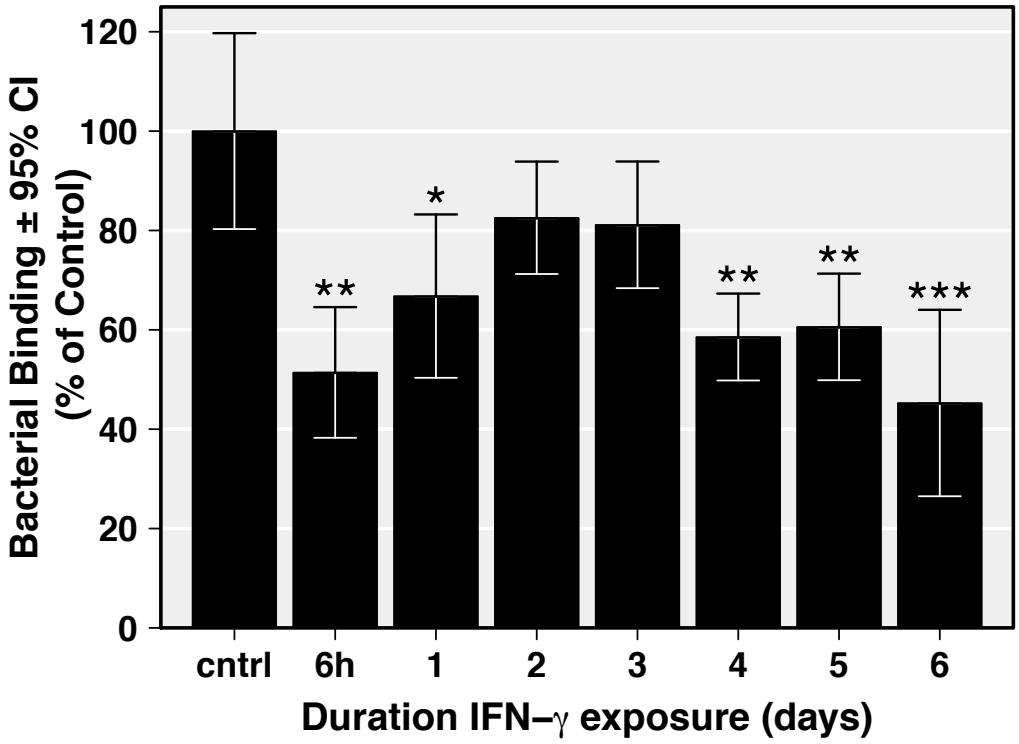

Figure 6.1 Non-linear effects of IFN-γ exposure on bacterial binding.

MH-S cells were seeded and grown in media with or without IFN-γ (25ng/ml/day) for between 0 and 6 days prior to inoculation at an MOI of 15 with the type 2 Streptococcus pneumoniae D39. Bacterial binding was measured as the difference between total bacterial colony forming units (CFU's) internalized and bound (bacteria released from MH-S cells with saponin treatment only) vs. bacterial CFU's internalized (gentamicin protection assay to kill extracellular bound cells followed by saponin treatment to release internalized bacteria). Bacterial CFU's reflect bacteria bound per macrophage (ie: bound CFU/viable MH-S cells). Binding in each condition are then reported as a percent of the mean CFU's bound to IFN-γ unexposed cells. Each bar represents pooled results from 3 independent experiments and each experiment examining 3 distinct wells for each condition for a total of 9 data points per condition. Error bars represent 95% confidence intervals and asterisks (, **, ***) represent statistically significant differences (p<.05, .01, .001; two-tailed Dunnett's test) from IFN-γ naïve controls.*

Interestingly however, this steep reduction was transient and pneumococcal adherence following 1, 2 or 3 days of exposure to IFN-γ recovered to approximately 80% that of IFN-γ naïve controls. This increase was however also transient and

binding fell again to less than 60% that of controls after 4 days of exposure, respectively ($p < 0.05$).

6.4.2 IFN-GAMMA REDUCES PNEUMOCOCCAL INTERNALIZING CAPACITY

To determine effects of increasing durations of exposure to IFN-γ on pneumococcal internalization by MH-S cells, a gentamicin protection assay was performed to kill all extracellular bacteria while keeping internalized bacteria alive and viable for bacterial counting after mAM lysis. Surprisingly, the dynamics of internalization, as a function of IFN-γ exposure, were distinct from effects on binding. Unlike binding, pneumococcal internalization steadily decreased over the first 2 days of IFN-γ exposure, leveling off between days 3 and 5 at approximately 25% capacity before falling to approximately 5% that of controls after 6 days of exposure (Fig. 2; $p<.05$). These dynamics can be approximated, for the reduction in internalization over the first three days, by an exponential decay function, with a decay rate (r) of -0.48 per day of IFN-γ exposure ($R^2=0.92$).

6.4.3 IFN-GAMMA DIMINISHES PHAGOCYTIC CAPACITY AS MEASURED BY THE PHAGOCYTIC INDEX:

To confirm our findings above, which utilitized mAM lysis and serial dilution plating of pneumococci, we performed a series of similar experiments, measuring results using fluorescent microscopy and calculations of the phagocytic index (see methods above). GFP expressing pneumococci were added to MH-S cells and subsequently fixed and stained for relative quantitation of pneumococci via mean fluorescence intensity, or MFI. Importantly, the phagocytic index here is a measure of alveolar

macrophage capacity to associate with bacteria (including binding and internalization). We report our findings as a ratio of the phagocytic index (rPI) of

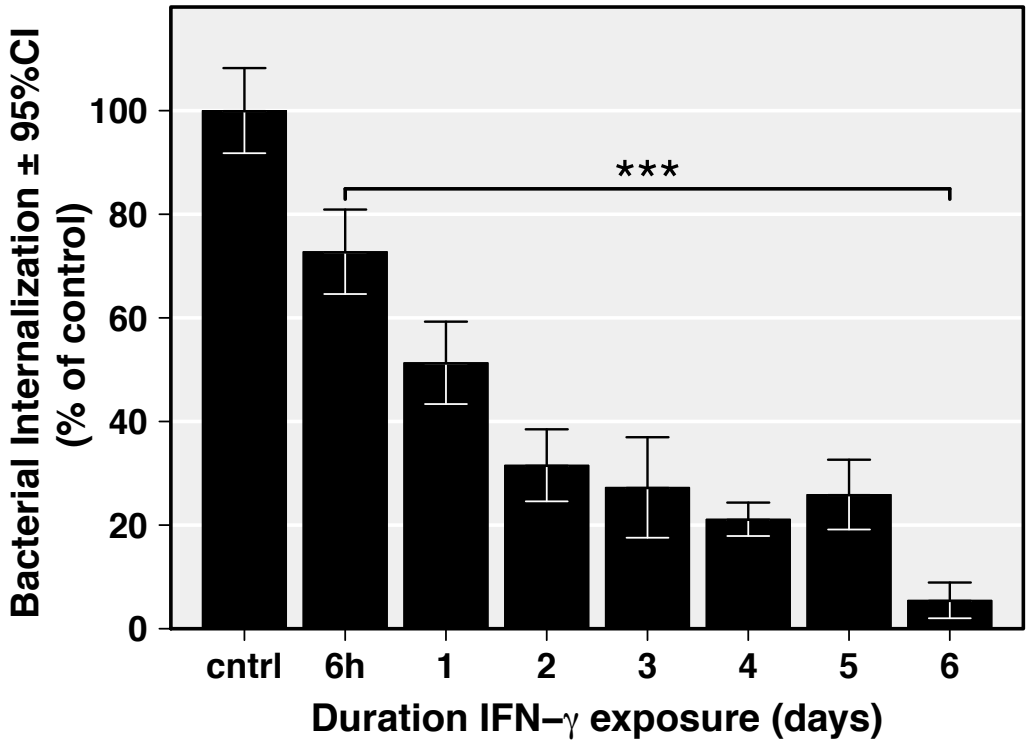

Figure 6.2 Bacterial internalization as a function of exposure to IFN-γ.

MH-S cells were seeded and grown in media with or without IFN-γ (25ng/ml/day) for between 0 and 6 days prior to inoculation with the type 2 Streptococcus pneumoniae D39. Bacterial internalization was measured as the number of internal bacterial colony forming units released from MH-S cells following sequential treatment with gentamicin and saponin. Bacterial internalization was quantified as bacteria internalized per macrophage and are reported here as percentages of the mean CFU's internalized in IFN-γ unexposed cells. Each bar represents pooled results from 3 independent experiments and each experiment measuring three distinct wells for each condition for a total of 9 data points per condition. Error bars represent 95% confidence intervals and asterisks (***) represent statistically significant differences (p<.001, respectively; two-tailed Dunnett's test) from IFN-γ naïve controls.

IFN-γ exposed macrophage vs. IFN-γ unexposed controls (Fig. 3). After only 6 hours of exposure to IFN-γ, the rPI fell from 1 (standardized control) to 0.46 (p=0.02). Similar to the binding results reported above, the PI of the exposed mAM recovered

to nearly 70% that of controls, before dropping significantly to to less than 0.3 after four days of exposure ($p<.001$). That the phagocyti ccapacity, determined by the rPI here, closely resemble effects of IFN-γ on pneumococcal binding (rather than Internalization) is unsurprising considering that MH-S cells here are much more efficient at binding than internalizing bacteria (see results above) and thus the phagocytic index will be dominated by numbers of bound bacteria.

6.5 DISCUSSION

Multiple reports have described a role for IFN-γ in the down-regulation of important AM cell surface receptors required for proper detection and phagocytosis of *Streptococcus pneumonia* and other bacterial pathogens. For example, Sun and Metzger have demonstrated that IFN-γ exposure reduces expression of the class A scavenger receptor, MARCO (macrophage receptor with collagenous structure), which is one of numerous receptors important for proper phagocytosis of Streptococcus pneumoniae 4, 14. Similarly, Chroneos et al. implicate IFN-γ in the reduction of the C-type lectin receptors (such as the mannose receptor), also important for pneumococcal phagocytosis [5] and Mosser et al. too have shown decreased phagocytosis of leishmania promastigotes in response to IFN-γ [6]. While these findings seem in contrast to the well known role of IFN-γ in the activation of resting macrophage [15], Mosser clearly points out that activated macrophage, while more apt to spread out and have higher pinocytic rates, are not necessarily more phagocytic than resident macropahge, due to the downregulation of important surface receptors required for phagocytosis[16].

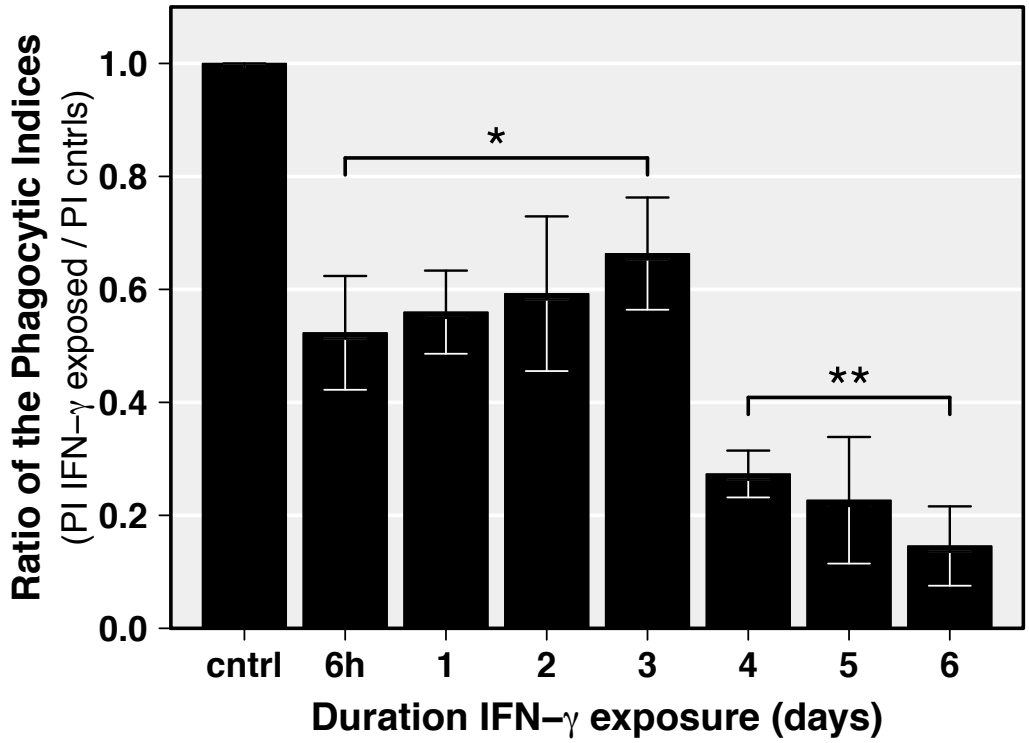

Figure 6.3 Changes in the phagocytic index with extended IFN-γ exposure.

MH-S cells were seeded on glass coverslips and grown in media with or without IFN-γ (25ng/ml/day) for between 0 and 6 days prior to inoculation with a GFP expressing D39 pneumococcus (MOI of 15). Fifty minutes following bacterial inoculation, cells were gently washed 3 times with 1 ml ice-cold PBS to remove unbound bacteria immediately before fixing with 4% paraformaldehyde. The phagocytic index was calculated as the fraction of GFP positive MH-S cells per field (e.g. % MH-S cells associated with at least one bacterium) multiplied by the mean fluorescence intensity (MFI; representing both bound and internalized bacteria) per cell. Data are representative of two independent experiments, and each experimental result calculated by enumerating at least 5 randomly selected fields (which were pooled for analysis) from each of 2 replicate wells at 100x magnification. Data was normalized for each experiment to the phagocytic index of the control cells. Error bars represent 95% confidence intervals. Asterisks (, **) indicate statistically significant differences from the control group (p<.05, <.001; two-tailed single sample t-test with Bonferroni correction for multiple comparisons).*

To further support and clarify downregulation of phagocytic capacity in response to IFN-γ exposure, Trost et al. [17] and Yates et al. [18] demonstrated that IFN-γ exposure leads to delayed lysosomal fusion and reductions of hydrolytic and proteolytic activities within the phagosome, respectively. Trost et al. hypothesized

that such a delay in lysosomal fusion, while seemingly contradictory to the activation of macrophage functions would ultimately enhance antigen presentation.

Although these investigations have each previously demonstrated effects of IFN-γ on reductions in bacterial phagocytosis, the kinetics of phagocytosis as a function of IFN-γ exposure have not been previously investigated. Such an understanding of these phenotypic dynamical changes in bacterial binding and internalization will help future investigations to better elucidate underlying mechanisms of various time-dependent disease processes, particularly with regard to co-infections that result in excess pneumococcal respiratory infections. For example, infection with an influenza virus upregulates expression of IFN-γ and is also known to contribute significantly to enhanced susceptibility to lower respiratory tract (LRT) bacterial infections in a time-dependent fashion (ie: most reports suggest maximal synergy when pneumococcal infection occurs around 6 days post influenza infection). [19] As Sun and Metzger reported [4], this may be due to effects of excess IFN-γ production on AM capacity to clear pneumococci. What is not reconciled in their report however is why IFN-γ exposure significantly reduces AM function ex-vivo within 24 hours while enhanced pneumococcal infections in the LRT is often not detected until around 6 or 7 days post influenza infection.

In agreement with the numerous reports mentioned above, we found that IFN-γ significantly reduces pneumococcal phagocytosis by the MH-S murine AM cell-line. We found that effects on binding, vs. internalization, are distinct and non-linear; whereas exposure to IFN-γ resulted in an initial exponential decay in the rate of pneumococcal internalization, pneumococcal binding paradoxically recovered to

near control levels by days 3 and 4, after a transient decrease was measured 6 hours post exposure.

We cannot conclude from the data described herein why binding demonstrates a transient recovery while internalization continues to fall. However, this phenotypic finding could aid in reconciling, for example, the findings of Metzger and Sun that internalization is reduced within 24 hours, but effects in terms of excess burden of bacterial disease are not detected until approximately 6 days. In this case, although internalization may be reduced, if near normal pneumococcal binding rates persist, binding alone may be sufficient to temporarily stave off severe excess bacterial disease until after both internalization and binding have fallen to sub-protective levels [20]. Such a finding would be further supported if macrophage, like neutrophils demonstrated an ability to kill bound pathogens in an extracellular fashion, without the need for ingestion.

Recently, Chow et al. discovered macrophage extracellular trap (MET) formation following treatment with statins in the RAW 264.7 murine macrophage cell line [20]. They report that METs led to normal bacterial clearance following statin use that was paradoxically coincident with reductions in bacterial internalization and suggest that MET formation induced by statins provides a mechanism whereby normal bacterial binding and clearance may persist in the absence of internalization. It is possible that excessive activation following extended exposure to IFN-γ may induce the development of MET-like phenotypes in AMs that could allow binding and bacterial killing in the absence of internalization.

While we have shown differential dynamics induced by IFN-γ on pneumococcal binding vs. internalization, our findings, which clearly support previous reports of IFN-γ induced reductions in pneumococcal clearance are limited to MH-S murine AM cell line. While this cell line is well described, with a rich history of use for in-vitro investigations of alveolar macrophage function, immortalized cell-lines clearly have distinct properties from cells in-vivo. Thus, we hope our report lays some groundwork for further investigators working with in-vivo or ex-vivo systems to study effects long-term exposure to IFN-γ on macrophage function.

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CHAPTER 7. MODELLING CROSS-KINGDOM EFFECTS OF VACCINATION: A LIVE VIRAL VACCINE PROFOUNDLY ALTERS POPULATION LEVEL BACTERIAL PATHOGEN DYNAMICS

Michael J. Mina

7.1 ABSTRACT

Vaccination campaigns have been unequivocally beneficial to reduce disease from vaccine-target species via direct (immune) and indirect (herd) effects[2, 3]. The consequences of wide-scale vaccination campaigns on the broad ecology and disease dynamics of entirely unrelated, non-vaccine-target pathogens remain however largely unexplored. Recently we demonstrated that vaccination with a live attenuated influenza virus vaccine (LAIV) significantly increased both the density and duration of bacterial colonization in a fashion nearly identical to that seen following WT influenza virus.[8] We have also found that, similar to WT virus, LAIV increases incidence and duration of bacterial acute otitis media. Previous reports have demonstrated that excess bacterial titers in the nasopharynx, whether due to influenza[9, 10] or other causes like non-viral inflammation or experimental neutrophil suppression,[11] are sufficient to increase bacterial transmission between colonized donor and non-colonized recipient hosts. Further, prior infection with influenza virus has the particularly potent effect of priming the nasopharynx for increased bacterial acquisition.[9] Considering the similar effects of live attenuated and wild-type influenza viruses to enhance bacterial density and duration of colonization in our previous investigations, live attenuated viruses may too increase bacterial transmission and prime the nasopharynx of vaccinated individuals for increased bacterial acquisition. Here, through a series of mathematical models we show that vaccination with live attenuated virus across a population can induce a novel class of herd-effects that we term '*generalized* herd-effects' which, unlike traditional herd-effects that reduce disease by vaccine-target pathogens by disrupting transmission, can significantly enhance, or reduce transmission of non-vaccine-target pathogens within divergent classes and ecological domains from the vaccine-target species. The

implications of such effects are vast. Our results suggest that wide-scale vaccination campaigns can drive prevalence of highly important human pathogens that are entirely unrelated to the vaccine-target species.

7.2 INTRODUCTION

Vaccination is intended to directly protect vaccinees against specific target pathogens through modulation of innate and adaptive immune responses[14]. Benefit often indirectly extends to unvaccinated individuals by herd protection, brought about by ecological crashes of target pathogen populations, initiated by interruption of transmission following sufficiently high vaccine coverage[15]. Through these direct and indirect effects, vaccines have been unequivocally beneficial by decreasing the incidence of disease from vaccine-target (VT) species.

Evaluations of vaccine effects usually focus on vaccine efficacy (ie: the reduction in disease incidence in a vaccinated vs. an unvaccinated population) against the targeted pathogen and occasionally extend to changes in closely related pathogen species or subspecies[16-18]. Increasingly however, multispecies interactions between phylogenetically distinct pathogens are observed to play important roles in modulating both health and disease. These interactions are likely to be most prevalent within mucosal surfaces that harbor complex communities of microorganisms and, importantly, include interactions between entirely phylogenetically distinct microbes.

Evaluation of vaccine herd-effects have thus far been restricted primarily to benefit conferred from reducing VT species, while the impacts of vaccination on pathogen dynamics from non-vaccine-target (NVT) species within and across species and

even taxonomic domains remain largely or wholly unexplored. With increasing awareness of the delicate balance underlying multispecies interaction and pathogen co-infection[19, 20], the potential for profound, unintended adverse changes in human pathogen transmission and disease by NVT species following vaccine campaigns should be actively considered.

Unintended ecological sequelae of vaccines have been observed, including replacement by non-vaccine genotypes within the same[21] or closely related[22] species, or via conversion of vaccine strains to virulent pathogens[23]. Replacement is driven, at least in part, by immune mediated reduction in competitive fitness of the VT strains, providing a competitive advantage to the NVT pathogens[24].

Recently, we demonstrated, in-vivo, that vaccination with a live attenuated influenza vaccine (LAIV) can profoundly increase bacterial carriage of at least six distinct human bacterial pathogens including four serotypes of both colonizing and invasive strains of Streptococcus pneumoniae (19F, 7F, D39 and A66.1) and two strains of Staphylococcus aureus (Wright and Newman).[8] The increases in bacterial carriage density and duration were seen irrespective of whether bacterial inoculation was subsequent to vaccination (within 28 days), or vaccine was introduced into pre-colonized mice. However, as time from vaccination to bacterial inoculation increased from -7 days (ie: precolonization) to subsequent colonization beyond a week and up to 28 days, the magnitude of response decreased. Maximum effect was however seen when LAIV preceded pneumococcal colonization by 7 days.[8] Importantly, effects of vaccination on bacterial carriage were nearly identical in all aspects to those changes observed following influenza infection with the wild-

type parent virus from which the LAIV strain was descended. Further we also have shown that LAIV increases incidence and duration of bacterial acute otitis media in a manner similar to the WT virus (See chapter 3).

Despite these unexpected adverse sequelae of LAIV on bacterial burden and disease in the upper respiratory tract, we have also found a transient but profound benefit of LAIV to provide significant protection against lethal bacterial infection with invasive pneumococcus. (See chapter 4) Indeed, when mice were vaccinated seven days prior to a 50% lethal dose of either D39 or A66.1 pneumococcus, LAIV reduced mortality by over 80% relative to PBS primed mice. In contrast, priming with the wild-type parent virus significantly increased mortality and 100% of mice died within only 2-3 days.

In another study, we also demonstrated an important benefit of early LAIV vaccination to prevent excess bacterial carriage in the nasopharynx secondary to influenza infection.[25] When LAIV vaccination preceded WT influenza virus infection by 35 days, LAIV vaccination completely abrogated excess post-influenza bacterial carriage.[25-27]

Bacterial carriage in the upper respiratory tract is the gateway for both horizontal (between-host) and vertical (within-host) bacterial transmission[28, 29] and there is a putative positive association between bacterial carriage density in the nasopharynx and bacterial dissemination. In mouse and ferret models (the latter considered to be among the most appropriate models for human respiratory infections) excess bacterial titers in the nasopharynx secondary to influenza infection significantly increased pneumococcal transmission.[9, 10] Further, the cause

for increased bacterial carriage density and transmission was demonstrated to be independent of influenza infection as non-viral mediated inflammation and chemical reduction of neutrophils each increased bacterial density and subsequent transmission between infant mice. These same processes also aid significantly to horizontal spread of bacterial pathogens by increasing enhancing bacterial acquisition. Indeed, priming of the nasopharynx of non-colonized mice and ferrets with influenza or non-viral mediated inflammation resulted in significant increases in bacterial dissemination from donor to recipient, independent of bacterial density in the donors.[9, 11, 30] Thus, influenza and inflammation synergistically increases bacterial transmission via two independent mechanisms: increasing bacterial titers in colonized 'donors' priming the nasopharynx for bacterial acquisition in uncolonized recipients.

Given the profound capacity for both wild-type influenza virus and LAIV vaccine to replicate within the nasopharyngeal tissue and increase bacterial carriage, and the positive associations between increased carriage density and transmission as well as viral replication and priming for bacterial acquisition, LAIV may too increase horizontal bacterial dissemination by both increasing bacterial density and enhancing rates of acquisition in LAIV vaccinated recipients. We sought here to explore potential impacts of LAIV vaccination on bacterial carriage rates and incidence of disease across a population over the course of a year, which included a single influenza epidemic.

Collecting samples and acquiring data to directly measure effects of LAIV on bacterial transmission across a population is difficult, if not impossible, in particular

owing to the current low levels of LAIV coverage of <4% of the US population (12 million doses of LAIV distributed in 2012-'13 in the US). Further, properly measuring transmission rates to capture effects amongst vaccinated and non-vaccinated bystanders across a population requires large sample sizes and longitudinal sampling that is both labour intensive and costly. Finally, measuring effects of LAIV on bacterial transmission in a population would be confounded by competing effects of LAIV to reduce influenza infections and prevent influenza mediated enhancements of bacterial transmission, potentially masking direct effects of LAIV on bacterial transmission. For these reasons we decided to investigate potential effects of wide-scale LAIV vaccination campaigns on bacterial transmission and disease using nested within- and between-host mathematical models that combine effects of LAIV and influenza virus on bacterial carriage density and disease with effects of density and virus priming on bacterial transmission and acquisition. Model development and parameterization are described in detail in the methods. Briefly, clinical and epidemiological data were used to parameterize model inputs including vaccine efficacy, baseline bacterial carriage and disease rates and pathogen invasivity. As well, timing of vaccine distribution was parameterized to reflect current vaccination strategy in the US based on information available from the US Centers for Disease Control and Prevention and influenza transmission accounts for known effects of environmental conditions and is parameterized in part using publicly available NOAA data. Finally experimental data from animal models were used to parameterize specific in-vivo interactions between wild-type influenza virus, LAIV and bacterial density and probability of transmission events.

Here, we describe a series of novel findings that a prominent and established live attenuated vaccine can profoundly impact population level dynamics of pathogens within taxonomic kingdoms distinct from the vaccine-target species. Whether that impact is generally beneficial or detrimental depends primarily on the proportion of LAIV coverage, vaccine efficacy and the basic reproductive number of the circulating influenza virus.

We show that the effects of LAIV vaccination on bacterial carriage and disease extend beyond the vaccinated individuals to unvaccinated 'bystander' populations. We detail a new term to describe this population level effect a *generalized herd effect*. In general, effects of LAIV on bacterial dynamics were robust to changes in prevalence of baseline bacterial colonization as well as variations in the within-host interactions between WT or LAIV viruses and bacterial replication and transmission. Our findings have considerable public health implications at both individual and human host population levels.

7.3 RESULTS

7.3.1 DEFINITION OF THE "GENERALIZED HERD-EFFECT"

While direct effects of a viral vaccination on bacterial colonizing density and duration of colonization are important in their own right, as previously described,[8] potential indirect effects of wide-scale vaccination on bacterial carriage throughout a population could be extensive. Vaccine induced inflammation within the nasopharynx, may increase bacterial density and probability of bacterial

transmission in colonized hosts or acquisition in uncolonized hosts[9, 11] and once acquired, our in-vivo data suggest these bacteria will proliferate more rapidly and to greater densities than in unvaccinated individuals, increasing probability of transmission.[11] Given sufficient vaccine coverage, such a chain of immunologic and microbial acquisition/transmission events may drive colonization rates and disease in both vaccinated and unvaccinated 'bystanders', particularly in populations with high baseline prevalence of bacterial pathogens. We term such a scenario a '*generalized* herd-effect' (GHE) where 'herd-effect' is defined as: *an alteration in the epidemiology of a microbial species in an unvaccinated segment of a population resulting from vaccination of a portion of the population*[31] and '*generalized*' refers to a relaxation on the assumption that the effect occurs at the level of the vaccine-target species. Further we refer to traditional herd-effects as 'directed herd-effects' (DHE). Whereas DHE's are normally advantageous, GHE's may be advantageous or adverse and such effects could be difficult or impossible to detect using current vaccine safety methodologies focused on individual vaccine recipients and target species.

We developed a mathematical compartment model to investigate potential impacts of LAIV on pneumococcal colonization over the course of a year, including a single influenza season with vaccine rollout similar to that which occurs annually in the United States[31] (see Model Development for full model descriptions).

The framework for the model considered here is described in figures 1 and 2 and in greater detail under 'Model Development'. Baseline colonization rates, initially at equilibrium, become disrupted when influenza virus or LAIV's are

introduced into the population. Simulations are run for increasing target vaccination rates (also termed population vaccinated). In the absence of influenza, vaccination with LAIV increases overall prevalence (Fig. 3a and Supplementary Fig. S1a) and incidence (Figs 3b and Supplementary Fig. S2a) of colonization under all target vaccination rates. Similarly, when influenza strains with relatively low basic reproductive numbers ($R_0 < 1.5$) are introduced, LAIV's always increase colonization rates over the excess already expected from the circulating virus (Fig. 3b and Supplementary Figs. S1b and S2b).

Under moderate seasonal influenza R_0 values (R_0: 1.5 – 1.9), LAIVs can reduce overall colonization by decreasing influenza related bacterial acquisition and transmission (Figs. 3b-e and Supplementary Figs. S1c, d and S2c, d). However, as target vaccine coverage (ie: proportion vaccinated) is increased, a threshold is reached where colonization is minimized and further expansion of vaccine coverage has a relatively detrimental effect. We term this minimum in colonization the "maximum *constructive* generalized herd-effect threshold" or *cGHE*. As proportion of the population vaccinated increases away from the maximum cGHE, prevalence rates eventually exceed that of an entirely unvaccinated population resulting in an absolute, rather than a relative, detrimental effect on acquisitions. We term this latter point the *destructive* generalized herd effect threshold because once vaccination increases beyond the dGHE, any increase in vaccination will have a net adverse effect on the population by increasing annual incidence of the secondary pathogen (pneumococcus). Of note, as R_0 increases, GHE thresholds too increase, requiring vaccination of higher proportions of the population to minimize

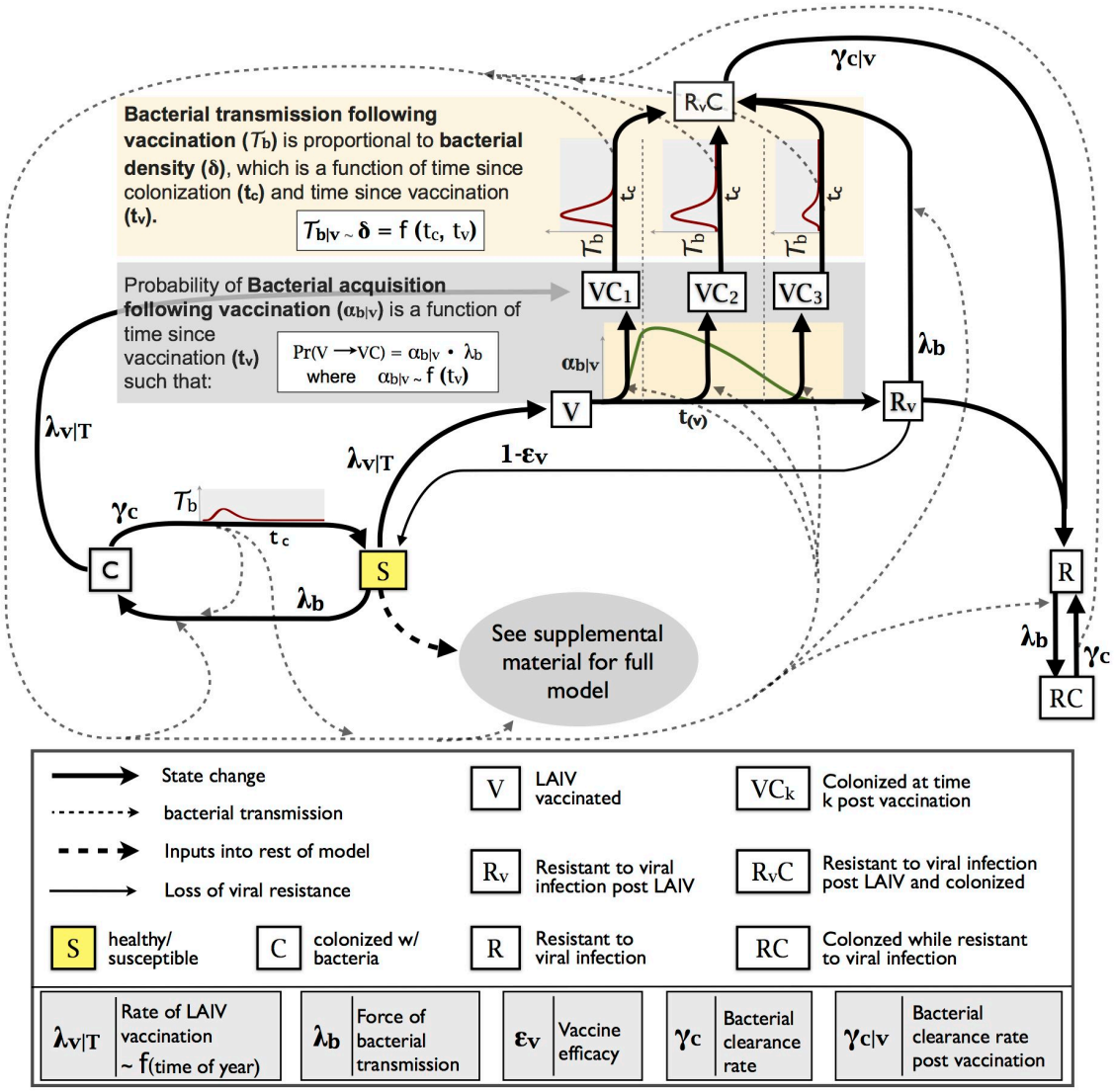

Figure 7.1 Partial framework for a mathematical compartment model linking within-host LAIV-influenza-pneumococcal dynamics with population dynamics of pneumococcal colonization and disease

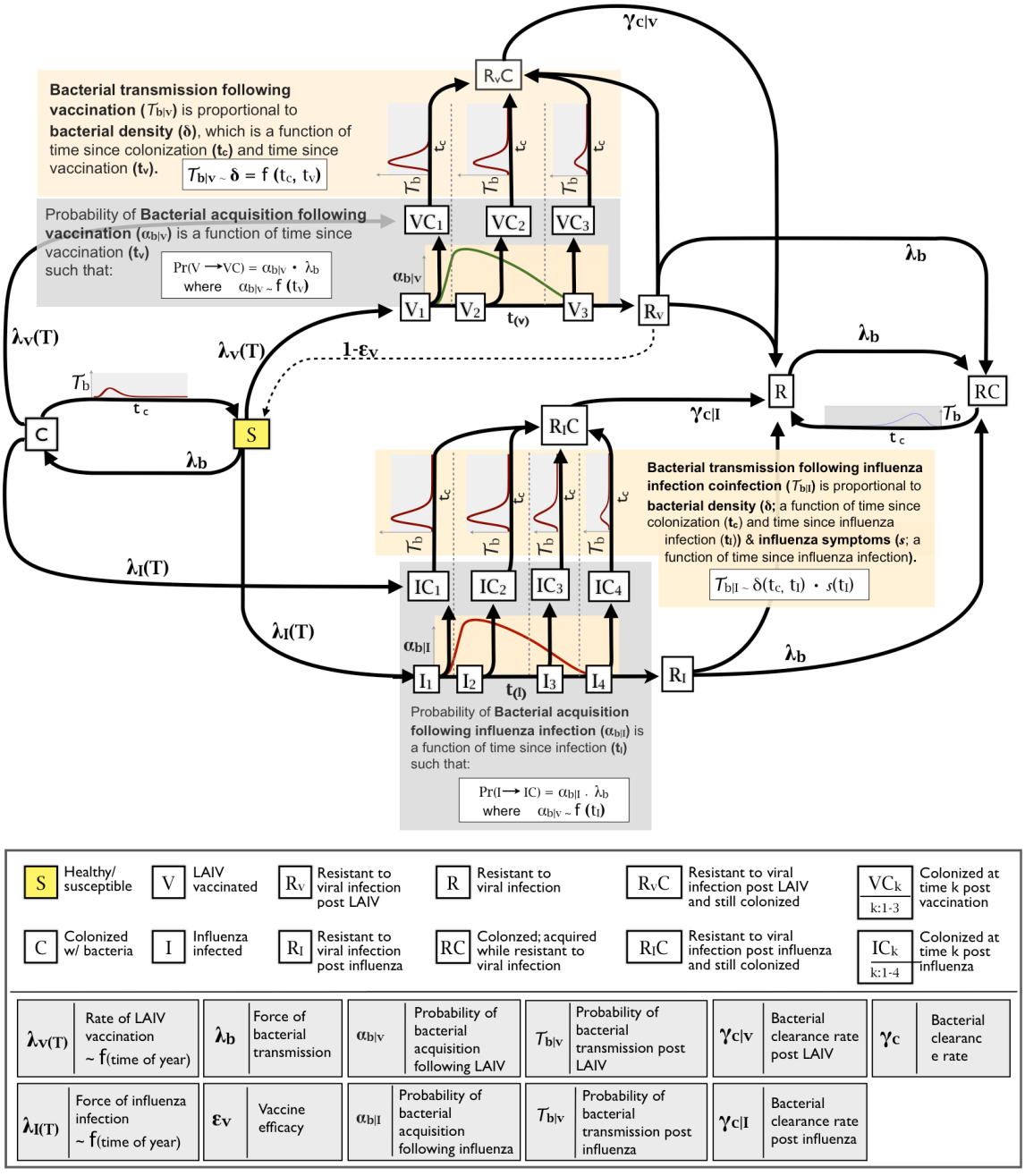

Figure 7.2 Partial framework for a mathematical compartment model linking within-host LAIV-influenza-pneumococcal dynamics with population dynamics of pneumococcal colonization and disease

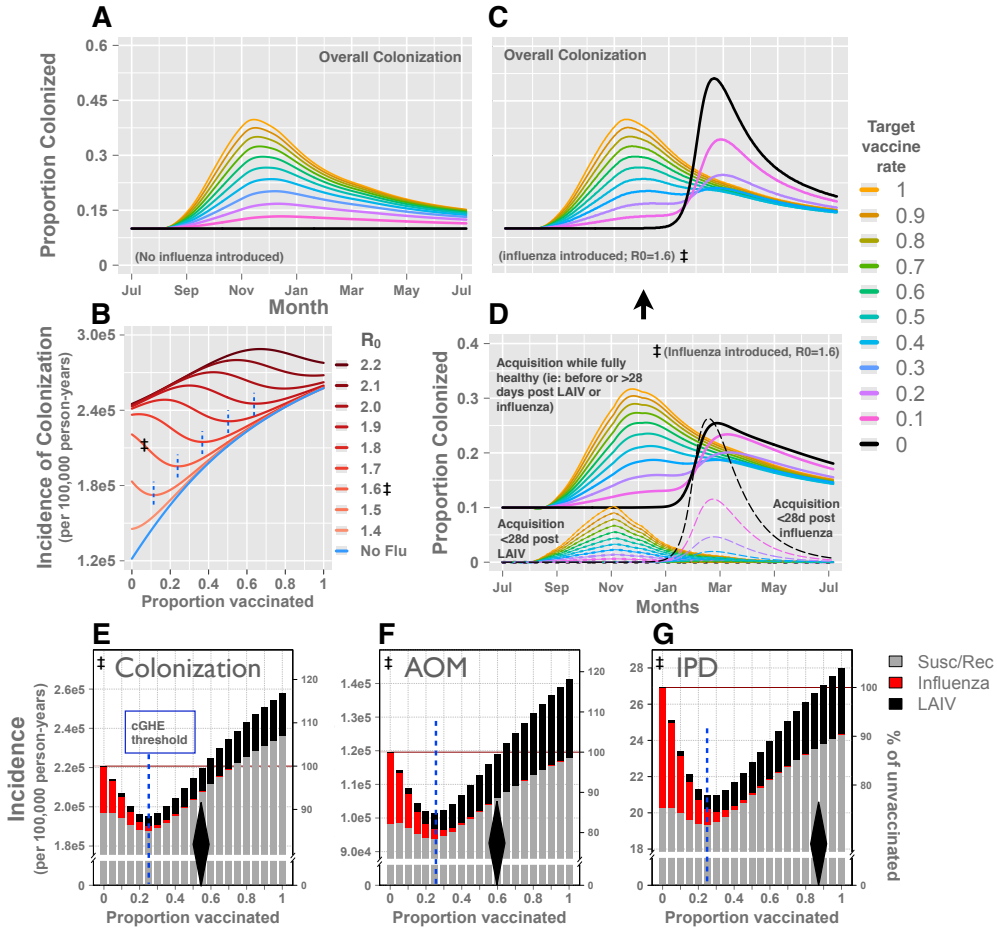

Figure 7.3 LAIV vaccination alters population dynamics of bacterial pathogens.

(A) Prevalence of pneumococcal colonization following LAIV vaccination campaigns is increased in the absence of influenza in all target vaccine rate scenarios. (B) Introduction of wild-type viruses with differing basic reproductive numbers (R_0) alter annual incidence of bacterial colonization, dependent on proportion of population vaccinated (equivalent to target vaccine rate). (C) Overall prevalence of colonization increases with an influenza epidemic (R_0=1.6) and can be abrogated or exacerbated by LAIV vaccine campaigns. (D, E) The greatest changes in prevalence (D) and incidence (E) are generalized herd effects where colonization is altered in unvaccinated, healthy individuals susceptible or recovered individuals – driven by direct effects on colonization of vaccinated individuals. The three distinct groups in (D) indicate contribution to overall prevalence of colonization shown in C by viral status at time of bacterial acquisition: bacterial acquisition while: fully susceptible (ie: generalized herd immunity group; solid lines), within 28 days post LAIV vaccination (dotted lines) or within 28 days following influenza infection (broken lines). (E-G) Incidence of colonization, acute otitis media or invasive pneumococcal disease at increasing target vaccine rates (x-axis). Columns are color coded to depict contribution to incidence by each of the three respective groups described for D.(grey is incidence following acquisition by susceptible or fully recovered individuals, red is incidence following bacterial acquisition when infected with influenza and black is incidence in individuals who acquired bacterial within 28 days of LAIV. Constructive (blue dashed lines in B, E-G) and destructive (black diamonds; E-G) generalized herd effect thresholds of target vaccination rates (ie: proportion vaccinated) may be

reached that minimize or increase, respectively, incidence colonization (E) or disease (F, G) vs. an entirely unvaccinated population (prop. vaccinated = 0). ‡indicate simulations run with influenza R_0 value of 1.6.

colonization (see blue broken bars in Fig. 3b). At high R_0 values (≥ 2.0 in our model), target vaccine rates would have to be so high to sufficiently abrogate the influenza epidemic that excess colonization throughout the population as a result of vaccination overrides any benefit of reducing influenza mediated excess colonization, and a constructive GHE becomes unattainable (Supplementary Fig. 3e-f). Interesting, in certain scenarios, a third threshold exists that the target vaccinecoverage must exceed to enter into the territory of a constructive GHE (see proportion vaccinated of 0.3 in supplementary Fig S2d). We term this third threshold the *minimum constructive generalized herd effect threshold* or $cGHE_{min}$. When a $cGHE_{min}$ exists, any plan for vaccination rates either below the $cGHE_{min}$ or above the dGHE will be generally destructive with regard to the secondary pathogen.

Generalized herd effects imply changes in bacterial colonization rates that extend beyond vaccine recipients. Indeed, like traditional herd-effects where a large portion of benefit is at the level of the unvaccinated individuals[15] the greatest absolute changes in bacterial colonization as a result of GHEs often occur in the unvaccinated, uninfected 'bystander' population, despite being driven by individual within-host effects at the vaccine recipient levels. This is demonstrated in figure 3d, and see footnote[1] below for an illustrated example. Given a scenario with a baseline

[1] To illustrate the impact of the GHE on the bystander vs. vaccinated population, we direct the reader to figure 3e. In figure 3e, take the difference in height of the grey portion of the bar at proportion vaccinated of 0.8 relative to proportion vaccinated of zero (2.23e5 - 1.98e5 = 2.5e4) and

colonization rate across the population of 10% and a moderate influenza virus, figure 3c depicts the total prevalence of colonization during the year, subset by target vaccination rate. In total, early increases in bacterial colonization following vaccination abrogate later increases in prevalence of bacterial that would otherwise arise as a result of an influenza epidemic, particularly in the extreme case of not vaccination coverage (black curve in figure 3c). To better understand how vaccination and wild-type influenza virus infections contribute to overall prevalence, the total prevalence curves in figure 3c are broken down by vaccination and influenza virus infection status at the time of bacterial acquisition in figure 3d. Prevalence of colonization due to acquisition following shortly after vaccination is generally in the lower left quadrant owing entirely to the time of vaccine rollout beginning with no vaccination in July, and hence a prevalence of zero in this group during July. Vaccination largely tapered off into the spring, with clearance of bacteria following acquisition in vaccinated individuals lagging up to three months behind. Prevalence of colonization in individuals acquiring bacteria within 28 days following influenza infection is generally in the lower right quadrant of figure 3d, primarily a result of when the influenza epidemic picked up. Finally, in the upper center of figure 3d is prevalence of colonization due to bacterial acquisition in the

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compare this value to the height of just the black portion of the bar at 0.8, representing the number of bacterial acquisitions directly related to vaccination (2.41e5 – 2.23e5 = 1.8e4). Thus, in a population of one million, providing 80% of the population with LAIV over the course of the 'flu vaccine season' (generally September to February) led indirectly to an additional 25,000 bacterial acquisition events in the 'bystander' population (unvaccinated, uninfected with influenza), which is greater than the 18,000 extra bacterial acquisition events that were directly associated with vaccination (ie: acquisition during the 28 days post-LAIV). That said, this simple calculation is for illustrative purposes only, as the calculation fails to consider a number of other factors such as the number of bacterial acquisitions averted in either group due to reduced influenza virus circulation following such a robust vaccination campaign reaching 80% of the population.

'bystander' population. Prevalence in this group is initially at 10% (this group encompassed the entire population prior to seasonal LAIV rollout and introduction of seasonal influenza virus) and increases as a result of increased transmission amongst the virus inoculated groups spilling over into the bystander population. This is the generalized herd-effect. The magnitude of the generalized herd effect is usually larger than direct effect on prevalence seen in the vaccine recipients but is not always larger than the direct effect that would occur from influenza virus in the absence of vaccination (compare black solid to black broken curve).

Acute otitis media (AOM) and pneumococcal invasive disease (IPD) follow from bacterial acquisition, thus LAIV, like influenza virus infection, may increase incidence of both AOM and IPD amongst a population by increasing rates of bacterial acquisition, as just discussed. Further, also like WT influenza virus, LAIV can increase AOM within the vaccinated individual by increasing the attack rate of colonizing bacteria to invade the middle ear (See chapter 3 and model development details). Unlike influenza virus infection however, LAIV vaccination does not increase attack rate of IPD in vaccinated individuals, and may instead confer protection against bacterial IPD and pneumonia, as discussed in chapter 4. These distinct differences in the within-host dynamics of LAIV vaccination and influenza virus infection on bacterial AOM and IPD are apparent at the population level (Fig. 3F and G and supplementary Figs. S3 and S4.). Because LAIV transiently increases the attack rate of AOM in vaccinated individuals who are already colonized or who acquire bacteria shortly following LAIV, the relative reduction in annual incidence of AOM at the cGHE due to abrogating the influenza epidemic is countered by the

increased attack rate of AOM. On the other hand, because LAIV does not predispose to increased IPD at the individual level, the relative reduction in IPD at the cGHE is increased over the reduction in AOM. As well, because infection with influenza virus greatly increases attack rate of IPD, the benefit of LAIV conferred by reducing the influenza epidemic is even further enhanced. In fact, although no direct protection of LAIV against IPD was modeled at the individual levels and IPD attack rates amongst each of the three individual acquisition groups were constant, as proportion of the population vaccinated increased to 1, IPD attack rate (measured as incidence of IPD/incidence of bacterial acquisition) decreased, owing to a reduction in influenza infections (Supplementary Fig S4a). At the population level, this two-fold benefit to reduce IPD is also demonstrated by a large rightward shift of the dGHE threshold for IPD relative to AOM and colonization (0.88 vs. 0.61 and 0.55, respectively). This shift demonstrates that up to 88% of the population could receive LAIV before incidence of IPD would become elevated over the unvaccinated scenario.

To more formally assess the role of vaccination amongst the vaccinated and bystander populations, the relative risk (RR) of bacterial acquisition and AOM were calculated as function of proportion vaccinated, and stratified by different influenza R_0 scenarios (Fig 4). Amongst the bystander population, the RR of colonization generally increased as the target population to be vaccinated increased, compared to the no vaccination scenario, although slight reductions were noted around at lower levels of vaccination cGHE (Fig 4a). RR of bacterial AOM in this group (Fig 4b) were nearly exactly the same as the RR of bacterial acquisition, which was to be expected because the attack rates were held constant. Very slight deviations were

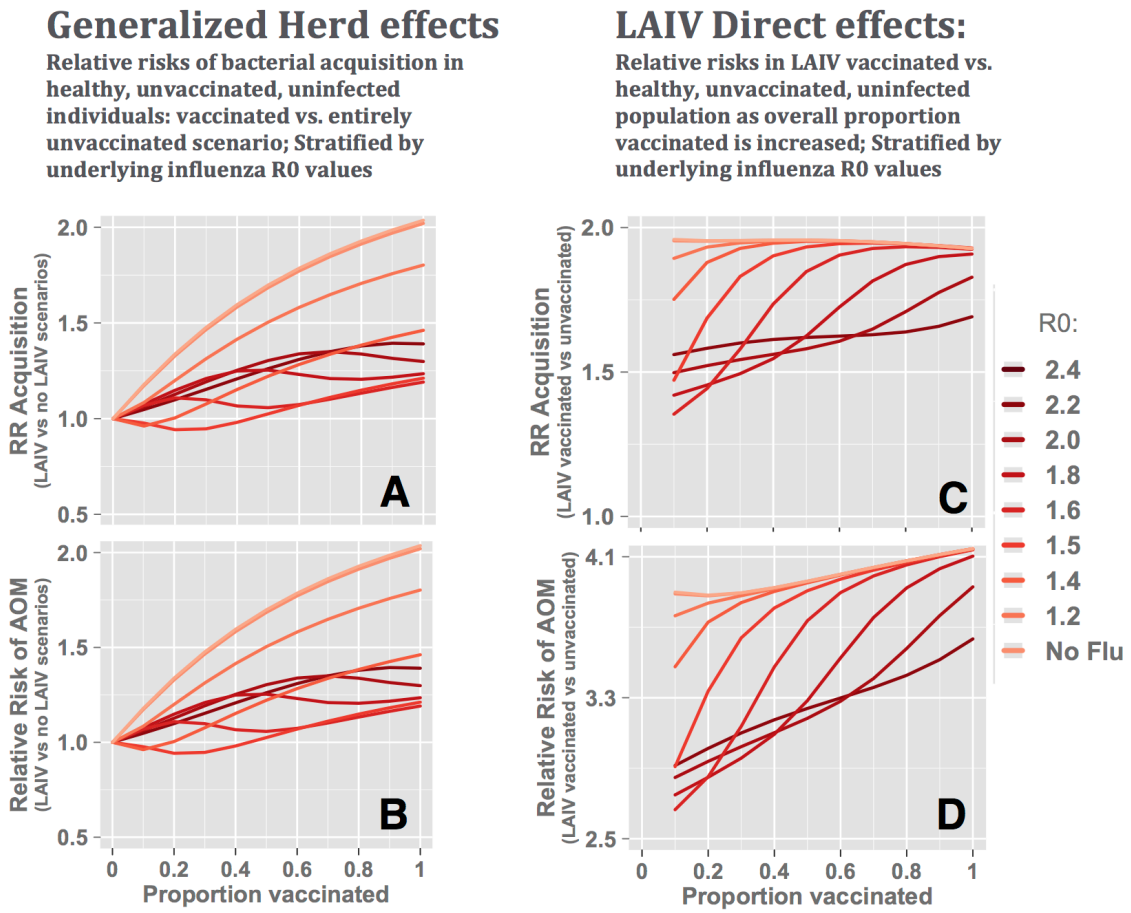

Figure 7.4 Relative risks of bacterial colonization and acute otitis media following LAIV vaccination campaigns

Relative risks (RR) of colonization (ie :bacterial acquisition) and bacterial acute otitis media are calculated for increasing target vaccine rates and stratified by underlying influenza R0 values. Relative risks of colonization in entirely susceptible (ie: unvaccinated, influenza uninfected and not colonized) individuals are affected by LAIV vaccination through generalized herd effects (A). Similarly, RR of AOM in colonized, but otherwise susceptible (ie: unvaccinated, influenza uninfected), individuals are affected by the proportion targeted for vaccination (B). RR's in (A) and (B) are relative to the scenario where no LAIV vaccination is distributed. Direct effects of LAIV on bacterial acquisition and AOM in LAIV vaccinated individuals are shown (C and D). RR of acquisition following LAIV vaccination (C) refer to the risk of acquiring bacteria within 28 days following LAIV vs. the risk of acquiring bacteria within any 28 day period for any unvaccinated, influenza uninfected susceptible individual. Similarly, RR of AOM following LAIV (D) refers to the risk of colonization evolving into an AOM episode in a vaccinated and colonized individual vs an unvaccinated, influenza uninfected but colonized individual.

however noted due to a relatively few individuals that were categorized as bystanders at the time of bacterial acquisition but were subsequently vaccinated or infected with influenza virus during colonization. In this case, the attack rate for these individuals was elevated, slightly altering the overall RR of AOM in the bystander population as vaccination levels increased. Graphically, these differences were imperceptible.

A similar analysis was performed to assess RR of acquisition and AOM in LAIV recipients as compared to the 'bystander' population. Because relative risk was zero in the case of no vaccination, curves are drawn beginning with target vaccination rates of 10% (Fig 4c and d). RR of bacterial acquisition and AOM were always elevated for vaccine recipients vs. 'bystanders', however the degree to which they were elevated varied by both influenza R_0 and target population to be vaccinated. Interestingly, at the extremes of the R_0 scenarios (ie: no influenza or 1.2 and R_0 of 2.2 and 2.4) relatively little change in RRs was detected as proportion targeted for vaccination increased. On the other hand, given relatively modest R_0's the RR for both acquisition and AOM increased dramatically as proportion vaccinated increased. We attribute this to the relatively small effect of vaccination on overall bacterial carriage at very high R_0's (see supplementary figure S2e and f) and the large GHE upon the bystanders, particularly at low R_0 values where there is little benefit for the bystanders in terms of reduced influenza infections at high levels of LAIV coverage.

7.4 DISCUSSION

Our results highlight, an important but almost entirely overlooked aspect of vaccine biology, ecology and safety, namely the potential unintended effects of vaccination on the broad disease ecology of unrelated, non-targeted human pathogens across species and ecological taxonomic kingdoms. We have previously demonstrated that an established intranasal vaccine against a virus can have profound and long-lasting adverse effects on the proliferation, duration of carriage and virulence of multiple, highly relevant human bacterial pathogens that add significantly to the global burden of LRI's, the leading killer of children globally[8, 32], and significantly contribute to the burden of antimicrobial resistant organisms in circulation[33, 34]. That this effect occurs through a process of ecological facilitation (vs. diminished competition, as is often seen with serotype replacement following vaccination[35, 36]) is of importance; while competition often assumes reliance on a common limiting resource (eg: energy, niche space), facilitation requires only that one biologically relevant entity (ie: microbe, pathogen, vaccine, etc.) enhance or *facilitate* another microbe's survival, either directly or indirectly, while placing no constraints such as shared resources. By relaxing an assumption of shared resources, microbial facilitation has increased potential to effect entirely unrelated pathogens across species and kingdoms that often share no common limiting resource.

To date, the effects that we have described through our in-vivo experiments and mathematical models have not been noted, despite numerous pre- and post-

licensure studies on the safety of the FluMist® vaccine, and we attribute this to at least three important findings. As stated, traditional vaccine safety and efficacy methodologies rarely consider disease due to entirely distinct infectious pathogens, and while LAIV safety studies have looked at incidence of AOM following vaccination there has not been a distinction between bacterial and viral (ie: influenza) etiologies. Therefore, the well-described benefits of reducing influenza associated AOM [37] could mask any true increases in bacterial AOM. Second, due to the asymptomatic nature of bacterial carriage, a proxy for increased bacterial prevalence is often invasive disease. In our experiments, we found no increases in bacterial invasive disease following vaccination, at the individual level, and thus modern vaccine safety trials, focused on individual vaccine recipient safety [38] would fail to pick up population level increases in disease. Finally, and perhaps most importantly, LAIV vaccination thus far has covered only a small fraction of the population (~0.5%-2%). At these rates, our model suggest that any generalized herd effects would either remain below detectable levels or would likely prove beneficial to reduce bacterial disease, as rates of 0.5 – 2% are well below dGHE thresholds. Nevertheless, the broad implications of the current study are a warning that vaccine policy and evaluation should strongly consider potential generalized herd effects that may alter a broad array of pathogen populations across species, classes and ecological kingdoms. Further, our findings are somewhat of a 'best case scenario' where the target pathogen and the live vaccine have similar effects on the cross kingdom species, in this case *Streptococcus pneumoniae* and *Staphylococcus aureus.* Thus, a central 'saving grace' of our findings is that LAIV vaccination at

levels below the destructive generalized herd effect (dGHE) thresholds can actually reduce bacterial disease by sufficiently abrogating the influenza virus itself – and thus the common secondary bacterial infections following influenza virus infection. With an increasing interest in nasally delivered vaccines and drugs for prophylaxis and treatment of numerous pathogens and conditions [39-43], which themselves do not predispose to secondary infections, future vaccine campaigns will run the risk of increasing upper respiratory tract pathogen populations in the setting where constructive (ie: beneficial) generalized herd effects (cGHEs) are not biologically plausible while cross species facilitation (immune modulated or otherwise) may well result in destructive GHEs across the population. For example, human papillomavirus (HPV) is not known to induce susceptibility to upper respiratory tract bacterial pathogens. However, a nasally administered vaccine [44] has been proposed as an effective needle-free alternative to the current HPV vaccination. In this case, administration of an intranasal HPV vaccine will induce an innate immune response and consequent inflammation. Inflammation alone has been demonstrated to be sufficient to increase pneumococcal density, transmission and acquisition and thus such a nasal vaccine to protect from HPV may too induce increased susceptibility to pneumococcal colonization. Unlike an LAIV vaccine, an HPV vaccine will not have the benefit of preventing a disease known to induce secondary bacterial transmission, and thus no plausible constructive generalized herd effect can occur. Rather, increases in bacterial density as well as transmission and acquisition may increase at any level of vaccination within the population such as that seen in simulations where influenza was not present. The benefits, in terms

of convenience, safety and immunogenicity of a needle-free intranasal vaccine should be weighed against the potential adverse effects of increasing, in this case, an entirely distinct pathogen not often associated in any way with the target vaccine species.

Our findings are likely to be most relevant when vaccine campaigns target high proportions of a population and particularly in less developed regions where likelihood of transmission and acquisition events is increased (see part III of supplementary text). Vaccine design, policy and evaluation should strongly consider potential for *generalized* herd effects that may alter broad arrays of pathogen populations across species, classes and ecological kingdoms not currently monitored within current vaccine safety and monitoring methodologies.

7.5 MODEL DESCRIPTION

7.5.1 OVERALL MODEL STRUCTURE AND FUNCTION:

A mathematical compartment model was developed to link the within-host interactions between LAIV vaccination, influenza infection and pneumococcal colonization with the population level effects of LAIV vaccination on pneumococcal colonization rates and disease. Our model is a time dependent compartment model that spans the course of a full calendar year (July-July) and includes at most 1 seasonal influenza epidemic. Model simulations are run for a population (N) of one million individuals using a range of target vaccination rates (ie: proportions of the population targeted for vaccination, pv) from 0.0 (no vaccine) to 1.0 (100% of the population targeted for vaccination). As well, simulations are run to account for a range of potential basic reproductive number ($\mathcal{R}_0$) values for influenza, and for the (unlikely) case of no influenza introduction into the population.

Figures 1 and 2 are graphical depictions of the basic framework upon which our model is based. For clarity, Figure 1 contains only the LAIV and pneumococcal portions of the model but also depicts bacterial transmission (dotted lines) while figure 2 attempts to depict all major components of the full model (see Table 1 for model variables). Parameter values were estimated either from clinical and epidemiological reports or from ours and others in-vivo animal data (see Table 2 for parameter names, values, references, etc).

Table 1: Model compartments (variables)

Compartment	Description	Compartment specific parameter values				
		γ^{-1}	δ	σ	α	Influenza infectious (1=infectious)
S	Fully susceptible	-	-	-	-	-
C_1, C_2	Colonized; susceptible to influenza; eligible for LAIV	15	1	-	-	-
RC_1, RC_2	Colonized; Resistant to Influenza; no longer eligible for LAIV	15	-	-	-	-
R	Susceptible to bacterial acquisition; resistant to Influenza; no longer eligible for LAIV	-	-	-	-	-
V_1	First stage of vaccination	3			1.1	-
V_1C_{1a}	**Co-colonization track V1:**	2	1	1	-	-
V_1C_{2a}	Acquisition during first 3 days post vaccination	1	10	1	-	-
V_2C_{2a}		2	10	1.3	-	-
V_2C_{3a}		4	3	1.3	-	-
V_2C_{4a}		4	2	1.3	-	-
V_2C_{5a}		3	2	1.3	-	-
V_3C_{6a}		13	1	1	-	-
RC_{6av}		27	1	1	-	-
C_1V_1	Vaccinated within 1-15 or 16-28 days post bacterial acquisition	2	2	1	-	-
C_2V_1		2	1	1	-	-
V_2	Second stage of vaccination	12			3	-
V_2C_{2b}	**Co-colonization track V_2:**	1	10	1.3	-	-
V_2C_{3b}	Bacterial acquisition between days 4 and 15	4	2	1.3	-	-
V_2C_{4b}	(inclusive) post LAIV	2	2	1.3	-	-
V_3C_{4b}		2	2	1	-	-
V_3C_{5b}		3	2	1	-	-
V_3C_{6b}		8	1	1	-	-
RC_{6bv}		32	1	1	-	-
V_3	Third stage of vaccination	13	-	-	3	-
V_3C_{4c}	**Co-Colonization track V_3:**	5	3	1	-	-
V_3C_{5c}	Bacterial acquisition between days 16 and 28 post	1	2	1	-	-
RC_{5cv}	LAIV	9	2	1	-	-
RC_{6cv}		40	1	1	-	-
I_1	Incubation period of influenza	3	-	-	1.1	0
I_1C_{1a}	**Coinfection track I_1:**	1	1	1	-	0
I_1C_{2a}	Bacterial acquisition within 3 days of infection with	1	10	1	-	0
I_2C_{2a}	influenza	1	10	3	-	1
I_2C_{3a}		3	3	3	-	1
I_3C_{3a}		1	3	2	-	0
I_3C_{4a}		4	2	2	-	0
I_3C_{5a}		3	2	2	-	0
I_4C_{6a}		13	1	1	-	0
RC_{6aI}		27	1	1	-	0
C_1I_1	Infection with influenza 1-15 or 16-28 days post	2	2	1	-	0
C_2I_1	bacterial acquisition	2	1	1	-	0
I_2	Infectious period of influenza	4			3	1
I_2C_{2b}	**Coinfection track I_2:**	1	10	3	-	1
I_2C_{3b}	Bacterial acquisition during the infectious phase of	1	3	3	-	1
I_3C_{3b}	influenza.	3	3	2	-	0
I_3C_{4b}		4	2	2	-	0
I_3C_{5b}		1	2	2	-	0
I_4C_{5b}		2	2	1	-	0
I_4C_{6b}		11	1	1	-	0
RC_{6aI}		32	1	1	-	0
I_3		8			3	0
I_3C_{2c}	**Coinfection track I_3:**	1	10	2	-	0
I_3C_{3c}	Bacterial acquisition during between days 8 and 15	3	3	2	-	0
I_4C_{3c}	post infection with influenza	1	3	1	-	0
I_4C_{4c}		4	2	1	-	0
I_4C_{5c}		3	2	1	-	0
I_4C_{6c}		5	1	1	-	0
RC_{6cI}		38	1	1	-	0
I_4		13			2	0
I_4C_{4d}	**Coinfection track I_3:**	5	3	1	-	0
I_4C_{5d}	Bacterial acquisition during between days 16 and 28	1	2	1	-	0
RC_{5d}	post infection with influenza	9	2	1	-	0
RC_{6dI}		40	1	1	-	0
RCPV	Transiently resistant to new acquisition following LAIV or influenza co-colonizations	10	-	-	0	-

Table 2: Parameter values used to build model

Symbol	Description	Value	Alternate Interpretation/Description	Source / Justification / Assumption
γ_B	Rate of normal bacterial clearance	1/30	Inverse of duration of colonization (days^{-1}) represents cumulative time spent in C_1/C_2 or RC_1/RC_2)	In vivo data and refs: [1-3]
γ_{CV}, γ_{CI}	Overall rate of bacterial clearance following LAIV or influenza coinfection	1/55	Inverse of duration of colonization for colonization occurring concurrently with influenza or LAIV (Days^{-1})	In vivo data and refs: [1-3]. (Not explicitly in our model but represents cumulative time spent in co-colonized tracks.)
p_c	Baseline colonization rate in the population	0.1	Proportion of the population colonized - in the absence of LAIVs or influenza virus	Assuming homogeneous mixing of indivuals >2 years of age, refs [2, 4-8]
β_B	Bacterial transmission rate constant	$\beta_B = \frac{\gamma_B}{(1\text{-}p_c)}$	Probability of transmission from a colonized to a susceptible individual given contact between the two individuals.	Assume equilibrium state for calculation
λ_B^*	Force of bacterial infection (at equilibrium)	$\beta_B * p_c$	Probability that a susceptible individual will acquire bacteria in the absence of LAIV or influenza introduction into the population	See section 2.2 *(eq 4)*
λ_B	Force of bacterial infection	Dynamically changes	Probability that a fully susceptible individual will acquire bacteria	Dependent on prevalence of colonization and structure of colonized population at time (T); See section 2.8
$\phi_{AOM\|C}$	Attack rate of AOM | colonization-only	0.5	Attack rate of AOM per colonizing event, in the absence of LAIV or influenza	See section 2.10
$\phi_{AOM\|CI}$	Attack rate of AOM given influenza and bacterial coinfection	0.7	Attack rate of AOM in influenza-bacteria coinfected individuals when bacterial acquisition is prior-to or within 15 days following influenza infection	See section 2.11
$\phi_{AOM\|CV}$	Attack rate of AOM given vaccination and concurrent colonization	0.7	Rate of AOM in colonized individuals who receive LAIV either while colonized or within 15 days following bacterial acquisition.	See section 2.11
$\phi_{IPD\|C}$	Attack rate of IPD given colonization	1.03e^{-4}	Attack rate of IPD per colonizing event, irrespective of vaccination or influenza status	See section 2.12
$\psi_{IPD\|I}$	Scaling factor for risk of IPD given influenza coinfection	2	Relative risk of progression from colonization to IPD in influenza coinfected individuals (vs. influenza uninfected colonized individuals)	See section 2.13
β_I	Influenza transmission rate constant	$R_0 \times \gamma_{I2}$	Probability of transmission to an influenza susceptible host given contact with an infectious (compartment I_2) individual	See Box 1
$\lambda_I(\mathrm{T})$	Force of influenza infection	Dynamically changes	Probability that an influenza susceptible individual will become infected with influenza, at time T	Dependent on number of influenza infectious individuals and on absolute humidity, both at time T of the year; See section 2.4
$\lambda_V(T)$	Force of vaccination	$p_v \times \psi_{V\|month}$	Probability that a susceptible or colonized-only individual will be vaccinated, at time T	Varies with target vaccination rate and by See section 2.3
$\psi_{R_0\|week}$	Weekly scaling factor for R_0	Function of time	Environmental scaling factor to account for seasonal variation in virus viability and transmissibility.	See section 2.4 and Box 1
δ	Density of bacteria	1-10	Bacterial carriage density in the nasopharynx following colonization. Changes with time since acquisition and time since LAIV or influenza infection	See Table 2 and sections 2.7-8
σ_v, σ_I	Scaling factor for bacterial transmissibility due to URT symptoms	1-1.3, 1-3	Parameter to reflect changes in bacterial transmissibility due to LAIV or influenza induced URT symptoms.	See section 2.8 and refs [9-11]
τ	Bacterial transmission parameter	$\sigma \times \delta$	Bacterial transmissibility per co-colonized compartment	See section 2.7-8
R_0	Basic reproductive number for influenza	Varies per scenario	Number of secondary infections per primary infection	See section 2.4 and Box 1
$\psi_{V\|month}$	Monthly scaling factor for rate of vaccination	Varies per month of simulation	Scaling factor to account for heterogeneity in the rollout of vaccinations per month over time	See section 2.3; Table 3
α	Bacterial acquisition parameter	1-3	Varies with time since vaccination or influenza infection	See section 2.5
$\mathcal{E}_v$	LAIV vaccine efficacy	.7	Proportion of vaccinated individuals who gain full immunity to influenza virus	Ref [12, 13]
$\gamma_{CV_{lkv}}$, $\gamma_{CI_{lkI}}$	Rate of bacterial clearance from co-colonized compartments	1/40 – 1/1	Inverse of duration spent in individual compartments along the various co-colonization tracks (days^{-1})	See section Table 2 and section 2.7-8
γ_{C1}, γ_{C2} $\gamma_{RC1}, \gamma_{RC2}$	Rate of bacterial clearance from 'colonized-only' compartments	1/15	Inverse duration spent in colonized compartments in absence of LAIV or influenza infection (days^{-1})	In vivo data and refs: [1-3]
p_v	Proportion targeted for vaccination	Varies		
γ_{I1}, γ_{I2}, $\gamma_{I3}, \gamma_{I4I}$	Rate of removal from influenza infected compartment	1/3,1/4, 1/8, 1/13	Inverse of duration spent in each respective influenza infected compartment	In vivo data and refs [14-17]
γ_{V1}, γ_{V2}, γ_{V3}	Rate of removal from LAIV vaccinated compartments	1/3, 1/12, 1/13	Inverse of duration spent in each vaccination compartment	In vivo data

7.5.2 BASELINE COLONIZATION AT EQUILIBRIUM:

Simulations begin with 'N' individuals[2] and a baseline colonization rate (proportion colonized or p_c) such that the number of initial fully susceptible individuals (S_0; ie: healthy, uninfected, unvaccinated) and colonized individuals (C_0) are, respecitvely:

$$S_0 = N \bullet (1-p_c) \qquad \textit{(Eq. 1)}$$

$$C_0 = N \bullet p_c \qquad \textit{(Eq. 2)}$$

In the absence of, or prior to any LAIV vaccinations or seasonal influenza virus introduction, colonization is at equilibrium and individuals acquire bacteria at a rate (α_0) given by:

$$\alpha_{B,0} = S_0 \bullet \lambda_B \qquad \textit{(Eq. 3)}$$

where λ_B is the force of bacterial infection:

$$\lambda_B^* = \beta_B(C_1 + C_2) \bullet N^{-1}. \qquad \textit{(Eq. 4)}$$

Here, C_1 and C_2 are bacterial carriage compartments such that after acquiring bacteria, susceptibles move from S_0 to C_1, where they remain until moving to C_2 before bacterial clearance and a shift back to a susceptible state. (The utility of the two-compartment system becomes apparent once LAIV vaccinations or seasonal

---

[2] For ease of explanation throughout our model description, we often refer to *individuals* and the paths that individuals take between compartments, etc. The use of the word 'individual' however is purely for illustrative purposes to enhance the readers ability to visualize the model dynamics. As a compartment model implies, our model does not truly track individual movement, but rather assumes homogeneity and mass action within compartments and thus measures populations moving between compartments, based on some probability for that specific compartment shift.

influenza viruses are introduced.) Assuming an equilibrium state, the coefficient of bacterial transmission (β_B), is calculated by:

$$\beta_B = {\gamma_B}/{(1\text{-}p_c)} \qquad \textit{(Eq. 5)}$$

where γ_B is the bacterial clearance rate, equal to the inverse of the duration of normal (ie: virus free) bacterial colonization. Taken together, we can formulate baseline equilibrium (denoted by '*') equations for changes in susceptible and colonized states ($\frac{S_0^*}{dt}$ and $\frac{C^*}{dt}$), respectively:

$$\frac{dS_0^*}{dt} = -S_0^*\lambda_B + 2\gamma_B C_2^* \qquad \textit{(dEq. 1)}$$

$$\frac{dC_1^*}{dt} = S_0^*\lambda_B - 2\gamma_B C_1^* \qquad \textit{(dEq. 2)}$$

$$\frac{dC_2^*}{dt} = 2\gamma_B C_1^* - 2\gamma_B C_2^* \qquad \textit{(dEq. 3)}$$

7.5.3 INTRODUCTION OF LAIV VACCINATION:

Susceptible and colonized individuals can become vaccinated at a rate $\lambda_V(T)$, which is dependent on the proportion targeted for vaccination annually (p_v) and time (T) of year via a monthly scaling factor $\psi_{V|month}$ that accounts for relative differences in monthly vaccination rates over the course of an influenza vaccination campaign. $\psi_{V|month}$ was developed for each month by dividing the monthly rate of vaccination, from reference [31], by the rate of vaccination over the *primary vaccine campaign*, where we define the *primary campaign* as the 90 day period with the

greatest mean vaccination rate. From this, we get monthly scaling factors for vaccination as in Table 3.

Table 3: Scaling factor values for monthly rate of vaccination		
$\psi_{V\|Jul} = 0$	$\psi_{V\|Dec} = 0.2$	$\psi_{V\|Jan} = 0.05$
$\psi_{V\|Aug} = 0.8$	$\psi_{V\|Oct} = 1.1$	$\psi_{V\|Feb} = 0.01$
$\psi_{V\|Sep} = 1.2$	$\psi_{V\|Nov} = 0.5$	

The figure below shows the simulation results for vaccine coverage over time vs. the true rates of vaccination within the United States, reported by the US Centers for Disease Control and Prevention, [31].

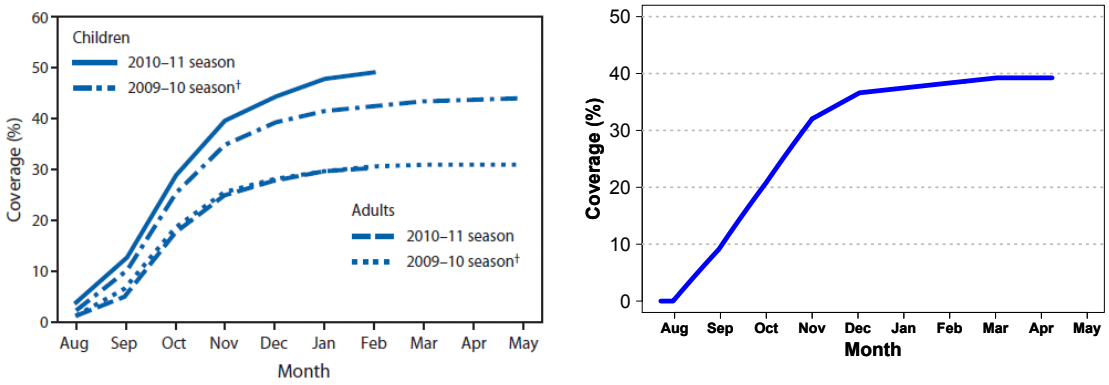

Figure 7.5 Cumulative vaccine coverage USA vs. simulation data.

Cumulative influenza vaccine coverage in the United States (A) and simulated coverage from model runs at a proportion targeted for vaccination of pv=0.4 (B). Data in (A) from[1]. runs at a proportion targeted for vaccination of pv=0.4 (B). Data in (A) from[1].

7.5.4 INTRODUCTION OF INFLUENZA VIRUS INFECTIONS:

Susceptible and colonized individuals can become infected with influenza at a rate determined by the force of influenza infection $\lambda_I(T)$, where:

$$\lambda_I(T) = B_I \left(I_2 + \sum I_2 C\right) N^{-1} \qquad \textit{(Eq. 6)}$$

and B_I is the coefficient of influenza transmission, I_2 is the number of influenza infectious individuals (who are only infectious while in compartment I_2; below for more details) and $\sum I_2 C$ represents the sum of the co-colonized-influenza infectious individuals (see section 7.5.7 below for details). B_I can be derived from the inverse of the influenza recovery rate (γ^{-1}) and the basic reproductive number ($\mathcal{R}_0$). In a departure from most SIR models for influenza, we chose here to model $\mathcal{R}_0$ with a base annual value $\mathcal{R}_{0,annual}$ that gets scaled weekly by $\psi_{R_0|week}$, which is a function of environment conditions over time (via $\psi_{R_0|week}$), which are believed to play important roles in influenza virus transmission and viability (see Box 1 for details).

Once vaccinated with LAIV or infected with influenza virus, a susceptible individual begins to move through a series of compartments each with elevated susceptibility to bacterial acquisition vs. healthy fully susceptible individuals.

Box 1: $\mathcal{R}_0$ as function of time (T):

Whereas a majority of mathematical compartment models of influenza epidemics consider a single value for the basic reproductive number ($\mathcal{R}_0$) of the influenza virus, considerable evidence exists to show that the viability and transmissibility of the influenza virus, modeled using the transmission rate parameter ($\mathcal{B}_I$), is dependent on environmental factors [4-6]. A leading hypothesis in this regard is that influenza virus viability and transmissibility (τ_I) is dependent, at least in part, on environmental vapor pressure, or absolute humidity (AH) [4, 6, 7] such that:

$$\tau_I \propto AH$$

Because the transmission rate parameter $\mathcal{B}_I$ is directly proportional to transmissibility by the equation:

$$\mathcal{B}_I = C \times \tau_I$$

where C is the inter-individual contact rate, it follows that:

$$\mathcal{B}_I \propto \tau_I \propto AH \Rightarrow \mathcal{B}_I \propto AH.$$

Further, because $\mathcal{R}_0$ can be derived from the product of $\mathcal{B}_I$ and the average infectious period ($^1/_\gamma$; the inverse of the recovery rate) by:

$$\mathcal{R}_0 = {}^{\beta_I}/_\gamma$$

it follows that

$$\mathcal{R}_0 \propto AH.$$

Finally, because average AH fluctuates over time and influenza transmissibility is lowest during periods of high absolute humidity we developed a simple equation to determine $\mathcal{R}_0$ at time T where:

$\mathcal{R}_0(T) = \mathcal{R}_{0,annual} \times \psi_{R_0|week}$ and $\psi_{R_0|week}$ represents a scaling factor for $\mathcal{R}_{0,annual}$ such that:

$$\psi_{R_0|week=T} = \left(\frac{AH(T)}{\min\limits_{T,1\to44} (\frac{1}{n}\sum_T^{T+8} AH_{week})} - 1 \right).$$

The denominator here is simply the minimum mean AH of any consecutive 8-week period over the course of a given year, and the numerator is a weekly average of AH, thus T here is in units of weeks. $\mathcal{R}_{0,annual}$ is the annual estimated value of $\mathcal{R}_0$, often estimated through epidemic models and data on rates of influenza related morbidity and mortality [12].

*Note: for our model, we queried the NOAA database [13] for the weekly AH in Atlanta, GA, USA between the years 2000 and 2011 and used a mean AH over those years (calculated for each week) to calculate the weekly $\psi_{R_0|week}$.

7.5.5 BACTERIAL ACQUISITION FOLLOWING LAIV OR INFLUENZA VIRUS.

Vaccinated individuals move through three distinct compartments (V_1, V_2 and V_3) where each compartment carries with it a relative probability of acquisition such that:

$$\Pr(aquisition) \propto \alpha_v \mid \alpha_0 < \alpha_{v1} < \alpha_{v3} < \alpha_{v2}$$

where α_0=1 for the no-vaccine condition and α_{v1}, α_{v2} and α_{v3} refer to the added probabilities of acquisition in vaccine compartments $V_{1\text{-}3}$, respectively. In this manner, probability of new bacterial acquisition is a step function (Fig 7.6) of time since vaccination, where each compartment accounts for a single step of the function and each step approximates the mean change in probability of acquisition (vs. no vaccination) over the duration of time spent in that compartment [9, 10].

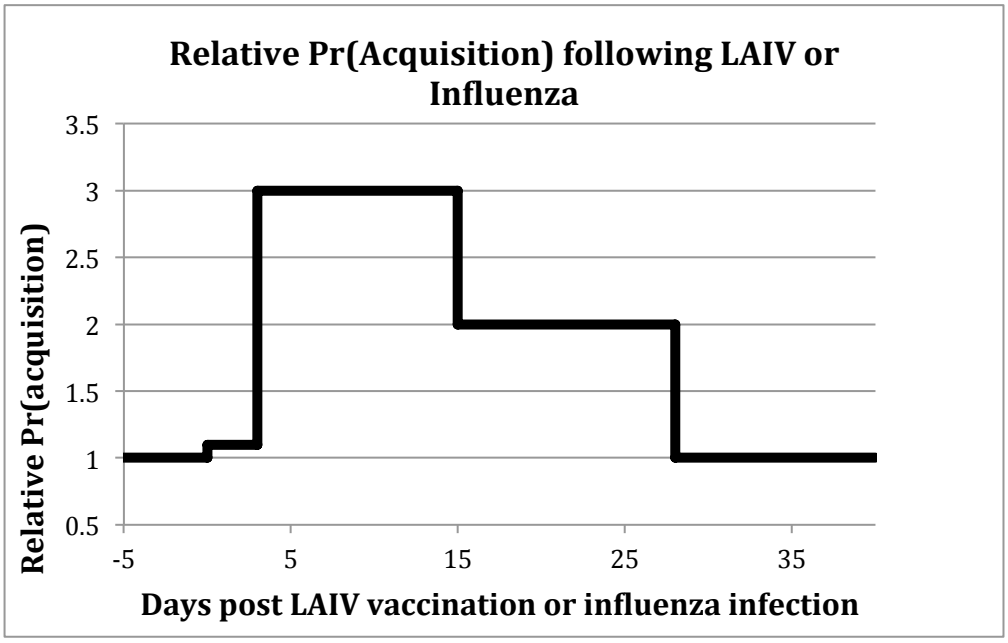

Figure 7.6 Step function for mean probability of bacterial acquisition following LAIV or influenza

An analogous situation holds true for susceptible individuals infected with influenza, however, following influenza infection, individuals move through four, rather than three influenza infected compartments. The step function for relative probability of bacterial acquisition vs. time since influenza infection is however no different than that for LAIV [9, 10, 45] such that:

$$\alpha_{0=}\alpha_0;$$

$$\alpha_{v1} = \alpha_{I1};$$

$$\alpha_{v2} = \alpha_{I2} = \alpha_{I3};$$

$$\alpha_{v3} = \alpha_{I4}$$

where the $\alpha_I's$ are the probabilities of acquisition following influenza infection in the 1st -4th influenza infected compartments.

The extra fourth compartments here is required purely to account for the changes in influenza infectiousness and bacterial transmissibility (see sections 7.5.7-7.5.8 below) but not acquisition following influenza infection, which is not necessary following LAIV vaccination. Specifically, we model the influenza infectious period (compartment I_2; Fig 2) as a four-day period following a three-day incubation period (I_1). Thus I_2 contains all influenza infectious individuals. And compartments I_2 and I_3 are comparable, in terms of relative probability of acquisition, to those in V_2. As well, combined time spent in I_2 and I_3 is the same as V_2.

7.5.6 IMMUNITY FOLLOWING LAIV OR INFLUENZA INFECTION AND VACCINE EFFICACY

Individuals who move through all three vaccinated (or four influenza infected) states, irrespective of colonization status, eventually move to an influenza resistant state. A proportion of vaccinated individuals however return to a fully susceptible state at rate 1-$\mathcal{E}_V$, where $\mathcal{E}_V$is vaccine efficacy.

7.5.7 MODELING THE WITHIN-HOST EFFECTS OF LAIVS (OR INFLUENZA) ON BACTERIAL COLONIZING DENSITY

If individuals become colonized with bacteria, they can then transmit bacteria at a rate proportional to the density of colonization. Further, density is dependent on both time of acquisition post vaccination (accounted for using compartments V_1, V_2, V_3 ,R_v) or influenza infection (similarly, using compartments I_1, I_2, I_3, I_4, R_v), and time since colonization. Depending on time of acquisition following vaccination or influenza infection, individuals move down one of four distinct 'coinfection' tracks following LAIV or one of five 'coinfection' tracks following influenza infection. Entrance onto these tracks is represented in figure 2 by arrows pointing towards VC_1, VC_2, V,C_3 and R_vC (for vaccination (V) or recovery post vaccination R_v and then colonization (C)) and arrows towards IC_1, IC_2, IC_3, IC_4 and R_IC for colonization following influenza infection. Initial acquisition within any particular compartment is assumed to occur at the time of entry into that compartment plus one-half the duration of time normally spent within that compartment. For example, if a susceptible is vaccinated such that they enter

compartment V_2 on day 100, acquisition occurring while in V_2, is assumed to occur on day 106 (normal duration of time spent in V_2 = 12 days).

Once on a specific track, individuals move through a series of 'coinfection' compartments to account for changes in bacterial transmissibility, which must be specifically accounted for when determining the force of bacterial infection (λ_B) in the population. For recent vaccinees that acquire bacteria (or colonized individuals who receive LAIV), changes in bacterial transmissibility (τ) depend almost entirely on bacterial density (δ), which itself is dependent on the time since colonization and time since vaccination (see "Bacterial transmission following vaccination" in Figs 1 or 2). We also account for a small effect on τ due to excess upper respiratory tract symptoms during the week following vaccination via a scaling factor σ_v (see section 1.3.8 below for details).

For influenza-bacteria co-infected individuals, bacterial transmissibility (τ) depends on both bacterial density and presence of symptomatic influenza [9, 10, 46] and we consider peak influenza symptoms to occur between 4 and 7 days (inclusive) following influenza infection and mild symptoms between days 8 and 15. Like the scaling factor (σ_v) used to account for mild upper respiratory tract symptoms following LAIV, we use an analogous scaling factor (σ_I) to account for excess τ while symptomatic.

To explicitly account for these differences from baseline transmissibility (ie; in the absence of influenza or LAIV) as a function of both time of acquisition following vaccination or influenza infection and a function of time since acquisition, each track (eg: VC_1, VC_2, IC_1, IC_2, etc…) is itself comprised of a series of sequential

compartments, each with a unique associated bacterial density. By 'tracking' along these paths we explicitly account for excess transmission over baseline that arises as a result of the putative interactions between influenza and bacteria to increase bacterial density and symptoms for influenza infected individuals, both have been demonstrated to increase transmission,[9-11] over time as individuals 'recover' away from the initial VC or IC compartment and towards the recovered (but still colonized) compartments, RC_v or RC_I, respectively. For instance, if an individual acquires bacteria while in V_1 (ie: within 3 days post vaccination), bacterial titers will rise rapidly, peaking approximately 3 days post bacterial acquisition and will remain at this highly elevated level (eg: δ=10 or density is 10-fold higher than baseline colonizing density) for 2 days before gradually (ie: through numerous compartments) returning to baseline (as demonstrated in our in-vivo experiments in chapter 2 and [8]. If a vaccinated individual acquires bacteria while in V_3 (ie: 16-28 days post vaccination), that individual will enter a different track (ie: VC_3) where bacterial titers will increase only modestly, but rapidly, peaking within just 1 day of bacterial acquisition and density will return to baseline colonization levels within only a few days. See for example the differences in amplitude and shape of density/transmission parameter functions in the NP density distribution curves leaving from VC1 vs. VC3 in figure 1 or 2). For clarity, we depict each of these co-colonization tracks with their respective sub-compartments to demonstrate time spent in each compartment (γ).

7.5.8 FORCE OF BACTERIAL TRANSMISSION (λ_B) IN THE PRESENCE OF LAIVS AND INFLUENZA

The force of bacterial transmission was mentioned earlier in our discussion of colonization at equilibrium, and was defined by:

$$\lambda_B = \beta_B(C_1 + C_2) \bullet N^{-1}. \qquad \textit{(Eq. 4)}$$

With the introduction of LAIVs and influenza virus into the population, the equilibrium state of pneumococcal colonization is disrupted and this definition for λ_B will no longer suffice. Rather, λ_B has to take into account all colonized individuals, and their respective bacterial transmissibility values (ie: bacterial density and influenza like symptoms - the latter important primarily for influenza infected individuals only). Therefore, in the setting of LAIV vaccination and/or seasonal influenza infections, the force of bacterial infection λ_B becomes:

$$\boldsymbol{\lambda_B} = \beta_B(C_1 + C_2 + \sum_{k_v=1}^{3}\sum \tau_{ik_v} VC_{ik_v} + \sum_{k_I=1}^{4}\sum \tau_{ik_I} IC_{ik_I} + R_vC + R_IC + RC_1 + RC_2) \bullet N^{-1} \qquad \textit{(Eq. 7)}$$

where the bacterial transmissibility parameters following vaccination (τ_{ik_v}) or influenza (τ_{ik_I}) are given by:

$$\tau_{ik_v} = \delta_{ik_v} \times \sigma_{ik_v} \qquad \textit{(Eq. 7.1)}$$

$$\tau_{ik_I} = \delta_{ik_I} \times \sigma_{ik_I} \qquad \textit{(Eq. 7.2)}$$

Here, δ_{ik_v} and δ_{ik_I} refer to the density of bacteria in compartment i of track k_v or k_I, following vaccination or influenza infection, resepectively. For example, track k_v is

the track that begins with the compartment VC_k, k being between 1 and 3 for the vaccinated tracks and 1 through 4 for the infected tracks. σ_{ik_v} is a scaling parameter for changes in bacterial transmissibility due to upper respiratory tract symptoms following LAIV vaccination. Similarly, σ_{ik_I} is a scaling factor for transmissibility due to influenza like symptoms (eg: excess coughing). Because symptoms are often short-lived and change dover time, σ_{ik_v} and σ_{ik_I} are set to 1(ie: no symptoms) for all combinations of i and k_I or k_v except for those combinations referring to the period of 4-15 days following vaccination (σ_{ik_v}= 1.2), 4-7 days following influenza infection (symptomatic period; σ_{ik_I}= 3) and 8-15 days following influenza infection (mild symptoms; $\sigma_{ik_I} = 2$).

7.5.9 Recovery and immunity following LAIV-bacteria and influenza-bacteria coinfections

Following recovery from vaccination or influenza infection, colonized individuals will move from the last VC or IC compartments to R_vC or R_IC, where they will remain until bacterial clearance almost 4 weeks later. The utility of these compartments is to account for the increased duration of bacterial colonization following LAIV or influenza infection, as we, and others[9, 47] have demonstrated (see chapter 2). At this stage, vaccinated individuals may also move back to a colonized, influenza susceptible state at a rate of 1-$\mathcal{E}_V$, where $\mathcal{E}_V$ is vaccine efficacy.

Once entirely resistant to influenza, individuals remain in a resistant state (R) where they may become colonized (RC_1 & RC_2) and recover in a manner no different than that described for colonization of fully susceptible individuals.

7.5.10 BACTERIAL ACUTE OTITIS MEDIA – COLONIZATION EQUILIBRIUM STATE

In the absence of LAIVs or influenza (eg: colonization at equilibrium), colonization can progress to acute otitis media (AOM) at a rate $\phi_{AOM|C}$ such that the incidence of AOM ($Inc_{AOM|C}$) in the population is a linear function of the number of acquisition events at any given time ($S_0^* \times \lambda_B^*$):

$$Inc_{AOM|C} = S_0^* \lambda_B^* \times \phi_{AOM|C} \qquad \textit{(Eq 8)}$$

The value $\phi_{AOM|C}$ is derived via two independent analysis, one from our experimental data (Fig 2) and the other mathematically derived from epidemiologic data as follows: References [48-51] demonstrate per capita annual incidence of AOM of approximately 1.6 unique episodes of AOM per person-year, of which 40-50% are of pneumococcal eitiology. These figures provide us with an overall per capital annual incidence rate of pneumococcal AOM (ϕ_{AOM}) of:

$$\phi_{AOM} = 1.6 \times 0.45 = 0.72. \qquad \textit{(Eq 9)}$$

Further, at baseline or equilibrium colonization rates, annual per capital incidence rate of bacterial acquisition can be given by:

$$Inc_{acquisition} = p_c \times \gamma_B \times 365 = 1.21 \qquad \textit{(Eq 10)}$$

Which allows us to calculate a risk of acute otitis media per bacterial colonizing event ($\phi_{AOM|C}$) such that:

$$\phi_{AOM|C} = \frac{\phi_{AOM}}{Inc_{acquisition}} = \frac{.72}{1.21} = .59 \qquad \textit{(Eq 11)}$$

which is very similar to, and well within the confidence limits of our experimentally derived value of 0.5; see PBS controls in Fig 2a & c.

7.5.11 BACTERIAL AOM FOLLOWING LAIV AND INFLUENZA VIRUS

INTRODUCTION

Based on our experimental data and other laboratory and epidemiologic studies [9, 45, 52-57], viral upper respiratory tract infections increase attack rate of AOM in colonized individuals. As well, and central to our findings, LAIV increases attack rates of AOM (see chapter 3). From our observed in-vivo data, we assign a risk of colonization progressing to AOM of 0.7 following LAIV ($\phi_{AOM|CV}$) or influenza infection ($\phi_{AOM|CI}$). Of note, in our model, this increased risk of AOM is only relevant if bacterial acquisition occurs during the first 15 days following LAIV or influenza infection (eg: $VC_{1\text{-}2}$ and $IC_{1\text{-}3}$), after which risk returns to baseline risk, $\phi_{AOM|C}$ = 0.5.

Because the proportion of individuals colonized, vaccinated, infected with influenza and coinfected is a dynamic process, there is no closed form solution to calculate the incidence of AOM, as in the baseline, equilibrium scenario. Thus, we quantify annual incidence of AOM as:

$$Inc_{AOM} = Inc_{AOM|c} + Inc_{AOM|CV} + Inc_{AOM|CI} \quad \textit{(Eq 12)}$$

where:

$$Inc_{AOM|C} = \sum_T \phi_{AOM|C}\, \lambda_B (S + R) \quad \textit{(Eq 13)}$$

$$Inc_{AOM|CV} = \sum_T (\phi_{AOM|CV}(\lambda_V(T)(C_1 + C_2) + \lambda_B(\alpha_{v1}V_1 + \alpha_{v1}V_2)) + \phi_{AOM|C}\lambda_B(\alpha_{v3}V_3)) \quad \textit{(Eq 14)}$$

$$Inc_{AOM|CI} = \sum_T (\phi_{AOM|CI}(\lambda_I(T)(C_1 + C_2) + \lambda_B(\alpha_{I1}I_1 + \alpha_{I2}I_2 + \alpha_{I3}I_3)) + \phi_{AOM|C}\lambda_B(\alpha_{I4}I_4)) + \quad \textit{(Eq 15)}$$

are the annual incidences of AOM for all individuals gaining AOM following normal colonization $(Inc_{AOM|C})$, concurrent colonization and vaccination ($Inc_{AOM|CV}$) or following bacterial-influenza coinfections ($Inc_{AOM|CI}$).

7.5.12 INVASIVE PNEUMOCOCCAL DISEASE – COLONIZATION EQUILIBRIUM STATE

Similar to AOM, invasive pneumococcal disease (IPD) follows pneumococcal colonization (albeit at much lower rates) and like AOM, IPD has an attack rate (given

colonization), of $\phi_{IPD|C}$, whose estimate has been the topic of numerous epidemiological investigations.[58-61] These investigations each aim to gain understanding of specific capsular serotype 'invasivity' or attack rate following colonization. However, because we do not account for serotype specific effects in our model, our aim in estimating $\phi_{IPD|C}$ was to approximate the mean attack rate of all pneumococcal serotypes. However, because not all pneumococcal serotypes are distributed evenly, we aimed further to calculate a weighted average, such that $\phi_{IPD|C}$ would more accurately reflect the 'true' overall attack rate of the pneumococcus throughout a population. To do this, we utilized national UK surveillance data in Table 1 of[61] and estimated $\phi_{IPD|C}$ as the weighted average of attack rates for 36 serotypes (representing 4652 unique acquisition events) by:

$$\phi_{IPD|C} = \frac{1}{\sum_i n_i} \sum_i \phi_{IPD|C_i} \times n_i \qquad \textit{(Eq. 16)}$$

where $\phi_{IPD|C_i}$ and n_i represent the attack rate and the number of acquisition events for the i^{th} serotype to provide an overall attack rate for IPD of $\phi_{IPD|C}$ = $1.03e^{-4}$ per bacterial acquisition event. Thus, while colonization is at equilibrium, the daily incidence of IPD can be described as:

$$Inc_{IPD|C} = S_0^* \lambda_B^* \times \phi_{IPD|C} \qquad \textit{(Eq 17)}$$

7.5.13 IPD FOLLOWING LAIV AND INFLUENZA VIRUS INTRODUCTION

In chapter 4 we described that we found no increased risk of IPD in LAIV vaccinated vs. unvaccinated mice and this is in agreement with numerous safety

reports of LAIVs in clinical and post-licensure safety studies.[62-64] In fact we found a short-term protective benefit of LAIV to reduce bacterial disease. Following influenza infection however, our in-vivo data suggests significant excess IPD and mortality following influenza-bacterial coinfection (see chapters 4 and 7), as has been reported previously [65-67]. To account for the increased within-host attack rate of IPD in the presence of influenza, we use a scaling factor $\psi_{IPD|I}$ which we set to 2, indicating that influenza-bacterial coinfected individuals are twice as likely as colonized-only individuals to develop IPD. Our estimate of 2 for $\psi_{IPD|I}$ was chosen by comparison against the literature [68, 69] of odds ratios for IPD of influenza infected vs. influenza free individuals for a number of $\psi_{IPD|I}$ values. For example we compared the OR of IPD for influenza vaccinated vs. unvaccinated individuals (Table 3 in [69], which are numerically calculated OR's for various values of $\psi_{IPD|I}$). A $\psi_{IPD|I}$ value of 2 provides an OR of 0.461, which almost exactly matches the OR of 0.46 from ref [69]. Although $\psi_{IPD|I} = 2$, this does not mean that the odds ratio (OR) for IPD in influenza vs influenza-free individuals is 2, but rather that the increase in attack rate of IPD in colonized individuals who are previously (within 28 days) or subsequently infected with influenza will be two-fold that seen in their colonized-only or LAIV-colonized counterparts. The OR of IPD must be calculated numerically from the model (for example, by comparison of any of the R_0 groups to the "no-Flu" group, at the desired vaccination level; Fig 4). However, this scaling parameter can be thought of as the relative risk (RR) of developing IPD for colonized individuals who are concurrently infected with influenza vs. colonized individuals who are free

of influenza (as can be seen by comparison of the upper vs. lower broken lines in Supplementary Fig. S4a).

To calculate total incidence of IPD, we take an approach very similar to our approach for AOM (*Eqs. 12-15)* such that:

$$Inc_{IPD} = Inc_{IPD|c} + Inc_{IPD|CV} + Inc_{IPD|CI} \quad \textit{(Eq 18)}$$

where:

$$Inc_{IPD|C} = \sum_T \phi_{IPD|C}\, \lambda_B (S + R) \quad \textit{(Eq 19)}$$

$$Inc_{IPD|CV} = \sum_T \phi_{IPD|C} (\lambda_V (T)(C_1 + C_2) + \lambda_B (\alpha_{v1} V_1 + \alpha_{v1} V_2 + \alpha_{v3} V_3) \quad \textit{(Eq 20)}$$

$$Inc_{IPD|CI} = \sum_T \psi_{IPD|I} \phi_{AOM|CI} (\lambda_B (\alpha_{I1} I_1 + \alpha_{I2} I_2 + \alpha_{I3} I_3 + \alpha_{I4} I_4) + \lambda_I (T)(C_1 + C_2)) \quad \textit{(Eq 21)}$$

and $Inc_{IPD|C}$, $Inc_{IPD|CV}$ and $Inc_{IPD|CI}$ represent the annual component incidencesmill of IPD due to individuals with: colonization-only, concurrent LAIV and colonization and influenza-bacteria coinfection, respectively.

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CHAPTER 8. CONCLUSION

Much of my PhD work has focused on the interactions between respiratory pathogens, the vaccines we utilize to combat these pathogens, and the immune mechanisms that facilitate a multitude of multi-species interactions. At the heart of this dissertation is a story about unintended consequences of live attenuated influenza vaccination on respiratory bacterial carriage and disease. Herein I have detailed numerous discoveries regarding live influenza vaccines and their beneficial and detrimental effects on the host, at least as far as infection with the bacterial species *Streptococcus pneumoniae* and *Staphylococcus aureus* are concerned – both of which are very important bacterial pathogens responsible for a large fraction of global morbidity and mortality.

Perhaps the most important discovery coming out of my PhD work is the unintended consequence of vaccination with a live attenuated influenza virus to increase bacterial carriage density and duration in mice. When mice were vaccinated with LAIV and inoculated with either *S. pneumoniae* or *S. aureus*, nasopharyngeal carriage density of the bacteria was increased between 10-fold and 1000-fold relative to unvaccinated controls, and duration of carriage was twice as long in the vaccinated mice. As well, LAIV enhanced bacterial otitis media and mice displayed increased incidence of bacterial AOM following vaccination and each

episode lasted, on average, 2-5 fold longer than bacterial AOM episodes in the unvaccinated mice. The effects of LAIV on bacterial carriage dynamics and AOM were nearly identical in all respects to the effects of the wild-type parent H3N2 influenza virus. Importantly however, the similarities were isolated to effects in the upper respiratory tract, where the LAIV replicates well. Unlike WT virus, LAIV did not enhance severe bacterial disease in the lower respiratory tract. In fact, when mice were given a lethal dose of bacteria sufficient to cause mortality in 50% of the infected mice, those that also received LAIV were much more likely to survive than controls. Although LAIV enhanced bacterial carriage in the upper respiratory tract, LAIV vaccination had the simultaneous effect of improving bacterial clearance from the lungs and increasing survival.

The results from mice suggest that the effects of LAIV at the level of the vaccine recipient are only minimally detrimental, increasing bacterial carriage, which is often asymptomatic, and enhancing bacterial otitis media, which while unpleasant, rarely results in long-lasting consequences to the host. On the other hand, the benefit of LAIV in terms of bacterial infection in the vaccine recipient was quite profound, conferring significant protection against lethal bacterial infections.

These results however led to the question of the larger impacts of LAIV vaccination on bacterial disease within the community at large, including unvaccinated bystanders. It is believed that influenza virus infections may enhance bacterial transmission and acquisition between individuals, and evidence exists from both ferrets and mice to support this idea. Although LAIV did not increase severe bacterial disease like that seen following WT influenza virus infection, it did

act very much like a WT virus in the URT, where bacterial transmission and acquisitions events take place. In this case, it is possible that, like a WT virus epidemic or pandemic, mass distribution of LAIV vaccines may too increase bacterial transmission throughout the community, and the effects may extend beyond the vaccinated hosts. A study to determine the impact of such unintended effects at the population level would be highly cost prohibitive, requiring massive numbers of nasal swab samples to be collected from the population at large, particularly from unvaccinated individuals surrounding vaccine recipients. Because such a study was not feasible during the course of my PhD work, I set out to understand the potential implications of mass vaccination with LAIV on bacterial transmission and disease using mathematical models. The details of the mathematical models are described in detail in the previous chapter. The main outcome was that vaccination with LAIV could be beneficial or detrimental in terms of bacterial transmission. At low levels of vaccination across the population, LAIV reduced excess post-influenza bacterial transmission by abrogating the seasonal influenza epidemic and in so doing, was beneficial to reduce overall incidence of colonization across the population. However, as greater fractions of the population were vaccinated with LAIV, the beneficial effects to reduce bacterial acquisition events, conferred by reducing the number of influenza infections, was outweighed by excess bacterial transmission and acquisition following LAIV vaccination. I found that effects of LAIV to both reduce and increase bacterial acquisition across the population extended beyond the vaccinated hosts and impacted the 'bystander' population who were neither vaccinated nor infected with influenza virus. I coined

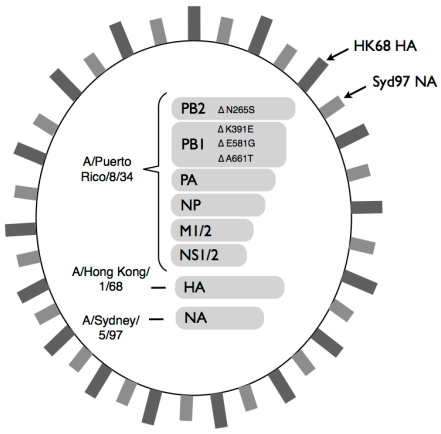
the term '*generalized herd effects'* to describe the alterations in the epidemiology of bacterial acquisition events in this 'bystander' population that was driven by mass vaccination with a viral vaccine. Further, I discovered both constructive and destructive generalized herd effect thresholds, termed cGHEs and dGHEs, respectively, which describe the points at which vaccination can be most beneficial to reduce incidence of bacterial acquisition by abrogating the influenza epidemic and at which incidence of bacterial acquisition is increased above the unvaccinated scenario, respectively. The idea of a generalized herd effect, coupled with these two thresholds, may help to provide a framework within which vaccine monitoring and policy may start to address the large scale implications of vaccinations on pathogen species that are phylogenetically distinct from the vaccine target species.

In conclusion, my PhD work has spanned from the laboratory where I performed numerous in-vitro experiments with cell line alveolar macrophage and in-vivo experiments with wild type and live attenuated influenza viruses and bacterial pathogens, through to mathematical models to understand the population level effects that may be driven by individual level effects of vaccination. In the future I will continue this work to better understand the effects of LAIV on bacterial carriage in humans by sampling children just prior to and following LAIV vaccination and measuring alterations in bacterial density within the nasopharynx that occurs as a result of vaccination. These types of studies will be prudent to further understand the ramifications of LAIV on bacterial disease and transmission and will lay the groundwork for my future as a physician scientist. I hope to continue investigating broad ecological effects of both pathogen epidemics and mass

vaccination strategies through epidemiological, mathematical and theoretical frameworks and feel that this can be developed into an area within which I can have considerable expertise and develop myself as a leader in the field. My PhD has encompassed a wonderful five years and I look forward to continue pursuing my work both within the medical setting and in the laboratories of academia.

CHAPTER 9. SUPPLEMENTARY FIGURES

Chapter 2:

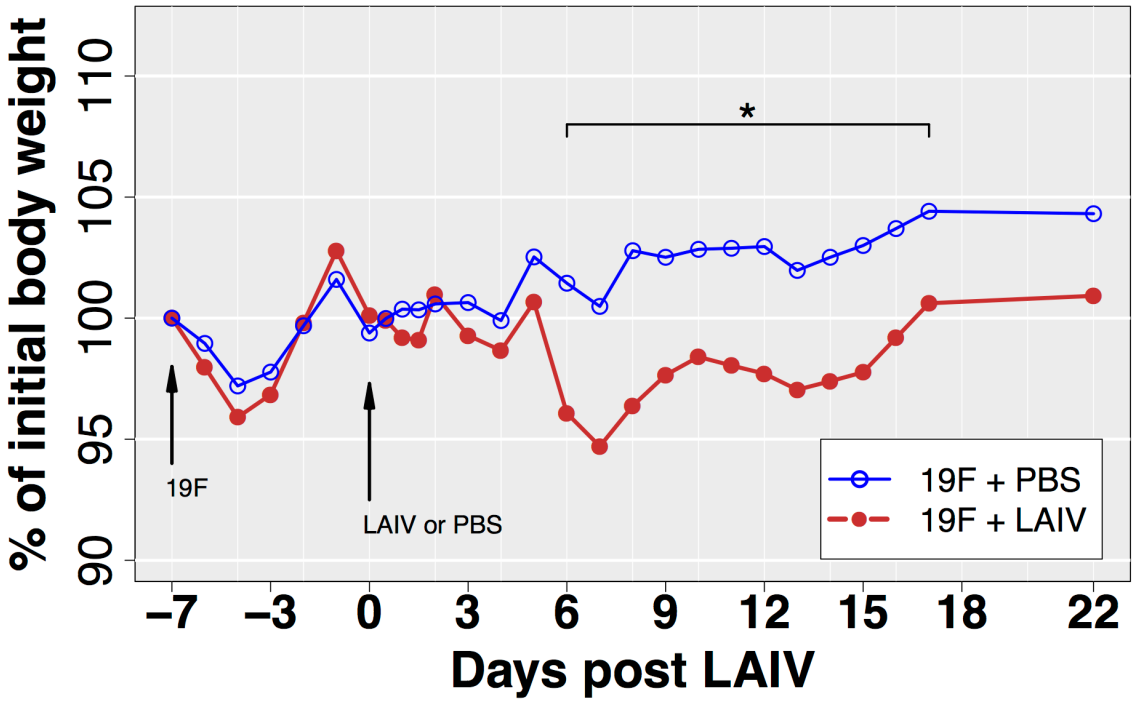


Substitutions conferring *ts* and *att* phenotype

Gene	Nucleotides (wt→*ts/att*)[a]	Amino acid (wt→*ts/att*)[b]
PB2	A794G	N265S
PB1	A1171G, A1173G	K391E
	A1742G, G1743A	E581G
	G1981A	A661T

Supplementary Figure S2.1: H3N2 HK/Syd 1:1:6 live attenuated influenza virus vaccine.

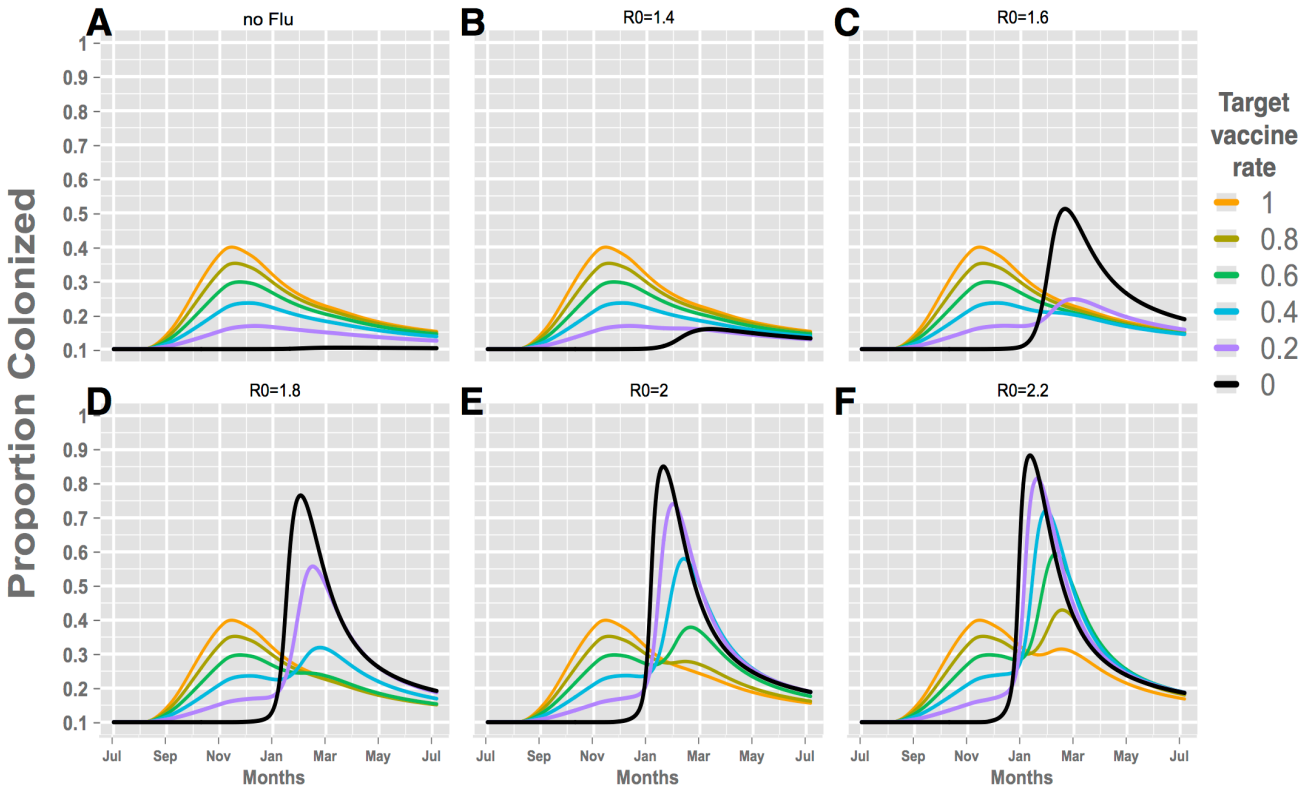
LAIV vaccine was developed via site specific mutagenesis of a parent H3N2 1:1:6 reassortant virus (HK/Syd) as described in [1], containing the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) from A/Hong Kong/1/68 (HK68) and A/Sydney/5/97 (Syd97) isolates, respectively, and the six internal protein gene segments from A/Puerto Rico/8/34 or PR8. LAIV consisted of a temperature sensitive (ts) attenuated variant of HK/Syd (HK/Syd$_{ts}$) that contains site-specific mutations in the PB1 and PB2 RNA segments of the genome as described in [1]. These mutations are found in the attenuated A/Ann Arbor/6/60 master donor strain used to produce the commercial product known as FluMist® [2].



Supplementary Figure S2.2: Weight changes in colonized mice following LAIV or PBS vehicle.

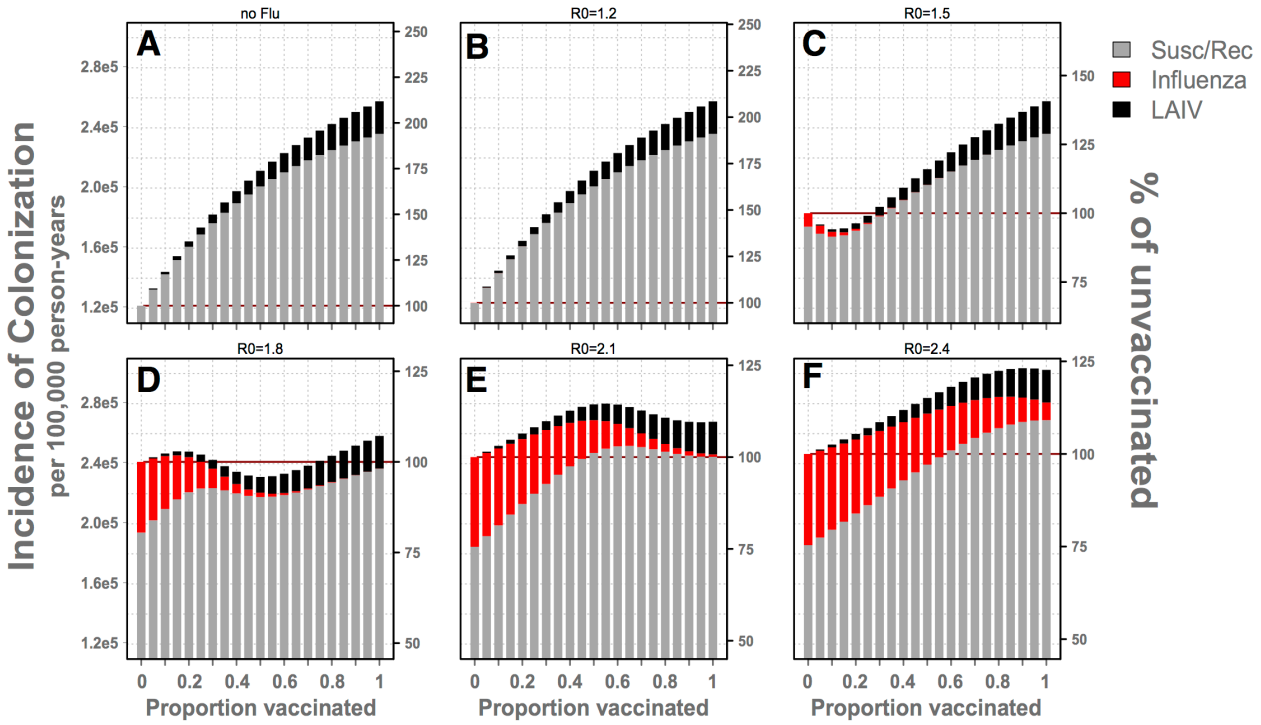
Groups of 12-14 BALB/c mice were inoculated with a colonizing dose (1e5 CFU) 19F Streptococcus pneumoniae at 7 days prior to receipt of 2e6 $TCID_{50}$ LAIV vaccination or PBS vehicle control and body weight was monitored. Asterisks indicate statistically significant differences between LAIV vaccinated and PBS controls (two-sided student's t-test; p<.05).

Chapter 8:



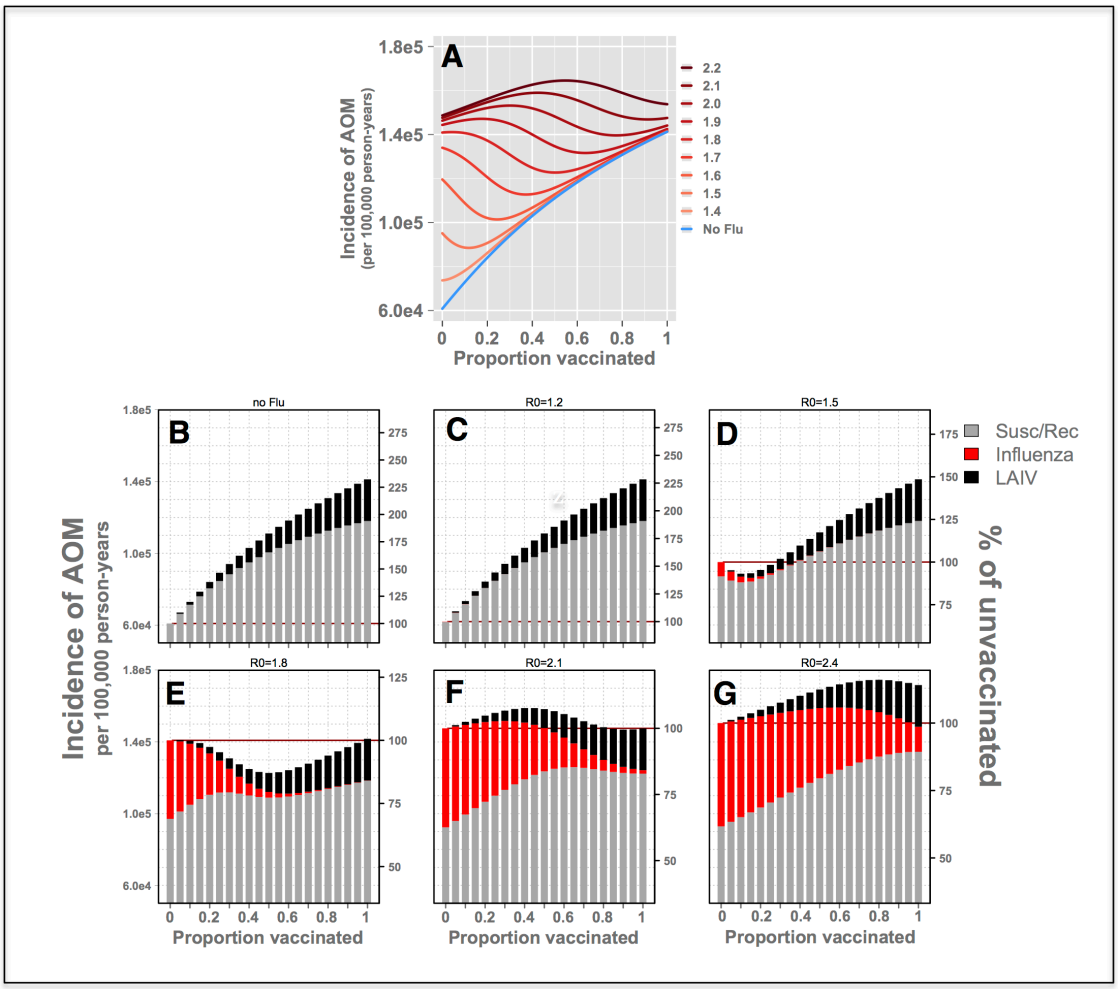
Supplementary Figure S7.1 Changing prevalence of pneumococcal colonization following LAIV vaccination campaigns.

Simulations were run and total proportion of the population colonized is shown over time for increasing target vaccination rates (0% - black lines to 100% of the population - orange lines). Each plot demonstrates a scenario with an underlying influenza epidemic ranging from no influenza introduction (A), R_0 of 1.4 (B) and up to an influenza virus with an R_0=2.2 (F), as noted.



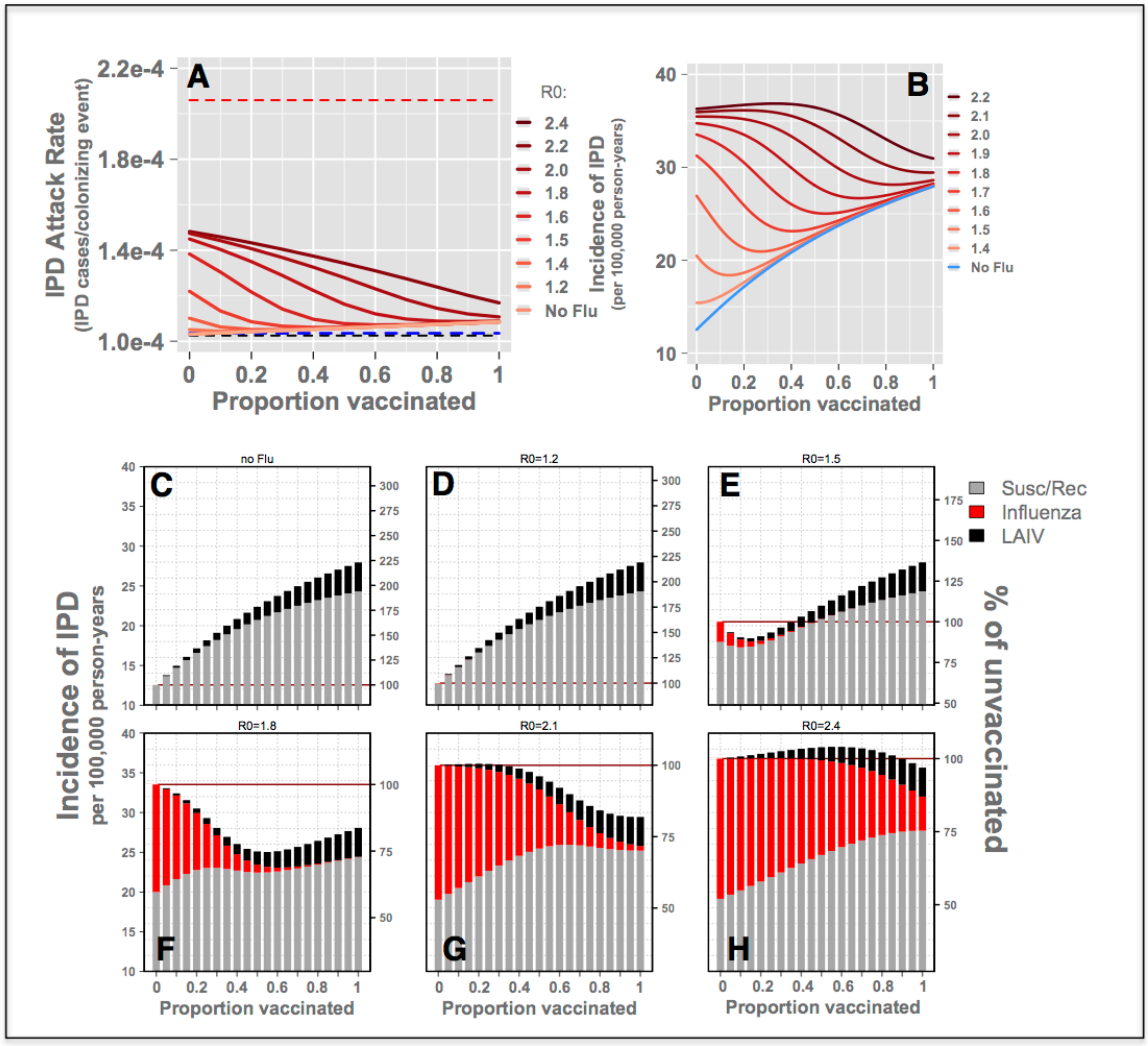
Supplementary Figure S7.2. Incidence of colonization following LAIV vaccination campaigns.

Incidence of colonization is calculated depending on when bacterial acquisition occurred: Grey bars indicate bacterial acquisition in entirely healthy individuals either in the absence of LAIV vaccination or influenza infection or >28 days following LAIV or influenza infection. Incidence of acquisition within 28 days from the time of LAIV vaccination (black bars) or within 28 days from time of influenza infection (red bars) are stacked as well. Scenarios are run for increasing target vaccination rates from zero (left most bar in each graph) to 100% population targeted for vaccination (right most bars) and each plot represents scenarios with unique values for the basic reproductive number of the underlying seasonal influenza epidemic ranging from no influenza introduction in (A) and up to 2.4 in B-F, respectively.



Supplementary Figure S7.3. Incidence of bacterial AOM following LAIV vaccination campaigns.

(A) Overall incidence of AOM throughout the population is plotted as a function of proportion vaccinated and stratified by influenza R0 values. (B-G) Incidence of AOM is plotted against proportion vaccinated (as in each respective line in A) however stacked bars indicate contribution to total incidence of AOM by vaccination and influenza status at time of IPD: Grey bars indicate transition from colonization-only to IPD in colonized but otherwise susceptible individuals. Red bars indicate IPD arising within 28 days from the time of influenza infection and, similarly, black bars indicate incidence of episodes of AOM arising within 28 days following LAIV. Scenarios are run for different R0 values.



Supplementary Figure S7.4. Attack rate and incidence of invasive pneumococcal disease (IPD):

(A) Overall attack rates of IPD, given bacterial acquisition, are shown in solid red hued lines. Specific attack rates given colonization following influenza (red broken line at top of A), LAIV (blue broken line at bottom of A) or colonization-only (Black broken line at bottom of A) are also shown and do not change with proportion vaccinated. (B) Overall incidence of IPD throughout the population is plotted as a function of proportion vaccinated and stratified by influenza R0 values. (C-H) Incidence of IPD is plotted against proportion vaccinated (as in each respective line in B) however stacked bars indicate contribution to total incidence of IPD by vaccination and influenza status at time of IPD: Grey bars indicate transition from colonization-only to IPD in colonized but otherwise susceptible individuals. Red bars indicate IPD arising within 28 days from the time of influenza infection and, similarly, black bars indicate incidence of episodes of AOM arising within 28 days following LAIV. Scenarios are run for different R0 values.

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