A Murine Model for the Transition of *Streptococcus pneumoniae* from Asymptomatic Colonizer to Systemic Pathogen

Basma H. Joma*1, 2, Nalat Siwapornchai*1, Vijay K. Vanguri3, Anishma Shrestha1, Sara E. Roggensack1, 4, Bruce A. Davidson5, Albert K. Tai6, Anders P. Hakansson7, Simin N. Meydani8, John M. Leong#1 and Elsa N. Bou Ghanem#9

* Co-first authors; B.H.J. and N.S. contributed equally to this work; the order of names was chosen alphabetically

# Co-corresponding authors

1 Department of Molecular Biology and Microbiology at Tufts University School of Medicine, Boston, Massachusetts, USA

2 Graduate Program in Immunology, Tufts Graduate School of Biomedical Sciences, Boston, USA

3 UMass Memorial Health Care, University of Massachusetts Medical School, Worcester, Massachusetts, USA

4 Graduate Program in Molecular Microbiology, Tufts Graduate School of Biomedical Sciences, Boston, USA

5 Department of Anesthesiology, University at Buffalo School of Medicine, Buffalo, New York, USA

6 Department of Immunology, Tufts University School of Medicine, Boston, Massachusetts, USA

7 Department of Translational Medicine, Lund University, Malmö, Sweden

8 Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts, USA
Department of Microbiology and Immunology, University at Buffalo School of Medicine, Buffalo, New York, USA

Running Title: Murine Model of Influenza-Pneumococcal Co-infection

Address correspondence and reprint requests to John M. Leong (J.M.L.), Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02114 and Elsa Bou Ghanem (E.N.B.G.), Department of Microbiology and Immunology, University at Buffalo School of Medicine, 955 Main Street, Buffalo, New York, USA. E-mail addresses: john.leong@tufts.edu (J.M.L.) and elsaboug@buffalo.edu (E.N.B.G.)

Keywords: Co-infection, secondary bacterial pneumonia, Streptococcus pneumoniae, Influenza A, colonization, aging, neutrophils, inflammation
ABSTRACT

Streptococcus pneumoniae (pneumococcus) resides asymptotically in the nasopharynx but, as a pathobiont, can progress from benign colonizer to lethal pulmonary or systemic pathogen. Both viral infection and aging are risk factors for serious pneumococcal infections. Previous work established a murine model that featured the movement of pneumococcus from the nasopharynx to the lung upon nasopharyngeal inoculation with influenza A virus (IAV) but did not fully recapitulate the severe disease associated with human co-infection. We built upon this model by establishing pneumococcal nasopharyngeal colonization in mice, then inoculating both the nasopharynx and lungs with IAV. In young (2 months) mice, co-infection triggered bacterial dispersal from the nasopharynx into the lungs, pulmonary inflammation, concomitant disease and mortality in a fraction of mice. Although neutrophils are critical for pneumococcal clearance, IAV infection was associated with inefficient killing of pneumococci by neutrophils ex vivo and these cells were dispensable for host resistance during co-infection. In old mice (20-22 months), co-infection resulted in earlier and more severe disease compared to young mice. Aging was not associated with greater bacterial spread from the nasopharynx to the lungs or bloodstream. Rather, old mice displayed a more rapid proinflammatory cytokine response, earlier pulmonary neutrophil influx, lung damage and abrogated neutrophil function. Thus, in this model, IAV fosters the transition of pneumococci from commensals to pathogens through modifying bacterial behavior in the nasopharynx, enhancing pulmonary infection, and compromising neutrophil function. This model faithfully replicates age-associated susceptibility and can provide insight into factors that influence pathobiont behavior in different hosts.
INTRODUCTION

*Streptococcus pneumoniae* (pneumococcus) is a Gram-positive pathobiont that typically resides asymptptomatically in the nasopharynx of healthy individuals (1). It is hypothesized *S. pneumoniae* establishes an asymptomatic biofilm on the nasopharyngeal epithelium by attenuating the production of virulence factors and concomitant inflammation (2-5). However, when immunity is compromised, a common occurrence in aging (6), pneumococci can cause serious disease such as otitis media, pneumonia, meningitis and bacteremia (7). In humans, pneumococcal carriage is believed to be a prerequisite to invasive disease (8, 9). Bacterial isolates from invasive infections are genetically identical to those found in the nasopharynx of patients (9); this and other longitudinal studies have led to the suggestion that invasive disease often involves pneumococcal carriage in the upper respiratory tract (8, 9).

The rate of reported colonization is quite variable among adults and is confounded by differences in detection methods, but colonization may be more prevalent in the elderly (10). A meta-analysis of twenty nine published studies found that in individuals above 60 years of age, conventional culture showed 0-39% carriage, while 3-23% carriage was detected by molecular methods (11). Importantly carriage was higher among nursing home residents (11). In older adults, conventional culture methods estimated carriage to be <5% (12-14) but more recent data from European studies using molecular detection methods indicated that carriage in the elderly ranges from 10-22% (10, 15-17). Thus, carriage rates may be much higher than what was previously estimated in elderly individuals (12-14).

Advanced age increases the risk of invasive pneumococcal disease and pneumococcal pneumonia (7). Aging is associated with immunosenescence, the overall decline in immunity that accompanies aging as well as inflammaging, a low-grade chronic
inflammation that render the elderly more susceptible to pulmonary infections (18).

Polymorphonuclear leukocytes (PMNs), also known as neutrophils, are a crucial determinant of age-related susceptibility to primary pneumococcal infection (19). These cells are required to control bacterial burden at the start of infection (20-22). However, aging is accompanied by impaired PMN anti-bacterial function (23, 24). In addition, aged hosts experience exacerbated PMN pulmonary influx during primary pneumococcal pneumonia (19, 25, 26), and persistence of PMNs in the airways beyond the first few days leads to tissue destruction and systemic spread of infection (27, 28).

In addition to advanced age, epidemiological and experimental data show that invasive pneumococcal infections are strongly associated with viral infection (29-31). The risk of pneumococcal pneumonia is enhanced 100-fold by influenza A virus (IAV) infection (32), resulting in the seasonal peak of pneumococcal disease during influenza outbreaks (33). Further, S. pneumoniae is historically among the most common etiologies of secondary bacterial pneumonia following influenza and associated with the most severe outcomes (30, 34-36). Symptoms of secondary bacterial pneumonia include cough, dyspnea, fever, muscle aches and when severe result in hospitalizations, respiratory failure, mechanical ventilation, and can lead to death (30, 34-36).

IAV commonly infects the upper respiratory tract, and at this location viral infection can enhance the nutritional environment for pneumococcus in the nasopharynx, leading to greater bacterial loads and/or higher rates of bacterial acquisition (4, 37). IAV can also directly bind the pneumococcal surface and enhance bacterial binding to the pulmonary epithelium leading to increased colonization (38). In addition, viral infection of the pulmonary epithelium induces the release of host components, such as adenosine triphosphate and norepinephrine, that are sensed by biofilm-associated pneumococci,
triggering both the production of pneumococcal virulence factors and the dispersal of bacteria (4, 37, 39, 40). This in turn facilitates bacterial spread to and colonization of the lower respiratory tract (4). Murine models featuring sequential lung challenge with IAV first followed by pneumococcus show the virus can also compromise pulmonary immune defense against pneumococcus (29, 34, 41). In these models, IAV alters both the lung environment and the immune response to enhance subsequent bacterial colonization and tissue damage. For example, IAV pre-infection increases mucus production and fibrosis and dysregulates ciliary function (34, 42, 43), thus impairing mechanical clearance of invading bacteria. Viral enzymes, along with virus-elicited inflammation, result in the exposure of epithelial proteins that promote pneumococcal adherence to and invasion of host cells (41, 44-46). Further, IAV triggers type I and II interferon (IFN) responses that impair both the recruitment and antibacterial function of phagocytes key to defense against pneumococcus (47-50). The combined tissue damage and the compromise in immune function render the lung more permissive for invasive S. pneumoniae infection (34, 45, 47).

These studies indicate that bacterial-viral synergy is multi-factorial and can occur at different sites with the host.

Notably, advanced age and IAV infection appear to synergistically enhance susceptibility to pneumococcal lung infections (6, 18, 51, 52). Indeed, individuals ≥ 65 years old account for 70-85% of deaths due to pneumonia and influenza (52). Interestingly, elderly individuals with influenza-like symptoms were reported to have an increased pneumococcal carriage rate of 30% (53). In animal models in which bacteria are directly instilled into the lungs following influenza infection, aging is associated with increase susceptibility of secondary pneumococcal pneumonia (51). Age-dependent changes in the expression of key components of innate immune signaling contribute to disease in this co-
infection model (51). However, the factors, including those that are age-dependent, that
trigger the transition of pneumococci from benign colonizer to pathogen are poorly defined,
in part because small animal models that recapitulate the transition from asymptomatic
colonization to overt clinical illness are lacking.

Insight into events that occur in both the nasopharynx and lung and contribute to the
heightened susceptibility of the aged to serious disease upon pneumococcal/IAV
coinfection is needed to develop better therapeutic and preventative approaches. A current
murine model for the spread of nasopharyngeal pneumococci to the lung after viral
infection of the upper respiratory tract relies on initial bacterial colonization of the
nasopharynx followed by viral infection, but does not recapitulate the severe signs of
human clinical disease (4). To better investigate the transition of S. pneumoniae from
asymptomatic colonizers to invasive pathogens following IAV infection, as well as the
effect of host age on the disease process, we built upon this mouse model of S. pneumoniae/IAV co-infection. The enhanced model incorporates the sequential
introduction of bacteria and virus and recapitulates the severe and age-exacerbated
clinical disease observed in humans.

RESULTS
Intranasal IAV inoculation of mice pre-colonized with S. pneumoniae strain TIGR4
does not result in disease.

Biofilm-grown S. pneumoniae are relatively less virulent and thus adapted to host
colonization rather than disease (2-4). A previously established murine model in
BALB/cByJ mice utilizes biofilm-grown S. pneumoniae strains D39 and 19F EF3030 to
establish heavy carriage in the nasopharynx (4). Then, two days after bacterial inoculation,
IAV is introduced into the nasal cavity and results in the spread of pneumococci from the nasopharynx (NP) to the lung (4). We first recapitulated this model with S. pneumoniae strain TIGR4, an invasive serotype 4 strain (54, 55) that we previously found to be highly virulent in aged C57BL/6 mice (19). We intranasally (i.n.) inoculated young (8-10 weeks) C57BL/6 (B6) mice with $1 \times 10^6$ colony forming units (CFU) of biofilm-generated S. pneumoniae TIGR4 by delivering the bacteria in 10 µl (a volume that is unlikely to inoculate the lung (56)) to the nares of non-anesthetized mice. Forty-eight hours later, mice were i.n. inoculated with 10 µl containing 20 PFU of Influenza A (IAV) virus PR8. (See Fig. S1A for general scheme). Control groups of mice were either infected with S. pneumoniae or IAV alone as controls. However, under these conditions, mice did not display signs of sickness, nor did bacteria spread into the lungs after seven days (not shown).

To increase the likelihood of clinical disease, we repeated the experiment with a five-fold higher ($5 \times 10^6$ CFU) dose of bacteria and a 25-fold higher dose (500 PFU) of IAV. Similar to earlier reports for other S. pneumoniae strains (D39 and 19F) (4), IAV co-infection resulted in a 10-fold increase in S. pneumoniae TIGR4 in the nasal lavage fluid at 2 days post-IAV infection (Fig. S1B). In addition, IAV co-infection was associated with the detection of bacteria in the lungs of 40% of mice, compared to none in the control group infected with S. pneumoniae TIGR4 alone. This trend is consistent with the previous BALB/cByJ mice model of coinfection (4), but did not reach statistical significance. Furthermore, co-infection was not associated with weight loss when assessed over the course of 4 days post-infection (Fig. S1C). We also scored mice for clinical signs of the disease based on weight loss, activity, posture and breathing and ranging from healthy [score = 0] to moribund [score = 25] and requiring euthanasia if the score was >9 as
previously described (57). As secondary pneumonia can occur several days following IAV
(41), we monitored the disease course up to 7 days, but did not detect disease symptoms
or death in any of the co-infected mice (100% survival and 0 daily clinical score for all
mice). Therefore, despite promoting bacterial dispersal from the nasopharynx into the
lungs, this model of S. pneumoniae TIGR4/IAV coinfection did not result in overt clinical
signs of disease.

Combined intranasal/intratracheal IAV inoculation of S. pneumoniae-colonized mice
results in bacterial dissemination and disease

IAV infection is not restricted to the upper respiratory tract, and can cause viral
pneumonia in a significant fraction of infected individuals (58, 59) that is likely to be crucial
for creating an environment in the lungs that is more permissive for bacterial infection (29,
34, 41). Indeed, viral lung infection diminishes pulmonary defenses against S. pneumoniae
and promotes secondary bacterial pneumonia (34, 41, 44-50). Delivery of IAV i.n. to
BALB/cByJ mice results in signs of viral pneumonia (4), but we found that pulmonary
access of inocula delivered via the nasopharynx is more restricted in B6 mice as compared
to BALB/c mice (unpublished observation), raising the possibility that the lack of disease
observed in co-infected B6 mice was due to the exclusive localization of virus in the nasal
cavity, with limited opportunity to alter systemic or pulmonary immunity.

To ensure delivery of IAV to both the nasopharynx and the lungs, we co-infected S.
pneumoniae-colonized B6 mice by delivering the virus by two routes. Mice were inoculated
i.n. with 5x10^6 CFU of biofilm grown S. pneumoniae TIGR4 and 48 hours later infected not
only with 500 PFU IAV i.n., but also 20 PFU i.t. to ensure pulmonary infection (Fig. 1A). No
mice in a control group inoculated i.n. with S. pneumoniae alone lost weight (Fig 1B),
displayed clinical signs of sickness (Fig 1C), or died (Fig 1D). A second control group, inoculated i.n. and i.t. with IAV alone displayed no disease until after day 4, when they exhibited weight loss (Fig 1B) and began succumbing to viral infection (Fig 1D). In contrast, inoculation of IAV to animals pre-colonized with *S. pneumoniae* caused a bacterial/viral co-infection that resulted in weight loss (Fig. 1B), clinical symptoms (Fig. 1C) and death (Fig. 1D) that were detected starting day 2 post IAV introduction. At this time point, a higher fraction of co-infected mice displayed signs of disease as compared to controls infected with IAV only (40% vs 64%), and disease was more severe in sick mice, although this did not reach statistical significance. In addition, the overall survival rate among co-infected mice was significantly lower than mice singly infected with *S. pneumoniae* alone (Fig. 1D). These signs of exacerbated disease were associated with significantly higher bacterial burdens in the nasopharynx as well as translocation of *S. pneumoniae* into the lung in comparison to mice colonized with the bacteria or mice infected with IAV alone (Fig. 1E). These findings demonstrated that the new co-infection model leads to disease in a fraction of young healthy mice, which may increase the likelihood of detecting enhanced susceptibility in vulnerable hosts.

**PMN depletion has no significant effect on the course of disease during IAV/S. pneumoniae co-infection**

We previously found that in primary pneumococcal pneumonia, PMNs are required to control bacterial numbers early in the infection process; however their persistence in the lungs is detrimental to the host and can promote the infection at later time points (20). To address the role of PMNs during co-infection, we treated young mice with PMN-depleting anti-Ly6G antibody (1A8) one day prior to pneumococcal colonization and throughout the
co-infection (based on timeline in Figure 1A). We then confirmed that the cells were depleted by staining with the RB6 antibody followed by flow cytometry (see materials and methods). Following infection, we measured bacterial burden, weight loss, clinical score and survival over time. Surprisingly, PMN depletion had no effect on bacterial burdens in the nasopharynx or bacterial spread to the lungs or blood following co-infection (Fig 2A and B). PMN-depletion was not associated with any significant differences in weight loss as compared to the control group (Fig. 2C). Additionally, untreated controls and PMN-depleted mice had comparable clinical signs of sickness at 18 hours and 48 hours post IAV infection (Fig. 2D). Lower survival was observed in the depletion group, where only 25% survived to day 7 compared to ~43% survival from untreated controls; however, this difference was not statistically significant (Fig. 2E). In conclusion, PMN depletion prior to infection appeared to have a slight but not significant effect on the course of co-infection in young mice in this model.

**Aging increases susceptibility to IAV/S. pneumoniae co-infection.**

We next tested if the new co-infection mouse model (Fig. 1A) recapitulates the age-associated increase in susceptibility to secondary pneumococcal pneumonia. Old (20-24 months) B6 mice were inoculated i.n. with 5x10^6 CFU of biofilm grown S. pneumoniae TIGR4 and 48 hours later were infected with 500 PFU IAV i.n. plus 20 PFU i.t. When compared to young co-infected controls, old mice displayed significantly more severe signs of disease, as indicated by a higher average clinical score (Fig. 3A). While seven of 16 (43.75%) young mice showed clinical symptoms (i.e., clinical score greater than 1; Fig. 3A), all 13 old mice showed at least some degree of illness by day 2 post co-infection ($p = 0.0012$, by Fisher’s exact test). Furthermore, whereas only 25% (4 out of 16) young mice
had a clinical score greater than 2, which is indicative of more severe disease, 92% (12 out of 13) aged mice fell into this category ($p = 0.005$, by Fisher’s exact test). In addition, old mice died at a significantly accelerated rate. By day 2 post co-infection, 60% of old mice had succumbed to the infection compared to only 25% of young mice. Differences in survival were observed at each successive time point, and at the end of the experiment on day 8, only 14% of old mice remained alive compared to 50% of young mice (Fig. 3B). Importantly, the accelerated death observed in co-infected old mice was not observed in old mice infected with *S. pneumoniae* alone or IAV alone (Fig S2).

We next tested whether these differences could be attributed to increased bacterial loads in the nasopharynx, lungs, or blood. As old mice got sicker at earlier time points post IAV co-infection, with the majority succumbing by day 4, we compared bacterial burden across age groups at 18 and 48 hours post IAV co-infection. We found no significant differences in the numbers of pneumococci in nasopharyngeal washes, pulmonary homogenates, or blood at either time point (Fig 3C-E). Taken together, these findings suggest that with aging there is an increased susceptibility to co-infection and an accelerated course of disease that could not be attributed to a more rapid bacterial dissemination or higher bacterial loads in the nasopharynx, lung, or bloodstream.

**Aging is associated with more rapid lung inflammation.**

We previously found that mice suffering from exacerbated PMN-mediated pulmonary inflammation during pneumococcal pneumonia did not display higher bacterial burdens in their lungs (27) despite a higher likelihood of severe disease (19, 20, 27). To test whether PMN influx was higher in old co-infected mice, we measured the percentage and number of pulmonary PMNs (Ly6G+) by flow cytometry. We found that old mice had
significantly (6-fold) higher percentages and numbers of PMNs in their lungs as compared to young controls at 18 hours post co-infection (Fig 4A). By 48 hours, although PMN percentages and numbers appeared to be higher in young mice, the differences were not statistically significant (Fig. 4A and B). Macrophages, which are important for host resistance to S. pneumoniae/IAV co-infection (49) and display age-driven changes (60, 61), displayed no significant age-dependent differences in either percentage or number (Fig. S3).

Next we tested the levels of inflammatory cytokines in co-infected young vs old mice. No significant differences between age groups were detected in baseline (uninfected) levels of any of the cytokines tested between the age groups (Fig 4B and not shown). However, consistent with the enhanced PMN influx at 18 h post-infection in aged mice, old mice had significantly higher levels of IL-10, IL-2, IL-1β and TNFα (Fig 4B) compared with young mice. Levels of IL-12p70, IL-17, IL-6 and IFNγ, were slightly but not significantly elevated 18 hours post co-infection (Fig S4A). By 48 hours there were no significant differences between young and old mice in cytokine levels except for IFNγ, which was higher in young mice (Fig S4B).

To determine if the pulmonary inflammation was associated with lung damage, we analyzed H&E stained lung sections for alveolar congestion, hemorrhage, alveolar thickness, neutrophils and lymphocytic infiltration (Fig. 4C). We found that the alveolar spaces of both uninfected old and young mice were clear and free of inflammatory or red blood cells (Fig. 4C). Similarly, at 18h hours post co-infection, the lungs of young mice did not show any overt signs of disease (Fig. 4C). However, co-infected old mice had significant pathology in the lungs by 18 hours post infection, including a loss of alveolar architecture, and spotty inflammation consisting of infiltrates composed of neutrophils,
alveolar macrophages and mononuclear cells that were mixed with red blood cells (Fig. 4C). At 48 hours post-infection, there were clear signs of lung pathology in both young and old co-infected mice (Fig. 4C). Taken together, these findings demonstrate that aging is associated with earlier pulmonary inflammation and damage following co-infection, which may account for the accelerated death observed in this mouse group.

**Aging and IAV infection diminish the ability of PMNs to kill** *S. pneumoniae* **ex vivo.**

Aged, co-infected mice experience an accelerated rate of pulmonary inflammation but bacterial loads in the lungs of aged mice were not lower than in young mice, indicating that PMN infiltration is not associated with bacterial clearance. Both aging (24) and IAV infection (62) have been reported to diminish antibacterial function of PMNs. To assess the ability of PMNs to kill *S. pneumoniae* in our co-infection model, we used a well-established opsonophagocytic (OPH) killing assay (19, 63). We first compared the bactericidal activity of bone marrow-derived PMNs from young or aged mice. The percentage of bacteria killed upon incubation with PMNs was determined by comparing surviving CFU to no PMN control reactions. We found that as previously reported for humans (23) and mice (24) the ability of PMNs isolated from uninfected old mice to kill pneumococci was reduced 5-fold compared to young controls (Fig. 5).

We next examined the bactericidal activity of PMNs isolated from young or old mice 2-days after i.t./i.n. IAV inoculation. As previously reported (47-50), PMNs from young IAV infected mice had a slight (2-fold) but significant reduction in their ability to kill pneumococci as compared to PMNs from uninfected controls (Fig. 5). Strikingly, IAV infection abolished the ability of PMNs from old mice to kill *S. pneumoniae*; instead, PMNs from IAV infected old mice promoted bacterial growth instead of death (Fig. 5). These
findings suggest that IAV infection completely abrogates the ability of PMNs from old mice to kill *S. pneumoniae*.

**DISCUSSION**

*S. pneumoniae* remains a leading cause of secondary bacterial pneumonia following influenza A virus infection and is associated with severe disease (30, 34-36), particularly in the elderly (52). The majority of *S. pneumoniae*/IAV co-infection studies have delivered bacteria into the lungs of mice pre-infected with IAV to reveal changes in the host lungs and immune response that are crucial for priming invasive pneumococcal disease (34, 41, 44-50). In this study, to investigate the transition of *S. pneumoniae* from colonizer to pathogen upon IAV co-infection, a process that has just started to be elucidated (4, 37), we have developed a modified murine infection model that recapitulates this transition and results in severe clinical disease. In a previously established model, female BALB/cByJ mice were first colonized intra-nasally with biofilm-grown pneumococci and then infected with IAV by delivering the virus to the nasopharynx (4, 40). This model showed that changes in the host environment in response to viral infection triggers the dispersal of pneumococci from colonizing biofilms and their spread to the lower respiratory tract (4).

Importantly, the dispersed bacteria expressed higher levels of virulence factors required for infection, thus, rendering them pathogenic (4). When we used this model to co-infect male C57BL/6 (B6) mice, we observed a significant increase in dispersed bacteria in the nasopharynx, but no disease and only a transient presence of *S. pneumoniae* in the lungs of B6 mice. This could be due to the differences in host susceptibility to bacterial infection between the different mouse strains, which has been well described for *S. pneumoniae* (64, 65) and other infections (66) or due to gender (67): Here we performed experiments in
male instead of female mice due to both the easier availability of aged male animals and the documented higher rate of pneumococcal pneumonia in men compared to women (68, 69).

Previous work of pneumococcal inoculation of IAV-infected lungs showed viral infection to be crucial for creating an environment in the lungs that is more permissive for bacterial infection (41, 44-50). The relatively low bacterial burden in co-infected male B6 mice observed here upon IAV inoculation only by the i.n. route suggested that the lung environment was not sustaining the bacteria. Therefore, we co-infected S. pneumoniae colonized B6 mice with IAV by delivering the virus not only i.n. to infect the nasopharynx, but also i.t. to ensure infection of the lungs. This modified model recapitulated the increase in non-adherent pneumococci in the nasopharynx observed upon viral co-infection (4, 70, 71), and resulted in bacterial spread into the lungs and circulation that increased over time. Importantly, this mode of dual infection recapitulated both the increased colonization burden (72, 73) as well as the clinical signs of severe disease observed in humans (30, 34-36), and resulted in death of approximately half of co-infected young controls.

Although the elderly are at higher risk for secondary pneumococcal pneumonia following IAV infection, animal studies exploring this age-driven susceptibility to co-infection are few (51, 74, 75). Here, using the modified model, we found that old mice were significantly more susceptible to S. pneumoniae/IAV co-infection. Old mice displayed more severe signs of disease as compared to young controls and the majority (>85%) failed to survive the co-infection. This increased susceptibility in old mice was not linked to higher bacterial dissemination from the nasopharynx, or greater establishment of infection in the lungs or systemic spread into the circulation at the time points tested. Although we did not directly measure viral loads, susceptibility to viral infection alone as measured by weight
loss, within the first 5 days following IAV was also similar between the age groups. Rather, the age-driven susceptibility to co-infection was associated with earlier and more severe pulmonary inflammation. Production of cytokines in the lungs has been reported in young mice infected with *S. pneumoniae* 7 days post IAV (76). Further, old mice displayed changes in the expression of pattern recognition receptors in the lungs leading to altered inflammatory responses (51). Similar to previous studies (51), we found here that co-infected old mice had higher levels of TNFα as compared to young controls. However, in contrast to previous reports that found reduced NLRP3 inflammasome expression in the lungs and lower production of IL-1β, we found higher levels of IL-1β in old mice as compared to young controls. This may be accounted for by differences in expression of bacterial factors. The expression of pneumolysin, which was found to activate NLRP3 inflammasomes and lead to production of IL-1β (77), was elevated in pneumococci dispersed from biofilms upon IAV infection (78) and therefore may have primed the IL-1β production we observed in old mice.

We previously found that PMNs were key determinants of disease during primary pneumococcal pneumonia and are required to initially control bacterial numbers (20, 79). Therefore, we explored here the role of PMNs in *S. pneumoniae*/IAV co-infection. Similar to other studies, we found that PMNs are recruited to the lungs of co-infected young mice (49). Previous reports indicate that the PMN-mediated anti-pneumococcal function in IAV-infected mice (62) and humans (73) is progressively reduced over time. For example, in mice, PMNs demonstrably contribute to host defense at 3 days but not at 6 days post-infection (62). Nevertheless, PMN depletion showed that these cells are important for control of bacterial transmission (80) and control of pulmonary bacterial numbers (49) in IAV co-infected mice. In contrast, here we found that PMN depletion starting prior to
bacterial colonization and continuing throughout viral co-infection had no significant effect on the number of dispersed *S. pneumoniae* in the nasopharynx or their spread to the lungs and blood. In this model IAV may rapidly impair PMN function, and in fact, we found that within 2 days following IAV infection, the ability of bone marrow-derived PMNs to kill *S. pneumoniae* was significantly blunted in young mice. Alternatively, the apparent inability of PMNs to limit bacterial numbers in this model could be due to the enhanced virulence of pneumococci dispersed from the nasopharyngeal environment (4) compared to broth-grown bacteria typically used in other models (62).

In this study we found that aging was associated with earlier influx of PMNs into the lungs of co-infected mice. We previously demonstrated that excessive PMN influx into the lungs is detrimental for the ability of old mice to control invasive disease following primary pneumococcal pneumonia (19) and that PMN depletion 18 hours after infection boosted host survival (20). Similarly, exacerbated PMN influx into the lungs of old mice singly infected with IAV was associated with host mortality, and depletion of these cells six days following viral infection significantly boosted host survival (81). Uncontrolled PMN influx can result in tissue damage, disruption of gaseous exchange and pulmonary failure. In fact, it was reported that in IAV singly infected old mice, PMNs mediated lung inflammation and damage and their depletion reduced the levels of inflammatory IL-1β and TNFα (81). Therefore, the early increase in these inflammatory cytokines and lung damage we observed here in *S. pneumoniae*/IAV co-infected old mice may be driven by the elevated levels of pulmonary PMNs.

In this model, aging was associated with significantly increased levels of pulmonary IL-10 during *S. pneumoniae*/IAV confection as compared to young controls, which may account for their enhanced susceptibility to co-infection. IL-10 was previously shown to
impair host resistance to *S. pneumoniae* following IAV infection (82). IL-10 levels were elevated in co-infected young mice as compared to those singly infected with *S. pneumoniae* and blocking this cytokine resulted in reduced bacterial numbers and extended host survival (82). The elevated IL-10 may also account for the complete inability of PMNs from IAV infected old mice to kill *S. pneumoniae*. We previously found that IL-10 impairs PMN anti-bacterial function by inhibiting ROS production in response to infection (79). Blocking this cytokine boosted PMN anti-bacterial function and importantly restored resistance of vulnerable hosts to primary pneumococcal pneumonia (79).

In summary, here we modified existing murine models to establish an experimental system that reflects the transition of *S. pneumoniae* from asymptomatic colonizer to invasive pulmonary pathogen upon IAV co-infection. In this model, IAV triggers the transition of a pathobiont from a commensal to a pathogenic state through modification of bacterial behavior in the nasopharynx, enhancement of bacterial colonization in the lung, and compromise of PMN-mediated anti-bacterial immunity. Importantly this model recapitulates the susceptibility of aging to co-infections. Moving forward, this model can be used to dissect the multiple phases of pneumococcal disease progression from commensals to pathogens, which can help inform specialized treatment options (70) tailored to the susceptible elderly population.

**MATERIALS AND METHODS**

**Mice.** Young (8-10 weeks) and aged (18-24 months) male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and the National Institute of Aging. Mice were housed in a pathogen-free facility at Tufts University. All procedures were performed in accordance with Institutional Animal Care and Use Committee guidelines.
**Bacterial biofilms.** NCI-H292 mucoepidermoid carcinoma cells (H292) were grown in 24-well plates in RPMI 1640 media with 10% FBS and 2mM L-glutamine until confluent. Cells were washed with 1x PBS and fixed in 4% paraformaldehyde for 1 hour on ice. *S. pneumoniae* TIGR4 (kind gift from Andrew Camilli) were grown on Tryptic Soy Agar plates supplemented with 5% sheep blood agar (blood agar plates) overnight, then diluted and grown in chemically defined liquid medium (CDM) (4, 40) supplemented with Oxyrase until OD$_{600nm}$ of 0.2. Bacteria were diluted 1:1000 in CDM and seeded on the fixed H292 cells. The bacteria/H292 cells were incubated at 34°C/ 5% CO$_2$ and media was changed every 12 hours. At 48 hours post-infection, the supernatant containing planktonic bacteria (non-adherent to NCI-H292 cells) cells was discarded, the cells gently washed with PBS and adherent biofilms collected in fresh CDM by vigorous pipetting. Biofilm aliquots were then frozen at -80°C in the CDM with 25% (v/v) glycerol.

**Intranasal inoculation.** Before use, biofilm aliquots were thawed on ice, washed once and diluted in PBS to the required concentration. The mice were restrained without anesthesia and infected i.n. with 10μl (5x10$^6$ CFU) of biofilm grown *S. pneumoniae*. The inoculum was equally distributed between the nostrils with a pipette. Bacterial titers were confirmed by serial dilution and plating on blood agar plates. To ensure stable colonization of the biofilm in the nasopharynx, groups of mice were euthanized at 18 and 48 hours post inoculation, and the nasal washes and tissue were collected and plated on blood agar plate for enumeration of *S. pneumoniae*.
**Viral infection.** The mouse-adapted H1N1 Influenza A virus PR8 (A/PR/8/34) was obtained from Dr. Bruce Davidson (4, 40) and stored at -80 °C. Before use, viral aliquots were thawed on ice, diluted in PBS and used to inoculate mice. At 48 hours following bacterial inoculation, mice were infected i.n. with 10μl of 500 plaque forming units (PFU) of virus by pipetting the inoculum into the nostrils of non-anesthetized mice. Following i.n. inoculation, mice were lightly anesthetized with isoflurane and challenged i.t. with 20 PFU of virus in a 50μl volume pipetted directly into the trachea with the tongue pulled out to facilitate delivery (20).

**Clinical scoring and bacterial burden.** Following co-infection, mice were monitored daily and blindly scored for signs of sickness including weight loss, activity, posture and breathing. Based on these criteria, the mice were given a clinical score of healthy [0] to moribund [25] as previously described (57). Any mice displaying a score above 9 are humanely euthanized in accordance with our protocol. Mice were euthanized at indicated time points and the lung, nasal lavages and blood collected and plated on blood agar for enumeration of bacterial loads, as previously described (20). For collection of sera, blood was collected via cardiac puncture into Microtainer® tubes (BD Biosciences) and centrifuged at 7607xg for 2 minutes to collect serum as per manufacturer’s instructions.

**Depletion of PMNs.** Mice were intraperitoneally (i.p.) injected with 100μl (50μg/mouse) of anti-Ly6G clone 1A8 (BD Biosciences) to deplete neutrophils. Mice were injected daily with the depleting antibodies starting one day prior and ending two days after bacterial inoculation, followed by every other day from day 1 post viral co-infection to the end of each experiment. Treatment resulted in >90% neutrophil depletion as described below.
Cell isolation and Flow Cytometry. Mice lungs were harvested, washed in PBS, and minced into small pieces. The sample was then digested for 45 minutes with RPMI 1640 1X supplemented with 10% FBS, 1 mg/ml Type II collagenase (Worthington, Lakewood, NJ), and 50 U/ml deoxyribonuclease I to obtain a single-cell suspension as previously described (20). The red blood cells were lysed using ACK lysis buffer (Gibco). Cells were then stained with anti-mouse Ly6G clone 1A8 (BD Biosciences), F480 clone BM8 (BioLegend), CD11c clone N418 (eBioscience), and CD11b clone M1/70 (eBioscience). For neutrophil depletion, cells isolated from the lungs at 18 and 48 h post co-infection were also stained with either Ly6G clone 1A8 or RB6 clone RB6-8C5 (BioLegend) antibodies to confirm cell depletion. The fluorescence intensities were measured on BD LSR II Flow Cytometer at Tufts FACS Core Facility (Boston, MA) to capture at least 25,000 cells and analyzed using FlowJo.

Isolation of PMNs and Opsonophagocytic Killing Assay (OPH). Femurs and tibias of uninfected mice were collected and flushed with RPMI, supplemented with 10% FBS and 2 mM EDTA to obtain bone marrow cells. Neutrophils were isolated by density gradient centrifugation, using Histopaque 1119 and Histopaque 1077 as previously described (83). The neutrophils were resuspended in Hank’s (Gibco) buffer/0.1% gelatin with no Ca+ or Mg+ and tested for purity by flow cytometry using anti-Ly6G antibodies (eBioscience) where 85-90% of enriched cells were Ly6G+. The ability of neutrophils to kill bacteria was measured using a well-established opsonophagocytic (OPH) killing assay as previously described (20). Briefly, 2.5x10^5 neutrophils were incubated in Hank’s buffer/0.1% gelatin with 10^3 CFU of S. pneumoniae pre-opsonized with 3% mouse sera. The reactions were
incubated in flat bottom 96-well non-binding plates for 45 minutes at 37°C. Each group was plated on blood agar to enumerate viable CFU. Percent bacterial killing was calculated in comparison to a no PMN control under the same conditions.

**Histology.** Whole lungs were harvested from groups of mice at 18 hours and at 48 hours post co-infection and fixed in 10% neutral buffered formalin for 2 days. The tissues were then embedded in paraffin, sectioned at 5 μm and stained with Hematoxylin and Eosin at the Animal Histology Core at Tufts University. Sections of lung from three mice per group were imaged using a Nikon Eclipse E400 microscope. Photomicrographs were captured using a SPOT Idea 5.0-megapixel color digital camera and SPOT software. Histopathologic scoring was performed by a board-certified anatomic pathologist experienced in murine pathology, from 0 (no damage) to 4+ (maximal damage) for alveolar congestion, hemorrhage, alveolar thickness, neutrophils, and lymphocytic infiltration (84).

**Cytokine Analysis.** Cytokines in the lungs and serum samples were measured using Mouse Cytokine 8-Plex Array (Quanterix, Billerica, MA) following the manufacturer's instructions. Frozen lung homogenates and serum samples were thawed on ice and mixed by gentle vortexing. Levels in lung supernatants and serum samples were measured using the Cirascan at the Imager at Tufts University Genomic Core (Boston, MA) and analyzed by the Cirascan/Cirasoft program. Quilcore Omic Explorer (version 3.5) was used for the generation of lung cytokine box plots. Concentrations of cytokines (IL-10, IL-2, IL-1β, TNFα, IL-6, IFNγ, IL-17 and IL-12p70) were log-transformed, and displayed as Log₂ pg/ml.
Statistical Analysis. Statistical analysis was performed using Graph Pad Prism7. CFU data were log-transformed to normalize distribution. Data are presented as mean values +/- SEM. Significant differences ($p < 0.05$) were determined by Student’s t-test. Differences in fractions of mice that got sick were measured using Fisher’s exact test. Survival analysis was performed using the log rank (Mantel-Cox) test. Asterisks indicate significant differences and $p$ values are noted in the figures.

ACKNOWLEDGEMENTS

We would like to acknowledge James Nicholas Lee, Summer Schmaling, and Ognjen Sekulovic for technical assistance with clinical score, virus preparation and nasal lavage respectively. We would also like to thank Andrew Camilli for bacterial strains and Marta Gaglia for protocols. They, along with Tim van Opijnen, Bharathi Sundaresh and Marcia Osburne provided important feedback on the manuscript.

FUNDING

Research reported in this publication was supported by the National Institute On Aging of the National Institutes of Health under Award Number R21 AG064215 to E.B.G. and F31 AI122615-01A1 to S.R.; King Abdullah Scholarship Program (KASP) implemented by the Ministry of Higher Education (MOHE) under Award Number 7896504 to B.H.J.
REFERENCES


**FIGURES & LEGENDS**

**A**

Sp. *i.n.**  
IAV *i.n.+ i.t.*

-2 -1 0 1 2  
Days

**B**

% Weight Change  

% Sp. + IAV  

-10 -5 0 5  10  
Days

**C**

Day 2  

Day 4  

% Clinical Score

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<th>Day 4</th>
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<td>7/11</td>
<td>3/5</td>
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<tr>
<td>IAV</td>
<td></td>
<td></td>
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<tr>
<td>Sp.+ IAV</td>
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**D**

% Survival  

Sp. *g/g*  
IAV 3/6  
Sp.+ IAV 4/12

0 2 4 6 8  100

Days

**E**

Log<sub>10</sub> CFU  

Nasal Wash  

Lung  

0/6  
3/6  
LOD

Day 2

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**Figure 1. Combined intranasal/intratracheal IAV inoculation of *S. pneumoniae*-colonized mice results in bacterial dissemination and disease.** (A) Timeline of co-infection; 8-10 weeks old male C57BL/6 (B6) mice were inoculated i.n. with 5x10<sup>6</sup> CFU of biofilm grown *S. pneumoniae* TIGR4 to establish colonization in the nasopharynx. 48 hours later, the mice were either mock treated (*Sp*) or received 500 PFU of Influenza A virus PR8 (IAV) i.n. and 20 PFU i.t. (B) Percent weight loss was monitored daily. (C) Blinded clinical scoring was performed on day 2 and day 4 post IAV infection. The fractions denote the number of sick mice observed over total number of mice. A score of 0 means no sign of sickness observed and a score above 1 indicates observable sickness. (D) Survival was monitored for 8 days post IAV infection with fractions denoting survivors over total number of mice. (E) The bacterial burden in the nasopharynx and lung were determined at day 2 post IAV infection. Pooled data from three separate experiments, n = 6-12 mice per group are shown. Statistically significant differences determined by Student’s t-test for bacterial
burden and clinical score and the log-rank (Mantel-Cox) test for survival are indicated by asterisks. #, indicates statistical significance ($p< 0.05$) between $Sp$ and $Sp+IAV$ groups by Fisher’s exact test.
Figure 2. PMN depletion does not significantly affect the course of disease during IAV/S. pneumoniae co-infection. 8-10 weeks old C57BL/6 mice were intraperitoneally (i.p.) injected with anti-Ly6G (clone 1A8) antibodies to deplete neutrophils or mock treated. The antibodies were given daily from day -3 to day 1, and every other day from day 3 to the end of each experiment (with respect to IAV-infection). (A) At 18 and 48 hours post IAV infection, bacterial numbers in the nasal wash and lungs were determined. Bacteremia (B) and weight loss (C) was monitored over time. (D) Mice were blindly scored for symptoms of diseases at 18 and 48 hours post IAV-infection. The fractions indicate number of mice with clinical sickness (clinical score above 1) over total number of mice. (E) Survival was monitored over time where the fractions denote survivors over total number of mice at 8 days post IAV infection. Data shown represent the means +/- SEM and are pooled from three separate experiments with n=16 mice per group. Statistically significant differences were determined by Student’s t-test for bacterial burden and clinical score and the log-rank (Mantel-Cox) and ns = not significant.
Figure 3. Aging increases susceptibility to IAV/S. pneumoniae co-infection. Young (8-10 weeks) and aged (18-24 months) C57BL/6 male mice were co-infected with S. pneumoniae TIGR4 i.n. and Influenza A virus PR8 i.n. and i.t. (as in Figure 1A). (A) Clinical score of co-infected young and aged mice at day 2 post IAV-infection is shown; the number of mice with demonstrable illness over total number of mice is indicated. Data shown are pooled from four experiments with n=13-16 mice per group. #, indicate statistical significance by Fisher’s exact test. (B) Survival of co-infected young and aged mice were monitored over time with fractions denoting survivors over total of mice. Data are pooled from four experiments with n=14 mice per group. Asterisks indicate statistical significance by the log–rank (Mantel-Cox) test. (C-E) Bacterial burdens in the nasal wash, lungs, and blood were determined at 18 and 48 hours post IAV inoculation. The mean +/- SEM pooled from three separate experiments with n=7-10 mice per age group are shown. LOD denotes the limit of detection.
Figure 4. Aging is associated with more rapid lung inflammation. Young and aged C57BL/6 male mice were co-infected with S. pneumoniae and Influenza A virus PR8. 18 and 48 hours following IAV-infection (see experimental design in Fig. 1A), the lungs were harvested. (A) The percentages and total number of PMNs (Ly6G+) in the lungs was measured by flow cytometry. Young mice are represented by open bars and aged mice by shaded bars. The mean +/- SEM pooled from three separate experiments with n = 6 mice and n=12 mice per group at 18 and 48 hours respectively are shown. Statistically significant differences determined by Student’s t-test are indicated by asterisks. (B) Cytokines in the supernatants of lung homogenates of young (n=4) or aged (n=5) mice at 18 hours post co-infection were measured by multiplex ELISA. Asterisks represent statistical significance by Student’s t-test. (C) Lungs were stained with Hematoxylin and Eosin and shown are representative photographs at 100x and 400X are shown (inset)
Figure 5. Aging and IAV infection diminish the ability of PMNs to kill *S. pneumoniae* ex vivo. PMNs were isolated from bone marrow of young (8-10 weeks) and aged (18-24 months) C57BL/6 male mice that were mock-infected (uninf.) or singly infected with IAV (i.n. + i.t.) for 2 days. PMNs were incubated with *S. pneumoniae* pre-opsonized with homologous sera from the same mouse for 45 minutes at 37°C. The percentages of *S. pneumoniae* killed upon incubation with PMNs were determined with respect to a no PMN control. Data shown represent the means +/- SEM pooled from two experiments (n=3 mice per group) where each condition was tested in quadruplicates per experiment. Asterisks represent statistical significance as determined by Student’s t-test.