A Critical, Nonlinear Threshold Dictates Bacterial Invasion and Initial Kinetics During Influenza

Amber M. Smith1,* and Amanda P. Smith1

1Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN 38105, USA
*amber.smith@stjude.org

ABSTRACT

Secondary bacterial infections increase morbidity and mortality of influenza A virus (IAV) infections. Bacteria are able to invade due to virus-induced depletion of alveolar macrophages (AMs), which are the host's first line of defense. However, this is not the only factor contributing to bacterial invasion during IAV infection. By analyzing a theoretical model describing the coinfection kinetics, we uncovered nonlinear initial dose threshold that is dependent on the amount of AM depletion caused by the virus. This threshold separates a growth phenotype from a clearance phenotype such that bacteria initially decline for dose-AM depletion combinations below the threshold, stay constant for combinations near the threshold, and increase for combinations above the threshold. In addition, the distance from the threshold correlates to the growth rate. Because AM depletion changes throughout an IAV infection, the dose requirement for bacterial invasion will also change according to the threshold. Using the threshold calculation to estimate the dynamical dose requirement, we found that the dose requirement drops dramatically during the first 7d of the IAV infection. We then validated these analytical predictions experimentally by infecting mice with doses below or above the predicted threshold over the course of IAV infection. These results identify the nonlinear way in which two simultaneous, independent factors work together to support successful post-influenza bacterial invasion. They provide insight into the timing of coinfections, the heterogeneity in outcome, the probability of acquiring a coinfection, and the use of new therapeutic strategies to combat viral-bacterial coinfections.

Introduction

Influenza A virus (IAV) poses a considerable threat to public health, resulting in 15-65 million infections and >200,000 hospitalizations each year during seasonal epidemics in the U.S.1,2. Morbidity and mortality increase when a pandemic strain emerges and/or when IAV infection is complicated by a bacterial pathogen like Streptococcus pneumoniae (pneumococcus), which has accounted for 40-95% of influenza-related mortality in past pandemics3-6. As the respiratory tract environment deteriorates during influenza, the physiological barriers and immune mechanisms that normally clear pathogens become compromised and bacteria are able to invade and grow rapidly. Several factors, including viral and bacterial strain, inoculum size, and bacterial infection timing, are thought to contribute to influenza-bacterial coinfection kinetics, pathogenicity, and the likelihood of severe pneumonia developing (reviewed in7-13). Understanding how each factor influences the virulence and interaction between influenza viruses and bacterial pathogens and how each is interrelated is pivotal to finding effective preventative and therapeutic strategies.
Although well-characterized animal models have allowed for the study of various factors that affect bacterial acquisition and pathogenicity after influenza (reviewed in11), the extraordinary complexity of host-pathogen and pathogen-pathogen interplay complicates investigating every possible interaction simultaneously. Quantitative analyses have made it possible to simultaneously assess the contributions of different components and identify critical mechanisms driving influenza-bacterial coinfection kinetics. We recently combined a mathematical model and data from animal models to establish dynamical host-pathogen feedbacks, quantify the contribution of various hypothesized mechanisms (e.g., virus enhanced bacterial attachment14,15 and alveolar macrophage (AM) inhibition16), and develop new hypotheses (i.e., bacteria enhanced virus production) about the relationship between influenza and pneumococcus17. Our model revealed that the rapid increase in bacterial loads, a hallmark of influenza-pneumococcal coinfection, is initiated solely by the virus removing the protective effect of alveolar macrophages (AMs) with 85-90% efficiency by 7d post-influenza infection (pii) and that bacterial clearance could be achieved with improved AM response. This was in correlation to one study suggesting that the phagocytic ability of these cells is inhibited16. It was initially unclear from either study if the effect accumulates over time and if it comprises several underlying mechanisms. However, a more recent study followed up these works by using an advanced gating strategy to better define the AM population throughout the course of an IAV infection and found that these cells are depleted18, rather than or in addition to their functional inhibition, by IAV and that the level of depletion corresponds to the amount of bacterial outgrowth18. While our model does not distinguish between these mechanisms, the AM data indicated that the maximum amount of depletion occurred 7d pii and matched our parameter estimate of 85-90%18. Further, our model did include a handling time effect on the rate of bacterial phagocytosis by AMs, which had only a minor role. This further supports AM depletion as the dominant mechanism driving bacterial establishment, with functional inhibition as a possible secondary mechanism, and the accuracy of our model. Remarkably, this also corresponds to the time when bacterial coinfections are the most lethal19.

Another important feature of influenza-pneumococcal coinfection biology is that bacteria grow rapidly for initial doses that would be rapidly cleared in the absence of virus17,19. In both naive and influenza-infected hosts, the trajectory of bacterial titers is dependent on the inoculating dose16,17,20–22. Further, in the context of the coinfection, a distinct dichotomous pattern emerged with a low dose (10^2 CFU) compared to a higher dose (10^3 CFU) such that some individuals had high bacterial titers, an indication of severe pneumonia, while others had low bacterial titers and, presumably, a more mild infection17. It is currently unclear what factors
contribute to the differential dynamics, although we hypothesized that this may be due to heterogeneity in the AM population because reducing the depletion parameter in our mathematical model could result in lower titers\textsuperscript{17} and because we previously related the dose dependent effect to the AM population in a naive host\textsuperscript{20}. However, the exact connection between dose and AM depletion during influenza-pneumococcal coinfection is unclear. Understanding this behavior in more detail will help elucidate what conditions lead to secondary pneumococcal infections after influenza and why only a proportion of IAV infections lead to severe bacterial pneumonia\textsuperscript{23–26}.

Here, we analyzed our coinfection model in more detail to quantify how the trajectory of bacterial growth changes with the level of AM depletion. In doing so, we uncovered a nonlinear initial dose threshold that is dependent on the number of AMs and found the critical number of AMs required to support a clearance phenotype. We then used this threshold together with data on the AM population\textsuperscript{18} to predict how the dose requirement declines over the course of an IAV infection. To validate the time-dependent threshold predictions, we examine bacterial growth/clearance rates in groups of BALB/cJ mice infected with influenza A virus A/Puerto Rico/8/34 (H1N1) (PR8) then 1, 3, 5, 7, 9, or 11 days later with pneumococcal strain D39 at a dose larger or smaller than the predicted threshold. The resulting data agree with our theoretical predictions and, in doing so, define an important, nonlinear relationship between bacterial dose and AM depletion that can be used a predictive tool. Taken together, our data gives new insight into the timing of coinfections, potential therapeutic strategies, and why bacterial coinfections occur more often during influenza pandemics compared to seasonal epidemics.

**Results**

**Coinfection Kinetics Depend on AM Depletion**

To examine how pneumococcal kinetics during IAV infection change with varying degrees of AM depletion, we simulated the coinfection model (Equations 2-6) with values of AM depletion ($\phi$) ranging between 0\% and 100\% ($0 \leq \phi \leq 1$). The resulting dynamics (Figure 1A) illustrate that distinct bacterial outcomes (i.e., maximum growth or clearance) exist depending on the degree that the IAV infection reduces the AM population. Bacterial resolution is predicted to occur with minor AM depletion (small $\phi$), but the length of time for bacterial loads to completely clear ($\log_{10} P(t) < 0$, where $P(t)$ denotes the solution to Equation 6) increases rapidly as depletion accumulates (increasing $\phi$) and saturates once these cells have declined by $\sim$80\% (Figure 1B).
**Initial Dose Threshold**

Because different levels of AM depletion result in different outcomes, we analyzed our coinfection model using mathematical steady-state and bifurcation analyses (details in the Supplementary Information). This analysis verified the two potential outcomes (stable steady states) as clearance ($P = 0$ CFU) and sustained growth to maximum carrying capacity ($P = K_P = 2.3 \times 10^8$ CFU) (Figure 2A). An intermediate, unstable state given by Equations 7-8 is dependent on the extent of AM depletion and governs which of these outcomes manifests. This state defines an initial dose threshold (Figure 2A) such that bacteria will exhibit a growth phenotype for doses above the threshold and a clearance phenotype for doses below the threshold for a given amount of AM depletion. Further, the rate at which bacteria grow or clear will increase as the distance from the threshold increases. As AMs become more depleted, the dose needed to elicit an infection drops rapidly in a nonlinear manner. Once AMs are reduced by $\sim 80\%$, as indicated by the near zero value of the threshold, any dose will support bacterial growth. This critical level of depletion ($\hat{\phi}_{crit}$) can be analytically calculated (see the Supplementary Information) in terms of the model parameters (Table S1) as

$$\hat{\phi}_{crit} = 1 - \frac{\text{bacterial growth rate}}{\text{bacterial clearance rate by AMs}} = \frac{r}{\gamma_{M_A} M_A} = 79.98\%.$$  

(1)

**Threshold Dynamics During IAV Infection**

Because AM depletion is dynamic during IAV infection$^{18}$ and the initial dose threshold is dependent on AM depletion, the initial dose threshold will change throughout the course of an IAV infection. To determine how the threshold changes with time, we used data on the number of AMs lost at 1, 3, 5, 9, and 11d pii (see Methods) (Figure 2B) as an approximation for the parameter $\phi$ in Equation 6 because our estimated parameter value$^{17}$ matched the empirical value$^{18}$ for a coinfection at 7d pii. We used the mean and the standard deviation of the AM data to obtain an estimated confidence interval for $\phi$ for each time point (Table 1). We then used these values together with the model solution for virus ($V(t)$) at each time point to calculate the overall effect of depletion, defined by $\hat{\phi} = \phi V/(K_P V + V)$ in Equation 6, and the corresponding initial dose threshold value at each coinfection time (Table 1). The resulting threshold drops rapidly between 1d and 3d, reducing the dose necessary for a secondary infection to establish by over 50% (Figure 2B). The threshold decreases by another 50% by 7d pii before increasing to near baseline level at 11d pii.
Bacterial Growth for Doses Below or Above the Threshold

The initial dose threshold indicates that bacteria will clear for doses lower than the threshold and grow for doses higher than the threshold. To test the predicted dynamic threshold, we infected groups of mice first with 50 TCID$_{50}$ PR8 then D39 at 1, 3, 5, 7, 9, or 11d pii at a bacterial dose below or above the predicted threshold value (Table 2). For bacterial infection 7d pii, a dose below the threshold was not examined due to the predicted value being less than 1 CFU. We then quantified bacterial growth at 4h post-bacterial infection (pbi).

When inoculating with a dose below the predicted threshold, we found that bacterial loads at 4h pbi were lower than the inoculum in all mice for all coinfection times tested (Figure 3A). This initial clearance supports our predicted threshold, which suggests that doses below the threshold will result in bacterial clearance. For a coinfection at 1d pii or 3d pii and for the doses used, bacteria were undetectable in 2 out of 5 mice. To investigate whether clearance could also be attained with a dose closer to the threshold value, we inoculated a group of influenza-infected mice with 7.55 × 10$^3$ CFU (compared to 3.55 × 10$^3$ CFU) D39 at 1d pii. At this dose, bacterial loads decreased in all mice but none resolved the infection within 4h pbi. Conversely, we also examined a dose lower than the low dose in Table 2 (1 × 10$^3$ CFU compared to 2.7 × 10$^3$ CFU) for bacterial infection 5d pii and found that the rate of clearance improved and that 1 out of 5 mice resolved the infection.

We next examined bacterial titers at 24h pbi in a separate group of mice using the low doses in Table 2 to determine if titers would continue to decline. For coinfection at 1d and 3d pii, where 40% had resolved the infection within 4h, the same proportion of individuals had undetectable titers at 24h pbi. If bacteria were not controlled within this time frame, then the initial clearance mechanisms were overcome and bacterial loads surpassed those at 4h and, in some cases, the inoculum (Figure 3B). The average rate of growth between 4h and 24h inversely correlates to the average clearance rate between 0h and 4h such that slower clearance supports faster growth (Figure 3C) and less titer heterogeneity (Figure 3B).

When mice were given a dose slightly above the threshold, bacterial loads at 4h pbi remained relatively constant and showed dichotomous behavior for all coinfection timings with 50-60% of individuals partially clearing the inoculum and the remaining 40-50% showed rapid growth (Figure 4A). To find the dose where 100% of infections result in immediate growth and to show that growth increases with the distance from the threshold, we infected groups of mice at 5d pii with doses incrementally higher than in Table 2 (7.0, 8.5, or 10.5 × 10$^3$ CFU) (Figure 4B-D). At 7 × 10$^3$ CFU, some individuals were still able to partially clear...
the inoculum. However, at $8.5 \times 10^3$ CFU and $10.5 \times 10^3$ CFU, bacteria readily grew in all individuals to levels higher than the inoculum. In addition, the strength of this growth increased and the titer heterogeneity decreased as the dose increased.

**Discussion**

Secondary bacterial infections increase the severity of influenza-associated illnesses and the mortality rates during influenza pandemics\textsuperscript{3–6}. However, not every IAV infection results in a bacterial invasion and only a proportion of successful coinfections lead to severe pneumonia\textsuperscript{23,24}. The probability of pneumonia manifesting during influenza is multifactorial and may involve several pathogen and host characteristics, including viral and bacterial strain, dose, and host immune status (reviewed in\textsuperscript{7–13}). Identifying which factors promote coinfection, how each affects the kinetics, the pathogenicity, and the likelihood of bacterial pneumonia following influenza, and how they are interrelated could identify new targets for treating or preventing secondary infections.

Our analysis and experiments pinpoint the way in which bacterial dynamics vary depending on the bacterial dose and the AM population size, and identified the combinations of these two factors that lead to distinct phenotypes. In particular, we showed that an early clearance phenotype exists for doses below an initial dose threshold, a growth phenotype for doses sufficiently above the threshold, and a dichotomous phenotype for doses close to the threshold. The nonlinearity of the relationship between dose and AM depletion is important and non-intuitive, even with our previous knowledge that these two effects are related\textsuperscript{16,20–22} and that they both influence coinfection kinetics\textsuperscript{16–19}. Knowing how the combination of two factors work together and quantifying the conditions that yield each of the outcomes in addition to the trajectory of bacterial titers is crucial to understanding coinfection kinetics. It also allows us to predict the conditions given the behavior and vice versa.

For example, we observed that initial bacterial clearance yields larger heterogeneity in bacterial loads as the infection progresses (Figure 3B). This knowledge has given us insight into the dynamics observed in other datasets that were previously unexplained. We noted two datasets in our earlier work\textsuperscript{17} where bacterial titers split into two groups such that $\sim 50\%$ were at high levels and $\sim 50\%$ were at low levels (Figure S3). In the first dataset (Figure S3A), groups of mice were infected with the same virus (PR8) but with different bacterial doses ($10^2$ or $10^3$ CFU) 7d pii\textsuperscript{17}. For the larger dose ($10^3$ CFU), little heterogeneity was observed in the data and bacterial growth at 4h pbi was at or above the inoculum (Figure S2B). This is consistent with a dose that
is above the threshold. Comparatively, the lower dose (10^2 CFU) resulted in some mice with high titers and some mice with low titers, which indicates a dose that is close to or below the threshold (Figure S3A). In the second dataset (Figure S3B), mice were infected with different viruses (PR8 or PR8-PB1-F2(1918)) and the same bacterial dose (10^3 CFU) 7d piii\textsuperscript{17}. Bacterial titers for a coinfection with the PR8-PB1-F2(1918) virus split into two groups (Figure S3B), which is consistent with an AM:dose ratio closer to the threshold, whereas little heterogeneity was observed for coinfection with the PR8 virus (Figure S2B). We hypothesize that this is due to a lower level of AM depletion caused by the PR8-PB1-F2(1918), which may be connected to the lower viral titer at 7d piii when the bacterial infection was initiated (Figure S3B inset). This is also in accordance with our model prediction that differences in bacterial dynamics were solely due to the antecedent viral infection\textsuperscript{17}.

With only a short window where the surviving AMs are able to control bacterial growth, the opportunity for successful treatment while the immune system has the upper hand may be limited. Our results indicate that one preventative strategy (replenishment of AMs) and one therapeutic strategy (early reduction in bacterial loads), used separately or in combination, could be effective because the ratio of AMs to bacteria is the critical quantity that needs to be increased to abrogate secondary pneumococcal pneumonia. Reducing the transmitted dose is ideal, but this is difficult to control in practice. To prevent a coinfection, the AM population can be partially restored via immune modulators such as granulocyte macrophage colony stimulating factor (GM-CSF)\textsuperscript{18}. In a study where mice were treated with recombinant GM-CSF (rGM-CSF) at -1d and 1d piii, the AM population increased by \~21% 2d after treatment (3d piii) and bacterial clearance in the first 3h after inoculation at this time point improved by \~14%. Further, pneumonia was reduced from 100% to 40% and 2 out of 11 of the treated mice were able to resolve the infection within 3h compared to 0 in the untreated group. These results are consistent with those presented here, where a decrease in dose and thus an increase in the distance from the threshold results in faster clearance and resolution in some mice. Thus, similar outcomes will manifest through therapeutically decreasing AM depletion or decreasing bacterial loads, however the therapeutic requirement may change because of the nonlinearity of the relationship. A more effective therapeutic approach may be to use antibiotics, which limit bacterial replication, either prophylactically or as an early treatment together with rGM-CSF.

The importance of the AM:bacteria ratio suggests that the bacterial dose should be chosen carefully when designing pneumococcal or influenza-pneumococcal infection experiments so that misinterpretation of the results is avoided. For example, if a particular mouse strain has a greater number of AMs at baseline, a larger
dose would be needed to examine a pneumococcal infection because a lower dose would be immediately cleared and the animal could be falsely regarded as protected. Further, sampling time should also be selected cautiously as important dynamics early in infection may be missed. However, based on our results, comparing the bacterial inoculum size to the final size and/or examining the heterogeneity in titers as we did here will help clarify where the experimental conditions are located along the AM:bacteria plane (Figure 2A).

Until recently, it was unknown why the morbidity and mortality from influenza-pneumococcal coinfection is maximal at 7d pii\(^{19}\). Connecting the strength of bacterial growth to the depletion of AMs, which is maximal at 7d pii, could provide the key to understanding coinfection kinetics\(^{17,18}\). Our work here quantitatively couples bacterial dose to these components and demonstrates that at least two simultaneous events (ample depletion combined with sufficient dose) are necessary for a coinfection to establish. Because both AM depletion and the dose effect are dynamic and likely dependent on other factors (e.g., AM depletion changes with viral strain), the probability of both events coinciding may be low. While a different virus strain is unlikely to impact the relationship we established between bacterial dose and AM depletion, strain dependent AM depletion may help to explain why only a proportion of influenza-infected individuals experience complications from secondary bacterial infections and why coinfections are less prevalent during seasonal epidemics\(^{27}\), where infections tend to be less severe compared to pandemic strains and may result in less AM depletion. When seasonal strains are in circulation, transmission of highly concentrated small particles (<10um), which are thought to deposit bacteria more readily into the lower airways (reviewed in\(^{28}\)), may be required. In contrast, less concentrated and/or larger particles may be sufficient to establish a bacterial infection with more virulent strains of IAV.

Extending our results to predict the time scale at which the pneumonia progresses, the intensity of pneumonia, and the probability of survival may be more complicated. Our mathematical model (Equations 2-6) is able to predict bacterial titer kinetics for a coinfection 7d pii and for infectious doses above or close to the initial dose threshold in which rapid growth ensues immediately\(^{17}\). In the case where bacterial loads reach maximum levels (> 10\(^8\) CFU), mortality occurs in 100% of mice within 48-72h pbi. The exact timing is dose-dependent and seems to be related to the rate at which this upper limit is achieved. However, predicting severity, outcome, and bacterial titers for infections with reduced doses, where bacterial titers may increase but remain low, is more challenging. Understanding the inverse relationship between the rates of early clearance (0→4h) and later growth (4→24h) (Figs. 3B-C) does aid our ability to predict the resulting bacterial burden, but more complex and time-dependent dynamics, including inflammation and...
tissue damage, likely contribute to pathogenicity without significantly impacting bacterial loads. Indeed, several other immune responses are elevated, dysfunctional, or otherwise altered during bacterial coinfection after influenza (reviewed in 7–13). For example, subsequent clearance mechanisms, such as neutrophils and macrophages, undergo influenza-induced apoptosis, become dysfunctional, and have reduced chemotactic and phagocytic functions15, 29–33. It is unclear the extent to which the inflated cytokine response (e.g., type I and II interferons (IFN-α, β, γ), TNF-α, IL-1β, IL-6, IL-12, and IL-10)16, 31, 34–43 facilitates these changes. With the resulting heightened state of inflammation and severe lung damage, determining how each of these events is interrelated, dose-dependent, and connected to AM depletion is critical to finding new therapeutic approaches.

Analyzing coinfection kinetics with a mathematical model provides a means to quantify and simultaneously assess multiple infection characteristics. This method has allowed us to make meaningful predictions, even when exact mechanisms were unknown, and design experiments to test these predictions. Carrying out targeted studies based on our analytical results, as done previously18 and here, provides new biological insight and pinpoints improvements that can be made to our analysis. It is only with these improvements that we will be able to further examine the interactions between influenza, pneumococcus, and the host. Determining the circumstances that lead to severe bacterial infections during influenza and quantifying in detail how epidemiological factors (e.g., transmission dose) and host immune status (e.g., AM depletion) work together provides important clinical insight into the threat these pathogens pose to public health. Further establishing how other pathogen (e.g., strain, viral dose) and host (e.g., neutrophils, cytokines) factors are related and contribute to infection probability and severity will aid the development of therapies that prevent or treat these diseases.

**Methods**

**Influenza-Pneumococcal Coinfection Model**

We previously developed a model to describe influenza-pneumococcal coinfection kinetics17. Briefly, the model couples single infection models for influenza virus44 and pneumococcus20 and includes terms that describe their interactions17. Five populations are tracked: susceptible epithelial (“target”) cells (T), two classes of infected cells (I1 and I2), virus (V), and bacteria (P).
\[
\frac{dT}{dt} = -\beta TV \\
\frac{dI_1}{dt} = \beta TV - kI_1 - \mu PI_1 \\
\frac{dI_2}{dt} = kI_1 - \delta I_2 - \mu PI_2 \\
\frac{dV}{dt} = pI_2(1 + \hat{a}(P)) - cV \\
\frac{dP}{dt} = rP \left(1 - \frac{P}{K_P(1 + \psi V)}\right) - \gamma_{MA}f(P, M_{A})M_{A}P(1 - \hat{\phi}(V))
\]

Target cells become infected with virus at rate $\beta V$ per cell. Once infected, these cells enter an eclipse phase ($I_1$) at rate $k$ per cell before transitioning to produce virus at rate $p$ per cell ($I_2$). Virus is cleared at rate $c$ and virus-producing infected cells ($I_2$) are cleared at rate $\delta$. Bacteria increases infected cell death at rate $\mu P$ and increases viral release from infected epithelial cells by an additional factor of $\hat{a}(P) = aP^c$. Bacteria replicate logistically with maximum rate $r$ and tissue carrying capacity of $K_P$. Alveolar macrophages ($M_A$) phagocytose bacteria at rate $\gamma_{MA}f(P, M_{A})$ per cell. This rate decreases as the number of pneumococci increase according to $f(P, M_{A}) = n^2M_{A}/(P^2 + n^2M_{A})$, where each AM is only able to phagocytose a maximum of $n$ bacteria. Virus further decreases this clearance rate ($\hat{\phi}(V) = \phi V/(K_{PV} + V)$) and increases the bacterial carrying capacity ($\psi V$). The model schematic is shown in Figure S1. Model fits to lung viral and bacterial titers from groups of mice infected 7d after influenza A/Puerto Rico/8/34 (H1N1) (PR8) with PBS (black line) or pneumococcal strain D39 (red line) are shown in Figure S2 and the model parameters are in Table S1\textsuperscript{17}.

**Derivation of the Initial Dose Threshold**

We used standard steady state and bifurcation analyses on the coinfection model to derive the initial dose threshold. Setting Equations 2-6 equal to zero and solving yields 4 equilibria: a disease-free state $(0,0,0,0,0)$ and three states with non-zero bacterial levels $(0,0,0,P^*)$. The non-zero $P^*$ values satisfy $P^3 + BP^2 + CP + D = 0$ and are defined by

\[
P^*_2 = M_1 + M_2 - \frac{B}{3} \\
P^*_3,4 = -\frac{1}{2}(M_1 + M_2) \pm \frac{\sqrt{3}}{2}(M_1 - M_2)i - \frac{B}{3}
\]
where

\[ M_{1,2} = \sqrt[3]{-\frac{q}{2} + \sqrt{\frac{u^3}{27} + \frac{q^2}{4}}} \]

\[ u = C - \frac{B^2}{3} \]

\[ q = D + \frac{2B^3 - 9BC}{27} \]

\[ B = -KP \]

\[ C = n^2 M_A \]

\[ D = n^2 M_A KP \left( \frac{\gamma M_A M_A}{r} - 1 \right) \]

For the parameter values in Table S1, three real solutions exist because \( \frac{\gamma M_A M_A}{r} > 1 \) and the discriminant, \( \Delta = B^2C^2 - 4B^3D - 4C^3 + 18BCD - 27D^2 \), is positive. One of the equilibria \( P^*_4 \) is excluded because it is negative and not biologically relevant. Both \( P^*_1 = 0 \) CFU and \( P^*_2 = KP \) CFU are stable while \( P^*_3 = 9993.5 \) CFU is unstable. The unstable state \( (P^*_3) \) indicates a threshold such that bacterial growth reaches the maximum carrying capacity when \( P(t) > P^*_3 \), while clearance occurs for \( P(t) < P^*_3 \). Because \( V^* = 0 \), these equilibria are equivalent to those in the single infection models and \( P^*_3 = 9993.5 \) CFU corresponds to the initial dose threshold in the absence of an antecedent viral infection\(^\text{20}\).

However, virus is non-zero \((V(t) > 0)\) at the initiation of the bacterial infection and the model solution, which is influenced by changes in the coinfection parameters\(^\text{17}\), is then perturbed away from its steady state. Re-solving for the equilibria yields non-zero state defined by Equation (7) but with

\[ D_\phi = n^2 M_A K_P \left( \frac{\gamma M_A M_A}{r} (1 - \phi) - 1 \right) . \]  

(8)

The two stable states remain unchanged \((P^*_1 = 0 \text{ CFU and } P^*_2 = KP \text{ CFU})\). The unstable, positive root, \( P^*_3 \), is dependent on \( \hat{\phi} \) and real if \( \frac{\gamma M_A M_A}{r} (1 - \hat{\phi}) > 1 \) (i.e., \( D_\phi > 0 \)). However, the root turns complex when \( \frac{\gamma M_A M_A}{r} (1 - \hat{\phi}) < 1 \) (i.e., \( D_\phi < 0 \)). The point where \( P^*_3 \) switches from being a real root to a complex root with real part less than 1 is found by solving \( D_\phi = 0 \) for \( \hat{\phi} \), which gives the critical value in Equation 1. The equilibria are plotted against \( \hat{\phi} \) in Figure 2A.
Mice

Adult (6 week old) female BALB/cJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in groups of 5 mice in high-temperature 31.2cm × 23.5cm × 15.2cm polycarbonate cages with isolator lids. Rooms used for housing mice were maintained on a 12:12-hour light:dark cycle at 22 ± 2°C with 50% humidity in the biosafety level 2 facility at St. Jude Children’s Research Hospital (Memphis, TN). Prior to inclusion in the experiments, mice were allowed at least 7 days to acclimate to the animal facility such that they were 7 weeks old at the time of infection. Laboratory Autoclavable Rodent Diet (PMI Nutrition International, St. Louis, MO) and autoclaved water were available ad libitum. All experiments were performed under an approved protocol and in accordance with the guidelines set forth by the Animal Care and Use Committee at St. Jude Children’s Research Hospital.

Infectious Agents

All experiments were done using the mouse adapted influenza A/Puerto Rico/8/34 (H1N1) (PR8) and type 2 pneumococcal strain D39 that was transformed with the lux operon (Xenogen) to make it bioluminescent.

Infection Experiments

The viral infectious dose (TCID\textsubscript{50}) was determined by interpolation using the method of Reed and Muench using serial dilutions of virus on Madin-Darby canine kidney (MDCK) cells. The bacterial infectious dose (CFU) were counted for serial dilutions of bacteria on tryptic soy-agar plates supplemented with 3% (vol/vol) sheep erythrocytes. Inocula were diluted in sterile PBS and administered intranasally to groups of 5 mice lightly anesthetized with 2.5% inhaled isoflurane (Baxter, Deerfield, IL) in a total volume of 100ul (50ul per nostril). Mice were inoculated with 50 TCID\textsubscript{50} PR8 at day 0 and with D39 at 1, 3, 5, 7, 9, or 11d pii at the doses listed in Table 2. Mice were weighed at the onset of infection and each subsequent day for illness and mortality. Mice were euthanized if they became moribund or lost 30% of their starting body weight.

Lung Titers

Mice were euthanized by CO\textsubscript{2} asphyxiation. Lungs were aseptically harvested, washed three times in PBS, and placed in 500ul PBS. Lungs were mechanically homogenized using the Ultra-Turrax T8 homogenizer (IKA-werke, Staufen, Germany). Lung homogenates were pelleted at 10,000 rpm for 5 minutes and the supernatants were used to determine the bacterial titer for each set of lungs using serial dilutions on tryptic soy-agar plates supplemented with 3% (vol/vol) sheep erythrocytes.
Alveolar Macrophage Data

The AM data used in this study is from Ref.18. In brief, groups of five 6-8 week old female BALB/cJ mice (Jackson Laboratory, Bar Harbor, ME) were lightly anesthetized with 2.5% inhaled isoflurane and 100ul of 10mM PKH26-phagocytic cell labeling dye was intranasally administered 5 days prior to infection. Mice were then infected intranasally with the PR8 virus at a dose of 25TCID\textsubscript{50} in 100ul. Note that the virus used in this study was from a different stock than in the current study, however the appropriate dose was chosen to result in \(~10\%\) weight loss in all experiments. After euthanasia by CO\textsubscript{2} inhalation, bronchoalveolar lavage fluid (BALF) was collected and whole lungs post-lavage were harvested and physically homogenized by syringe plunger against a 40um cell strainer. Cell suspensions were centrifuged and enumerated by trypan blue exclusion, and the cell pellets were incubated in antibodies against CD11c, F4/80, Ly6G, Ly6C, and CD11b. Flow cytometry was performed and the data were analyzed using FlowJo 8.8.6. Viable cells were gated from a forward scatter/side scatter plot. Neutrophils (CD11b\textsuperscript{hi}Ly6G\textsuperscript{hi}) were then gated out and AMs were gated as F4/80\textsuperscript{hi}CD11c\textsuperscript{hi}CD11b\textsuperscript{−}. Average mean fluorescence intensity of PKH26-PCL was measured to ensure accuracy of the gating strategy. Here, we use the AM data from the lung and obtain the percentage of AMs depleted by normalizing the absolute number of AMs at each time point to the average number of AMs in a naive mouse.

Author Contributions

A.M.S conceived the concept, planned the experiments, and wrote the manuscript. A.M.S. and A.P.S. performed the experiments. A.M.S. analyzed the model and data.

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Figures

Figure 1. Simulation of the coinfection model for various levels of AM depletion. (A) Simulation of the coinfection model (Equations 2-6) for infection using the parameter values in Table S1 that were optimized for an infection with $10^2$ TCID$_{50}$ PR8 followed 7d later by $10^3$ CFU D39 and values of AM depletion ($\phi$) ranging from no impairment ($\phi = 0$), which results in immediate clearance, to 100% impairment ($\phi = 1$), which results in immediate growth to the maximum carrying capacity. (B) The number of days until complete bacterial clearance ($\log_{10} P(t) < 0$, where $P(t)$ is the solution to Equation 6) occurs for AM depletion between 0% and 100%.
Figure 2. Initial dose threshold dependence on AM depletion. (A) Bacterial outcomes (steady states) for the coinfection model for different values of AM depletion ($\hat{\phi}$). Two possible outcomes exist, clearance (0 CFU) or growth to maximum carrying capacity ($K_p = 2.3 \times 10^8$ CFU), and are indicated by solid black lines. The intermediate state (dashed blue line, defined by Equations 7-8) is the initial dose threshold that dictates if bacterial loads will decline (doses below the threshold) or if they will increase (doses above the threshold). (B) The predicted threshold (black line, calculation in Table 1) over the course of an IAV infection given the amount of AM depletion (magenta bars).
Figure 3. Bacterial growth for doses below the threshold. (A) Percent bacterial growth after 4h compared to the inoculum for PR8 infection followed by D39 at 1, 3, 5, 9, or 11d pii at doses below the threshold (Table 2). Colored bars are the averages and each black dot represents an individual mouse (5 mice/group). (B) Bacterial loads (CFU) at 4h and 24h for the indicated coinfection time. The red line indicates the inoculum size. (C) Average log$_{10}$ rate of bacterial growth from 0h to 4h and from 4h to 24h for coinfection at 1, 3, 5, 9 or 11d post-influenza at the indicated coinfection time.
Figure 4. Bacterial growth with doses above the threshold. Percent bacterial growth after 4h compared to the inoculum for PR8 infection followed by D39 at (A) 1, 3, 5, 7, 9, or 11d pii at doses above than the threshold (Table 2) or at (B) 5d pii at the indicated dose with the corresponding (C) location relative to the threshold and (D) average log₁₀ bacterial growth rate from 0h to 4h. Colored bars are the averages and each black dot represents an individual mouse (5 mice/group).
Table 1. Calculation of the estimated initial dose threshold. The threshold (Equations 7-8) is calculated using the overall AM depletion estimate \( \hat{\phi} = \phi V(t) / (K_p V(t) + V(t)) \), where \( \phi \) is estimated by the percent AM depletion (Figure 2B) and \( V(t) \) is the coinfection model solution for virus.

| Coinfection Days | Coinfection Percent AM Depletion | \( \log V(t) \) | Virus \( \times 10^3 \) (CFU) | Percent Overall AM Depletion | Initial Dose Threshold \( \times 10^3 \) (CFU) |
|------------------|----------------------------------|----------------|--------------------------|-----------------------------|----------------------------------|
| 1                | 21.8 [10.2, 33.5]                | \( \log_{10} 2.2 \) | 9.89 [9.83, 9.94]         | 3.50                        | 10.7                             |
| 3                | 64.6 [51.4, 77.8]                | \( \log_{10} 6.7 \) | 6.46 [2.29, 6.49]         | 2.75                        | 9.40                             |
| 5                | 65.4 [60.9, 70.0]                | \( \log_{10} 6.1 \) | 65.3 [3.65, 4.91]         | 2.70                        | 5.90                             |
| 7                | 92.7 [83.9, 100]                 | \( \log_{10} 5.3 \) | 91.8 [4.39, 7.43]         | 5.2 \times 10^{-5}          | 3.69                             |
| 9                | 58.7 [40.3, 71.1]                | \( \log_{10} 4.5 \) | 52.9 [0.05, 3.17]         | 5.91                        | 3.90                             |
| 11               | 27.5 [23.3, 31.7]                | \( \log_{10} 3.7 \) | 20.9 [0.05, 3.17]         | 2.2 \times 10^{-5}          | 10.0                             |

Table 2. Bacterial doses used to test the predicted initial dose threshold.

| Coinfection Days | Threshold Dose Below Threshold \( \times 10^3 \) (CFU) | Dose Above Threshold \( \times 10^3 \) (CFU) |
|------------------|--------------------------------------------------------|------------------------------------------|
| 1                | 9.89                                                   | 3.50                                     |
| 3                | 6.46                                                   | 4.39                                     |
| 5                | 65.3                                                   | 6.46                                     |
| 7                | 91.8                                                   | 4.39                                     |
| 9                | 52.9                                                   | 4.39                                     |
| 11               | 20.9                                                   | 4.39                                     |
Supplementary Information

A Critical, Nonlinear Threshold Dictates Bacterial Invasion and Initial Kinetics During Influenza
Amber M. Smith\(^1\),*, Amanda P. Smith\(^1\)

\(^1\)Department of Infectious Diseases, St. Jude Children’s Research Hospital, Memphis, TN 38105, USA
*Email: amber.smith@stjude.org

Influenza-Pneumococcal Coinfection Model Schematic, Fits, and Parameters

The coinfection model schematic is shown in Figure S1. Model fits to lung viral and bacterial titers from groups of mice infected 7d after influenza A/Puerto Rico/8/34 (H1N1) (PR8) with PBS (black line) or pneumococcal strain D39 (red line) are shown in Figure S2 and the model parameters are in Table S1 (Smith et al., 2013).

**Figure S1: Schematic of the coinfection model (Smith et al., 2013).** Target cells (\(T\)) are infected with virus (\(V\)) at rate \(\beta V\). Infected cells enter an eclipse phase (\(I_1\)) and transition to producing virus (\(I_2\)) at rate \(k\). Productive infected cells (\(I_2\)) produce virus at rate \(p\), which is increased by \(aPz\) when bacteria are present. Infected cells are removed at rate \(\delta\) and killed by bacteria at rate \(\mu P\). Bacteria (\(P\)) replicate logistically with maximal rate \(r\) and carrying capacity \(K_P\), which is increased by \(\psi V\) when virus is present. Bacteria are phagocytosed by alveolar macrophages (\(M_A\)) at rate \(\gamma_{M}(P,M_A)\), which is decreased by \((\phi V/(K_{PV} + V))\) when virus is present.
Datasets With Heterogeneous Bacterial Titers

Our results suggest that heterogenous bacterial titers indicate an AM:dose ratio that lies close to or below the initial dose threshold. This heterogeneity lessens with increased dose or AM depletion. Two datasets (Figure S3) in our previous work exhibited heterogenous behavior (Smith et al., 2013). In the first dataset (Figure S3A), mice were infected with $10^2$ TCID$_{50}$ PR8 followed by $10^3$ CFU D39 7d pii. At both 24h and 48h pbi, bacterial titers split into two groups where ~50% at high levels and ~50% at low levels. Comparing these data with data from mice infected with 1 log$_{10}$ higher bacteria ($10^3$ CFU, Figure S2B) shows that decreasing the dose increases the heterogeneity. These data suggest that the lower dose ($10^2$ CFU) is close to the initial dose threshold and that the higher dose ($10^3$ CFU) is sufficiently above the threshold.

In the second dataset (Figure S3B), mice were infected with $10^2$ TCID$_{50}$ PR8-PB1-F2 (1918) followed by $10^3$ CFU D39 7d pii. At 8h and 16h pbi, some mice begin to clear the inoculum while others have bacterial titers that are 1-2 log$_{10}$ higher than the inoculum. At 16h pbi, two distinct groups emerge such that 50% have low bacterial loads and 50% have high bacterial loads. Comparing these data with data from mice infected with the PR8 virus and the same bacterial dose ($10^3$ CFU, Figure S2B) shows that altering the viral strain can change the heterogeneity and trajectory of bacterial titers. Our results here suggest that infection with the PR8-PB1-F2(1918) virus yields an AM:dose ratio closer to the threshold than the PR8 virus. Thus, we hypothesize that infection with PR8-PB1-F2(1918) results in less AM depletion. Although the extent of AM depletion is unknown for the PR8-PB1-F2(1918) virus, a lower viral titer 7d pii compared to infection with PR8 was observed (Figure S3B inset) (Smith et al., 2011a, 2013) and may indicate reduced AM depletion.
Table S1: Parameter values of the influenza model (Smith et al., 2011a), pneumococcal model (Smith et al., 2011b), and coinfection model (Equations (2)-(6) (main text)) (Smith et al., 2013).

| Parameter | Description                  | Value               | Units               |
|-----------|------------------------------|---------------------|---------------------|
| \( \beta \) | Virus infectivity            | \( 2.8 \times 10^{-6} \) (TCID\(_{50}\)) day\(^{-1} \) |
| \( k \)   | Eclipse phase                | 4.0                 | day\(^{-1} \)      |
| \( \delta \) | Infected cell death         | 0.89                | day\(^{-1} \)      |
| \( p \)   | Virus production             | 25.1                | (TCID\(_{50}\)) day\(^{-1} \) |
| \( c \)   | Virus clearance              | 28.4                | day\(^{-1} \)      |
| \( T(0) \) | Initial uninfected cells    | \( 10^7 \)          | cells               |
| \( I_1(0) \) | Initial infected cells     | 0                   | cells               |
| \( I_2(0) \) | Initial infected cells     | 0                   | cells               |
| \( V(0) \) | Initial virus               | 2.0                 | TCID\(_{50}\)      |
| \( r \)   | Bacterial growth rate       | 27.0                | day\(^{-1} \)      |
| \( K_P \) | Carrying capacity           | \( 2.3 \times 10^8 \) | CFU                |
| \( \gamma_{MA} \) | Phagocytosis rate       | \( 1.35 \times 10^{-4} \) | cell\(^{-1}\) day\(^{-1} \) |
| \( n \)   | Maximum bacteria per AM     | 5.0                 | (CFU)cell\(^{-1} \) |
| \( M^*_A \) | Number of AMs               | \( 10^6 \)          | cells               |
| \( P_0(7) \) | Initial bacteria at day 7 post-influenza | \( 10^3 \)          | CFU                |

| Parameter | Description                  | Value               | Units               |
|-----------|------------------------------|---------------------|---------------------|
| \( \phi \) | Decrease in phagocytosis rate | 0.87                |                     |
| \( K_{PV} \) | Half-saturation constant    | \( 1.8 \times 10^3 \) (TCID\(_{50}\)) |
| \( a \)   | Increase in virion production/release | \( 1.2 \times 10^{-3} \) (CFU)\(^{-2} \) |
| \( z \)   | Nonlinearity of virion production/release | 0.50                 |                     |
| \( \psi \) | Increase in carrying capacity | \( 1.2 \times 10^{-8} \) (TCID\(_{50}\))\(^{-1} \) |
| \( \mu \) | Toxic death of infected cells | \( 5.2 \times 10^{-10} \) (CFU)\(^{-1} \) |
Figure S3: Dichotomous Bacterial Titers. (A) Bacterial titers for infection with $10^2$ TCID$_{50}$ PR8 followed by $10^2$ CFU D39 7d ppi. (B) Bacterial titers for infection with $10^2$ TCID$_{50}$ PR8-PB1-F2(1918) followed by $10^3$ CFU D39 7d ppi. Inset: Viral titers 7d ppi for infection with $10^2$ TCID$_{50}$ PR8 or PR8-PB1-F2(1918).

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