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The inflammatory response to influenza A virus (H1N1): an experimental and mathematical study

Ian Price^{1,*}, Ericka D. Mochan-Keef^{2,*}, David Swigon^{1,2}, G. Bard Ermentrout^{1,2}, Sarah Lukens^{1,3,5}, Franklin R. Toapanta^{4,6}, Ted M. Ross^{4,7}, and Gilles Clermont³

¹Department of Mathematics, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

²Department of Computational and Systems Biology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

³Department of Critical Care Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

⁴Center for Vaccine Research, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

Abstract

Mortality from influenza infections continues as a global public health issue, with the host inflammatory response contributing to fatalities related to the primary infection. Based on Ordinary Differential Equation (ODE) formalism, a computational model was developed for the in-host response to influenza A virus, merging inflammatory, innate, adaptive and humoral responses to virus and linking severity of infection, the inflammatory response, and mortality. The model was calibrated using dense cytokine and cell data from adult BALB/c mice infected with the H1N1 influenza strain A/PR/8/34 in sublethal and lethal doses. Uncertainty in model parameters and disease mechanisms was quantified using Bayesian inference and ensemble model methodology that generates probabilistic predictions of survival, defined as viral clearance and recovery of the respiratory epithelium. The ensemble recovers the expected relationship between magnitude of viral exposure and the duration of survival, and suggests mechanisms primarily responsible for survival, which could guide the development of immunomodulatory interventions as adjuncts to current anti-viral treatments. The model is employed to extrapolate from available data survival curves for the population and their dependence on initial viral aliquot. In addition, the model allows us to illustrate the positive effect of controlled inflammation on influenza survival.

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Corresponding Author: Gilles Clermont, MD, University of Pittsburgh School of Medicine, Scaife 602 A, 3550 Terrace St, Pittsburgh, PA 15261, cler@pitt.edu.

⁵Current address: University of Notre Dame, South Bend, Indiana, USA

⁶Current address: Center for Vaccine Development, University of Maryland, Baltimore, USA

⁷Current address: Vaccine and Gene Therapy Institute, Port St. Lucie, FL, USA

*These authors contributed equally to this work

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Introduction

Influenza A virus (IAV) continues as a very serious public health problem in both its seasonal and pandemic expressions. Although mortality from seasonal influenza is often associated with secondary bacterial infection, emerging variants may trigger disease where the acute inflammatory response plays a major role in pathophysiology, either by triggering conditions particularly conducive for early severe secondary infection, or by triggering a cytokine storm with an ensuing organ dysfunction reminiscent of severe sepsis [1–6]. The 1918–19 H1N1 influenza virus pandemic infected an estimated one third of world population and caused the deaths of 50–100 million people [7]. Analysis of postmortem lung samples from 1918-H1N1 infected individuals showed severe histopathological changes indicative of bacterial pneumonia, suggesting that secondary infections were the primary cause of death [8]. In recent strains associated with severe disease, contrary to the 1918–19 H1N1 strain, inflammation and virus-induced cytokine dysregulation appears to be the major contributor to morbidity during influenza infections, although secondary infections were also prevalent [9]. Although many are not readily transmissible to and between humans, natural mutation could lead to enhanced human-to-human transmission of these inflammation-producing strains. Influenza-induced inflammation has the potential to cause aseptic death due to self-sustained damage of the lungs [9–11] and other tissue [12]. To better capture the role of inflammation induced by IAV pathophysiology, data obtained from mice infected by IAV is combined with mathematical modeling of the in-host immune response to this infection.

Many mathematical models of the immune response to IAV infection have been developed with varying degrees of detail [13–31]. Depending on the scope of the research and questions addressed by the studies, existing models range from those describing just the viral trajectory and epithelial cell dynamics [17,18], to those including one specific aspect of the immune response, such as type I interferons [32] or T cells [19,20], to those including several arms of immune response [21,22,27,33]. Likewise, systemic inflammation has been studied using mathematical modeling, also with varying degree of detail, ranging from conceptual models, to more detailed models leveraging rich biological datasets linking cytokine expression and organ dysfunction [1,5,10,34,35].

The model of in-host response to influenza presented in this paper is a comprehensive model that includes all major cellular and molecular components of immune response and inflammation. The model accounts for the classical mechanisms of antiviral immune response as represented by several arms of immunity described in the literature, including the innate, adaptive, and humoral (antibody) responses [36]. As a novel component, the model also includes the basic pathways of systemic inflammation, comprised of macrophages, pro-inflammatory and anti-inflammatory cytokines, chemokines attracting neutrophils, and toxins utilized by cytokines.

The model presented herein provides several new contributions to the literature that include (1) merging acute inflammation as part on the innate immune response to influenza A infection, (2) delving into the relationship between systemic inflammation and morbidity, (3) linking severity of infection to survivorship, and (4) inferring basic mechanisms

responsible for an adverse outcome to influenza A infection. In addition, the model is calibrated using Bayesian inference methods using a data rich murine study of influenza response [37], augmented by unpublished data (lethal infections) by the same group. The current model aims at describing murine influenza infection where the primary mode of death is overwhelming viral infection and inflammatory response, and does not consider secondary infections as contributing to illness severity. The model does not describe the inflammatory response associated with secondary infections in humans. The calibrated model could be used to evaluate the impact of interventions targeting different aspects of the innate or specific immune responses, or to investigate time-dependent vulnerability to secondary infections.

Materials and Methods

Experimental data

The full experimental procedures for sublethal infections have been previously reported in Toapanta *et al* [37]. Briefly, female BALB/c mice (Harlan-Sprague, Indianapolis, IN, USA), aged 12–16 weeks, were inoculated intranasally with a sublethal (50 plaque forming units (pfu)/ml,) or lethal (500 pfu/ml) dose of influenza A/8/PR/34 (H1N1) and observed for a maximum of 19 days. Animals were treated according to the guidelines of the IACUC of the University of Pittsburgh. The following data were collected at baseline and at several times following inoculation: viral concentration in lungs (plaque assay), multiplex assays (LuminexTM) and ELISA of cytokines in supernatants of lung homogenates, flow cytometry of lung mononuclear cells to determine cell counts, immunophenotype, and antigen specific CD8⁺ cells, weight and observed markers, and day of death for animals dying prior to planned sacrifice. A minimum of three animals were sacrificed at each time point. For mice given a sublethal inoculum, experimental data were collected for 16 of the 20 variables comprising the mathematical model over a course of 11 days. For mice given a lethal inoculum, data were collected for 13 of the 20 variables over a course of 7 days. Data for activated macrophage counts, antibody counts, and antigen presenting cell counts were reported only for the sublethal inoculum. There were no surviving subjects beyond day 7 for the lethal cohort. The data are shown in Table 1.

The following subset of cytokines in supernatants of lung homogenates was used in calibrating the mathematical model (variable name in parentheses): (T) TNF, (L) IL-10, (C) MCP and MIP-1B, (F) IFN- α and IFN- β , (W) IL-12, and (G) IFN- γ . Each cytokine multiplex assay (LuminexTM) sample was typically the average of duplicate fluorescence measurements, reported as a fluid concentration (pg/ml) based on a standard curve. Cell counts and flow cytometry data were combined to generate concentrations of the following cells in lung tissue. Where applicable, the percentage of cells with activation markers ($CD40^+$ or $CD69^+$) is considered for model parameter calibration. (M) Macrophages— $CD11c^- CD11b^+ Gr-1^{dim} F4/80^- CD40^+$, (N) Neutrophils— $Gr-1^{+(high)} CD11b^{(high)}$, (K) Natural Killer Cells— $CD49b(DX5)^+ CD69^+$, (P) Antigen Presenting Cells— $CD11c^+ CD11b^+ Gr-1^{dim} CD40^+$, (B) Mature B cells— $CD19^+ CD69^+$, (E) $CD8^+$ T cells— $CD3^+ CD8^+ CD69^+$, (O) $CD4^+$ T cells— $CD3^+ CD4^+ CD69^+$. $CD40^+$ cell marker data were not available in the lethal cohort – this includes macrophages and antigen presenting cells.

Mathematical model development

The present model of immune response to influenza A virus improves and extends the model presented in Hancioglu et al. [22]. The model now includes an inflammation component represented by macrophages, neutrophils, pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines. This inflammatory component contributes to defense against infection, yet also may injure epithelial tissue [11]. The target epithelial cell count for the mice is approximated as 2.5×10^5 cells. Cellular products from damaged epithelial cells, namely damage-associated molecular patterns molecules (DAMPs) further stimulate the inflammatory pathway, so that inflammation-induced damage can potentially enter a run-away positive feedback loop. If the viral infection resolves but inflammation does not, aseptic death can occur as a result.

Figure 1 depicts a schematic diagram of the interactions between model variables, and major regulatory pathways of the system. Briefly, the virus (V) infects target epithelial cells (H), leading to a population of infected cells (I) that in turn produce virus. Dead cells (D) and virus stimulate antigen presenting cells (P), which activate the immune response. Infected cells and antigen presenting cells produce type I interferons (F) which decreases production of the virus. Antigen presenting cells also stimulate production of effector $CD8^+$ T cells (E) which destroy infected cells. Together with helper $CD4^+$ T cells (O) and immune mediators, antigen presenting cells stimulate B cells (B) which mature and produce antibodies (A) that neutralize the virus.

An important and novel feature of our model is the inclusion of the inflammatory component of the immune response. The virus, dead cells, and TNF stimulate the inflammatory response through pro-inflammatory signals (Σ_1 and Σ_2). The pro-inflammatory signals stimulate production of cytokines and chemokines (C) that chemo-attract natural killer cells (K), which kill infected cells. The pro-inflammatory signals also trigger an innate immune response that stimulates an influx of neutrophils (N) which, in the process of producing reactive oxygen and nitrogen species (free radical; X), causes increased cell-death of infected and uninfected cells, ultimately decreasing the production of virus. The system of differential equations implementing these interactions in a dynamical model is presented in Supplemental Materials.

Model parameters are assumed to be constrained within ranges determined by analysis of literature or previous modeling efforts (see Supplement 3). In addition, three heuristic requirements impose further constraints on admissible model parameters. The requirements are that: (1) small perturbations from baseline (i.e. inocula smaller than one viable virion) will always heal; (2) after sublethal inocula viral titers, cytokine levels and the epithelial cell population all return to baseline within 15 days; (3) the target epithelial cell population vanishes after lethal inocula. The study defines time of death in this model as the point when epithelial cell count reaches less than 10% of the baseline value [21], although the precise value of the threshold does not alter asymptotic model trajectories.

Uncertainty analysis: Metropolis-Hastings Monte Carlo sampling

For each analyte and at each time point, the data available to calibrate the mathematical model are comprised of values obtained from three to six animals sacrificed at each time point and then averaged. Bayesian inference and ensemble model techniques [38–43], are used to compute a sample of the posterior distribution of parameter sets as a way of quantifying the uncertainty in the estimation of parameters. Each parameter set in the sample provides a parametrization of the system of differential equations that yields a potential predicted trajectory of analytes that is sufficiently close to experimental data and obeys the heuristics described above. The likelihood of occurrence of the parameter set in the distribution (or a sample) equals to the likelihood that the corresponding trajectory has contributed to the observed data. The *ensemble model* is defined to be the association of first a system of model equations and the sample distribution of parameter sets. By specifying a distribution of parameters, rather than the usual unique parameter set, we quantify the uncertainty in determination of parameter values. By sampling trajectories from this distribution we quantify the uncertainty in model predictions. More details on the computations of this distribution are found in Supplemental Materials.

Results

Marginal distributions of model parameters

The Metropolis-Hastings Monte Carlo sampling procedure simultaneously trains to two sets of data corresponding to two distinct inocula, 50 and 500 pfu/ml and produces a single ensemble of parameter sets that satisfy *both sets of data* and represents a sample of the posterior distribution. Histograms for full marginals of the posterior distribution of all 94 parameters are shown in Supplementary Figure 1. The bounds on each subplot represent the upper and lower bounds of the parameter as used in the MHMC procedure. These bounds are given explicitly in the table in Supplement 3.

Ensemble responses to viral infection

Graphs of the ensemble trajectories are shown for sublethal and lethal regimes, in Figure 2 left and right panels, respectively, for selected model variables: target epithelial cells (H), TNF (T), CD8⁺ T cells (E), virus (V), IL-10 (L), type II interferon (G), infected cells (I), antibodies (A), and type I interferon (F). Trajectories for all variables can be found in Supplementary Figures. The graphs show, at each time point, the median value over all trajectories of the ensemble as a solid black line, as well as the interquartile ranges with 25 – 75% of the trajectories shown in dark gray and 5 – 95% shown in light gray. Infection occurs at day 0. Where data are available, the mean and standard error are graphed with the trajectories.

Ensemble response to a sublethal challenge—In the sublethal regime (Figure 2, left panels), the ensemble predictions of most measured variables correspond well with experimental observations. The virus level rises and falls in the same time-frame as the data, and resolves between days 7 and 9. The peak of the simulated ensemble exceeds the peak anticipated by the data by almost a full log scale; this was accepted due to the large range given by the data. The model fits the data well for both pro-inflammatory (TNF) and anti-

inflammatory (IL-10) cytokines. The data show TNF levels begin at zero and start to rise immediately after infection. The ensemble TNF trajectory continues to rise until day 6, and then returns to baseline. The TNF data initially rise significantly before continuing to rise between days 3 and 5. The model hypothesis is that the first phase of this response is caused by sentry alveolar macrophages to viral particles, and the later rise is part of a more complex innate immune response. After the peak of TNF response, trajectories fall slightly faster than data. There are probably biological sources of TNF not explicitly modeled by our system that would have contributed to a greater production rate. The sublethal IL-10 trajectory fits the rise and fall of the data, with the most variation seen in the initial level of IL-10 present in the system. Antibody titers are low for the first five days of infection, until sufficient antigen presentation occurs, again in accord with the data. The population of CD8⁺ T cells rises between days 2 and 5, and, as exactly as the data suggest, does not fall during the experimental period. Adaptive immune system components remain engaged for weeks after the initial infection.

The inferred target cell trajectories show a decrease of 60% due to the infection, with the minimum between days 5 and 6, after which it returns to baseline. Importantly, the population of target cells does not approach zero at its minimum, implying that target limitation does not play a large role in the resolution of the infection. Some trajectories, notably activated macrophages (M) and epithelial damage (DH) are not well captured by the model (Supplemental Figure 3), suggesting that the immunophenotype we selected for active macrophages may not be accurate, and that using animals weight as a proxy for epithelial damage may not be appropriate.

Also of note, while the range of responses during the decline of the target cell population is tight, the recovery to baseline shows a wider range of behavior. The inferred infected cell trajectory stays close to zero until the end of day 2, when the infected cell population continues to rise until it peaks between days 3 and 4. The ensemble shows a large variation in the peak, from 10% to 20% of the epithelial cells being simultaneously live infected cells. However, neither the viral trajectory nor the target cell trajectory shows the same variation, suggesting variation in the response of the population as a whole to virus.

Ensemble response to a lethal challenge—In the lethal regime (Figure 2, right panels), the ensemble predictions agree with experimental observations for the most part, with some exceptions. Viral trajectories capture many qualitative features of the data, rising to peak on day 3 and remaining elevated until the end of the experiment at day 7. The viral trajectory does not capture the initial decline in virus seen on day 1. The data likely implies an eclipse period in virus activity which is not included in the model dynamics.

The trajectories for TNF, CD8⁺ T cells, and type II interferon generally follow the time series data well. IL-10 trajectories rise more quickly than the data. The data do not show a significant increase until day 7, unlike the sublethal case. A plausible explanation is an increase in receptors that bind to IL-10 in response to high levels of virus rather than a decline in anti-inflammatory cytokine production; however, it is not clear which cell types would be responsible. Type I interferon data peak on day 3, although the simulations underestimate this data point and continue to rise. Interferon data peaks earlier after lethal

infection than sublethal infection, with a very large standard error. The data fitting methods emphasize data with tight standard errors, making this feature difficult to capture.

The inferred target cell trajectories show a monotonically declining number of cells that falls below 10% between days 4 and 6; a loss of 90% of epithelial tissue represents here a high likelihood of respiratory failure. Lethal infected cells show a higher peak at an earlier time and a generally wider range of response than seen in the sublethal ensemble; target limitation prevents the infected cell population from remaining elevated. As infected cells apoptose without replacement, the infected cell population falls. There was no data for antibodies titers in lethal infection. Inferred antibody trajectories show no significant rise, as more virus is produced than the humoral immune system can neutralize. The model does not capture IL-10 and type I interferon trajectories well, possibly due to its inability to adequately characterize the (unmeasured) activated macrophage trajectory (Supplemental Figure 4).

Overall, we see some interesting trends in the data between the sublethal and lethal infections. The peak level of the virus is about three times higher in the lethal condition than in the sublethal. We also see higher peak values in the cytokines and chemokines after the lethal dose, and our model subsequently predicts a greater inflammatory responses in the lethal simulations. We also see greater levels of B cells after the lethal infection and a faster rise in the epithelial cell damage. The ensemble is generally able to capture these differences in the data seen between sublethal and lethal infections.

Ensemble predictions of host survival

In order to characterize the survivorship beyond what is known from the data, the study simulated each parameter set in the ensemble at various levels of initial inocula and determined the likelihood of host survival and other outcomes at each inoculum. In Figure 3A, we selected one representative parameter set from our ensemble and simulated the system of ODEs with these parameters at each of 11 different inocula. These simulations show that our model is capable of representing three distinct regimes of behavior: (i) For sufficiently low inocula (5 pfu/ml in this example) there is no detectable reduction in the target cell population. This type of behavior is defined as an asymptomatic infection; the virus is eliminated by only nonspecific clearance, without the need to stimulate the remainder of the immune system to fight the infection. The virus population will monotonically decay, thereby not causing any appreciable damage to target cells. (ii) For moderate inocula (between 25 pfu/ml and 150 pfu/ml), there is an initial decline in the target cell count followed by recovery. As the viral aliquot increases, the rate of decline in the target cell population and the disease duration also increase. This type of behavior is defined as a sublethal infection; while the infection is survivable, the number of cells that become infected is large, which would initiate a full immune response. (iii) For a sufficiently large inoculum (in this example, an inoculum above 175 pfu/ml), there is no recovery from the disease. For an individual parameter set, the sharp difference between sublethal and lethal disease trajectories can be ascribed to a saddle node in the phase space of the system, which gives rise to a stable manifold that separates the domains of attraction of the healthy fixed point (with $H = H_0$) and death fixed point (with $H = 0$). This manifold intersects the V axis

at a critical threshold where a small change in the initial virus leads to a large change in the outcome.

The ensemble model can be used to delineate how the probability of each of these three regimes depends on the initial viral aliquot. We now simulate the full ensemble of parameter sets at varied viral aliquots and compute the proportion of the ensemble that leads to a particular behavior at each aliquot. This proportion determines the likelihood of the particular behavior (asymptomatic, sublethal, or lethal) for given initial viral aliquot as determined by the ensemble model. Figure 3B shows the separation of these regimes, defined by two thresholds shown in the black lines. When the inoculum is less than 0.1 pfu/ml, all parameter sets lead to an asymptomatic infection. The viral trajectory will monotonically decay from its initial value to zero, suggesting that an animal given such a low aliquot would not experience any symptoms of the viral infection. As the inoculum increases, a greater proportion of the parameter sets will predict a sublethal, symptomatic infection, indicating the virus population will increase before eventually clearing from the body. The leftmost black line in Figure 3B shows the threshold between the asymptomatic infection and the sublethal infection. Lethal infections in the ensemble begin to occur at initial viral inocula greater than 100 pfu/ml. The threshold between the sublethal and lethal trajectories, shown by the rightmost line, is defined by the proportion of the ensemble that predicts a total depletion of target cells due to the infection.

We can also use the full ensemble to study at what time death will occur after a lethal aliquot. Survival curves (Figure 3C) are computed over 15 days, measuring the percentage of parameter vectors that lead to survival at a given time point. Survival of the ensemble is simulated for 13 initial viral loads ranging between 50 and 750 pfu/ml. Figure 3C shows that at 250 pfu/ml, the model predicts that mortality occurs between days 5 and 6. At 550 pfu/ml, death occurs between days 4 and 5. Therefore increasing the inoculum decreases time to death in our model. However, at 750 pfu/ml, the expected lifespan of the ensemble is only about half a day shorter. This leads to the prediction that increasing virus beyond the experimental dosage will only marginally shorten the expected lifespan.

Model-based differences between survivors and non-survivors

At an intermediate value of initial virus aliquot (210 pfu/ml), the ensemble model predicts equal likelihood of survival or non-survival (i.e., 50% mortality) (see Figure 3C). We use this outcome to divide the sample of parameter sets into two sub-ensembles: Sub-ensemble S (survivors) containing parameters that predict lethal course of disease and sub-ensemble N (non-survivors) containing parameter sets that predict to sublethal course of disease at that same initial virus. (Note that both sub-ensembles predict survival at 50 pfu/ml and non-survival at 500 pfu/ml). By comparing the two groups of parameter sets we uncover mechanistic differences in response to infection. Full marginal histograms of the survivor and non-survivor sub-ensembles sublethal were compared in univariate analysis using a t-test where the vast majority of parameters were significantly different between S and N (Supplement 5, Table 1). In multivariate analysis, model parameters most discriminatory of survivorship are identified using regularized logistic regression and greedy information gain

criterion, in a 10-fold cross-validation design. Regularized regression (balanced L_1 and L_2 penalty on the magnitude of parameters) is well adapted to deal with correlated predictors.

Regression models were consistent across L_1 and L_2 weights in ranking parameters indicative of a lethal phenotype (Supplement 5, Table 2). An increased ability of virions to infect healthy cells ranked consistently highest in predicting a lethal phenotype, followed by a higher level of chemokines beyond which neutrophils activation saturates, a lower affinity of cytotoxic lymphocytes to infected cells, an increased ability of virions to activate antigen presenting cells, and a decreased ability of IL-10 to curb the TNF response. In essence, increased virulence and a more robust innate inflammatory response were associated with a worse outcome through a variety of mechanisms.

Impact of inflammation on host survival

The symbols Σ_1 and Σ_2 represent combinations of various pro-inflammatory signals that provide the stimulus to the inflammatory cascade (see Supplement 2 for a full explanation of the equations). These signals sum pro-inflammatory effects from cytokines, dead epithelial cells, and virus, and they stimulate the activation of TNF, IL-10, and chemokines. The inflammation components of the model are vital for controlling host survival to infection. To demonstrate the role of the pro-inflammatory signal to model response, we compare the behavior of the full model to behaviors of the model with particular components of the immune response suppressed. The initial inoculum in all cases is 50 pfu/ml. Figure 4 shows the results of each of these tests. Column 1 demonstrates the behavior of the full model, which we deem the “wild-type” behavior. Each subsequent column shows the behavior of the model with one or more ODE right hand sides set to zero (i.e. $dY/dt = 0$ for some variable Y).

First, we examine the role of the inflammatory response as a whole. Two major effectors of the feedback from the inflammation to the remainder of the immune system are (1) free radicals produced by neutrophils (see Figure 1, bottom E) and (2) NK cells (Figure 1, bottom F). When both inflammation pathways are eliminated by suppressing signals Σ_1 and Σ_2 (Figure 4, column 2), all elements of the inflammatory response remain at their initial level and do not have significant contributions to the host’s reaction to the virus. Without inflammation, the ensemble is unable to heal, even though 50 pfu/ml is a sublethal dose. Infected cell levels are higher than in the baseline case, leading to more virus production and thus increased death rates. Even when only one of the inflammatory pathways is eliminated by keeping the effector components (X or NK) at their initial levels (Figure 4, columns 3 and 4), the result is an ensemble which is unable to heal. Since both of these components kill infected cells, we hypothesize their absence allows the infected cell population to grow faster. More virus is then produced, and the remaining immune components cannot control the virus, leading to the eventual death of the host [44,45].

To complement this study, we also study the ensemble behavior when the anti-inflammatory components are turned off. Suppressing the effects of IL-10 (Figure 4, column 5) removes a major antagonist of TNF, allowing the pro-inflammatory cytokines to increase. Suppressed IL-10 has two potential effects on the behavior of the ensemble. For most parameter sets, it results in faster virus killing, as the infected cell population is more effectively controlled. In

more than 5% of parameter sets, however, increased inflammation leads to death even at the originally sublethal inoculum level, due to inflammation-mediated damage to the epithelial layer.

Finally, we also investigate the behavior of the ensemble when antibodies are fixed to their initial level (Figure 4, column 6). Since antibodies do not begin to rise until day 7 (see Supplemental Figure 3), the impact of the fixed antibodies is not seen until later in the simulations. The ensemble predicts viral clearance for most trajectories even without high levels of antibodies, as most of the infected cells have been cleared by the natural killer cells by day 7. In more than 5% of the population, however, the lack of antibody up-regulation will lead to a resurgence of the viral infection.

Discussion

A quantitative ensemble model of in-host immune response to influenza virus infection was developed combining systemic inflammation with the innate, adaptive, and humoral immune responses seen in other published mathematical models. The model is supported by rich experimental data in combination with literature reports and provides insight into the role of inflammation as a core component of the host response to influenza A virus, a component not utilized in other published models. It is also the first time the ensemble approach is used to create a realistic survival analysis.

This model focuses on a comprehensive view of the immune response to influenza, rather than isolating the role of any one mechanism. Much work has gone into individually modeling the role of T cells [20,23], type I interferons [15,19,46], viral kinetics [17,18], and other effectors in the immune response. There is a large body of work describing the first 24 hours of the viral infection, detailing the stages a target cell undergoes in becoming an infected cell [36,47–50]. Many of these details, although known, were omitted from the model, because they were not suited to the chosen model granularity in terms of number of equations and parameters.

Though each part of the immune response is important to resolution of the infection, in this study, inflammation is the link between the severity of infection and survivorship. The inflammatory response provides an important component of the control of the population of infected cells, thus controlling the virus level in the host, but comes at a price of a potentially deleterious effect that must be tightly controlled by anti-inflammatory mediators. The study found that while a decrease in inflammation increases the probability of death in the entire ensemble, an increase in inflammation (due to absence of anti-inflammation) results in increased probability of death in a segment of the ensemble. The relatively high complexity of the model was necessitated by the exceptional quality and quantity of the data. Experimental data were available for 16 physiological variables in the sublethal study and 13 variables in the lethal study. The equations include just 4 additional variables for which data were not available, but which were essential for obtaining a closed loop model: target cell level, infected cell level, damaged cell level, and the level of reactive oxygen species. The model is constructed so that the influences between model variables account for

the most important mechanisms of immune response to IAV infection [36] and inflammation [51].

Naturally, such a comprehensive model results in a large number of parameters but this should not be viewed negatively – much larger models are commonly used in systems biology research [52]. Rather, the key question is whether the study has a sufficient amount of data to compute informed parameter distributions – and the answer is affirmative. The study combines the model with a sample of parameter distribution that characterizes the parameter uncertainty of the model, obtained using Bayesian inference to calibrate the model on data collected for naïve adult mice subjected to two different levels of initial viral aliquot, one which lead to recoverable disease and the other leading to mortality. The predicted trajectories are well focused, showing tight and confident prediction even for variables for which data were absent.

In this model, viral clearance and survival occur without sustained reduction in the target cell population. In the sublethal trajectories of target epithelial cells, approximately half remain uninfected through the course of the viral infection and the rest recover by the end of the studied time period. Comparable models of multifaceted immune response show significant persistent loss of target epithelial cells [17,33] which is inconsistent with experimental data and would lead to respiratory failure. This implies target limitation need not play a dominant role in viral clearance. This is an important biological feature of our model which distinguishes it from most other influenza models (e.g. [15,17,25,27,28]). Here, the infection is resolved largely by antibody activity and the removal of infected cells by NK cells. This is in contrast to target cell-limited models, in which the virus population decays over time because of much reduced availability of target epithelial cells to propagate infection. Autopsies of animals infected with IAV show a heterogeneous infection, with some areas of the lung more severely damaged than others, but certainly not all cells have been depleted in the lungs [53]. In this way, we believe our model reflects the biology of infection more closely than typical target cell-limited models.

The addition of inflammation to the system creates a saddle separatrix in the target cell trajectory, which allows the same model to produce survivorship in a low dose and mortality in a high dose. Again, including the inflammatory component of the model is crucial to fully understanding the immune dynamics responsible for survival of the infection.

Type I interferons have been of modeling interest in recent years [17,19], as these molecules represent a potential intervention to influenza infections. In the results, the model demonstrates a two-phase production of interferons, initially by infected cells and later by antigen presenting cells. The data indicate as the infection clears and the infected cell population declines, the unbound interferons remain elevated. The model accounts for this by including production by antigen presenting cells; however, the model produces a peak higher and later in the time course data of infection. This implies that the role of antigen presenting cells in the production of interferons may be more subtle than it appears. More studies are needed to clarify this point. These interferons perform the two-fold task of slowing cellular machinery to reduce production of new virions and increasing MHC presentation for immune-mediated lysis. This aligns better with the presentation of type I

interferons in Baccam et al. [17] where infection is slowed, than with Hancioglu et al. [22] which creates a separate category of resistant epithelial cells. In the present study, a resistance multiplier is introduced as a Michaelis-Menten function of the interferon concentration. The resistance multiplier, which varies from 0 to 1, reduces the number of virions that an infected cell can produce, increases the targeting by NK cells and CD8+ T cells, and reduces the production of type I interferons by infected cells. Interferons thus play a significant role in viral clearance, and contribute to the resilience of epithelial cells and recovery.

This model simplifies the biology for the purpose of creating a model that is both comprehensive in scope and small enough to be used in calibration and analysis. For example, there are cytokines that have either pro- or anti-inflammatory effects depending on the cell and receptor they bind to; this complexity is not represented in the model. Although this model was designed and calibrated to the time scale and response to influenza A virus, there is little in the structure of the model or equations that is specific to the viral strain. As an advantage, the model can be calibrated to different influenza strains. The model, beyond describing response to influenza, can describe immune responses that have properties such as a rebound in virus, viral fixation, oscillation in virus levels, and chronic inflammation. Heuristic rules are applied to the energy function to invalidate a model that demonstrates any of these behaviors.

Since this model is calibrated to mouse data and not longitudinal data in individual animals, care should be placed in drawing conclusions on how these data and analyses should be applied to humans. As mammals, humans and mice share a very large number of traits on cellular and physiological levels, but numerous differences exist in their immune responses to viral infection [54]. One major difference is in the makeup of the white blood cell population of each species. In humans, the majority of white blood cells are neutrophils, while in mice neutrophils make up only 10–25% of white blood cells [55]. In addition, influenza itself is not endemic in mice due to a difference in cellular receptors. Despite this, animal models continue to show promise in cultivating an understanding of how influenza affects human hosts. Mouse models continue to be the most popular choice for *in vivo* influenza studies, as mice are inexpensive and reagents are easily obtainable [56]. Swine and primates represent the best non-human animal models for understanding influenza. These studies would have the added benefit of longitudinal data, which would give greater insight still into how the role of cytokines changes from the onset of inflammation through the adaptive immune response and the course of infection. A quantitative model calibrated to these data could then be used to test hypotheses and suggest clinical interventions for human patients.

Conclusions

Quantitative modeling of systems provides an *in silico* platform for hypothesis testing. Inflammation has a direct link to morbidity from influenza infections, so models such as the one in this study can be used to develop clinical interventions that reduce morbidity without compromising the patient's immune response. Drugs that target drivers of inflammation may also delay the immune cascade. Interventions that target cytokines, such as biologics, may

be used in the future alongside antivirals to increase survivorship. Central to inflammation are the cytokines that drive the initial response. However, these cytokines are pluripotent and have various effects for different cells. This study of inflammation focuses on the relationship of inflammatory cytokines to macrophages, as this produces an important early feedback mechanism. Future studies should consider the role of these cytokines to all cells in the study, the adaptive immune cells in particular.

Until a reliable universal vaccine is developed, one path towards better treatment of viral infection relies on understanding how influenza A virus is neutralized by the host's immune system. This is especially important in cases with secondary pneumonia infection, where inflammation causes additional damage to the organism. Assembling the known mechanisms of immune response into models of host-pathogen interactions provides a platform for testing hypotheses of how proposed mechanisms of inflammation immune defense contribute to recovery. As more data becomes available for calibration, mathematical models of the infection can in turn shed light on the contributions of each immune pathway towards recovery [57]. Fully developed models can then be used to understand the qualities of patients that allow them to survive viral infections. Isolating these qualities, and understanding both the positive and negative effects of a viral infection, can lead to improved, more targeted clinical interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1 top

Figure 1. Model Schematic

(top) Schematic representation of a model of immune response to influenza A virus. Influences between the virus (diamond), epithelial and immune cell types (circles), inflammatory mediators and immune components (triangles) and combined inflammatory signals (hexagons) are shown as activating influences (thin arrows) and inhibitory influences (thin lines ending in cross-bar). The fluxes between different subtypes of cells are shown as thick gray arrows. For legend to model variables see Table 1. **(bottom)** Smaller panels show the major parts of the system: (A) primary reproduction pathway for the virus, (B) innate immunity and resistance, (C) cellular immunity, (D) humoral adaptive immunity, (E) general inflammation, and (F) natural killer cells pathway.

Figure 2. Ensemble Trajectories and Data

Ensemble simulations and data for disease response to sublethal inoculum of 50 pfu/ml (left column) and disease response to lethal inoculum of 500 pfu/ml (right column). Time on the horizontal axis extends over 11 days, with output shown to day 11 for sublethal and shown to day 7 for lethal, the last day with lethal data. The graphs show the interquartile ranges for the ensemble as a population level response. The black line shows the median of the trajectories at each time point; the dark grey shows the range from 25% of trajectories above and below the median; the light grey shows the range from 5% to 95%. The figure shows the outputs, from top to bottom, for the analytes: Target Epithelial Cells, Influenza A Virus, Infected Epithelial Cells, TNF, IL-10, IgM Antibodies, CD8+ T cells, Type II Interferon, Type I Interferons.

Figure 3. Response to Increasing Viral Aliquot

(A) Example trajectories of target epithelial cell levels for fixed set of parameters and different initial viral aliquots (in pfu/ml). Three outcomes can be observed: (i) no disease ($V_0 < 5$ pfu/ml in this example), (ii) sublethal disease ($25 < V_0 < 150$ pfu/ml in this example), or (iii) lethal disease ($V_0 > 175$ pfu/ml in this example) of the infection depends on then of epithelial cells as the size of the exposure to virus in outcome versus initial viral aliquot. (B) Ensemble response to varied viral aliquots. For very low levels of virus, there is no successful infection. As the amount of the initial virus rises, a decreasing proportion of the population resists becoming infected. Likewise, as the initial aliquot increases further, a decreasing proportion of the population survives the infection. These two effects are graphed continuously to predict how the threshold of infection and survivability changes with increased initial virus. (C) Survival curves, i.e., graphs of the surviving percentage of the ensemble population versus time. The ensemble shows approximately 50% survivorship at 210 pfu. For 500 pfu and above, there are no survivors.

Figure 4. Effect of immune components on behavior of the ensemble

The behavior of the full ensemble for hypothetical immune-compromised mutants is shown for a selected number of variables: IL-10, TNF, reactive oxygen species (ROS), infected epithelial cells, target epithelial cells, virus, and natural killer cells. Each row represents one of these variables and each column represents one iteration of the model. Columns are as follows: (1) baseline behavior, (2) system with no systemic inflammation or NK cell response, (3) fixed base level of NK cells, (4) fixed levels of ROS, and (5) fixed levels of IL-10.

Table 1

The experimental data collected for the system, and the corresponding variable used in the model.

Label	Variable	Units	Measurable	Measurement type
TNF	T	pg/ml	TNF- α	Luminex
IL-10	L	pg/ml	IL-10	Luminex
Chemokines	C	pg/ml	MCP and MIP-1 β	Luminex
Macrophages	M	cell count	CD11c- CD11b+ CD40+ GR-1 dim F4/80-	Flow cytometry
Blood neutrophils	$\tilde{N}$	cell count	None	N/A
Tissue neutrophils	N	cell count	Gr-1+ (high) CD11b+ (high)	Flow cytometry
Reactive oxygen species	X	pg/ml	None	N/A
Target epithelial cells	H	cell count	Recovery/mortality	Heuristic
Infected epithelial cells	I	cell count	None	N/A
Damaged epithelial cells	D _H	cell count	Weight loss	Weight
Virus	V	pfu/ml	Influenza A/PR/8/34	Plaque assay
Type I interferon	F	pg/ml	IFN- α and IFN- β	ELISA
Type II interferon	G	pg/ml	IFN- γ	Luminex
Natural killer cells	K	cell count	CD49b(DX5)+ CD69+	Flow cytometry
Antigen presenting cells	P	cell count	CD11c+ CD11b+ CD40+ GR-1 dim	Flow cytometry
B cells	B	cell count	CD19+ CD69+	Flow cytometry
CD8+ T cells	E	cell count	CD3+ CD8+ CD69+	Flow cytometry
IL-12	W	pg/ml	IL-12	Luminex
CD4+ T cells	O	cell count	CD3+ CD4+ CD69+	Flow cytometry
Antibodies	A	pg/ml	IgM antibodies	HAI