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Increasing Viral Dose Causes a Reversal in CD8+ T Cell Immunodominance during Primary Influenza Infection due to Differences in Antigen Presentation, T Cell Avidity, and Precursor Numbers

Fabio Luciani,* Megan T. Sanders,† Sara Oveissi,† Ken C. Pang,†,1,2 and Weisan Chen†,2,3

T cell responses are characterized by the phenomenon of immunodominance (ID), whereby peptide-specific T cells are elicited in a reproducible hierarchy of dominant and subdominant responses. However, the mechanisms that give rise to ID are not well understood. We investigated the effect of viral dose on primary CD8+ T cell (TCD8+) ID by injecting mice i.p. with various doses of influenza A virus and assessing the primary TCD8+ response to five dominant and subdominant peptides. Increasing viral dose enhanced the overall strength of the TCD8+ response, and it altered the ID hierarchy: specifically, NP366–374 TCD8+ were dominant at low viral doses but were supplanted by PA224–233 TCD8+ at high doses. To understand the basis for this reversal, we mathematically modeled these TCD8+ responses and used Bayesian statistics to obtain estimates for Ag presentation, TCD8+ precursor numbers, and avidity. Interestingly, at low viral doses, Ag presentation most critically shaped ID hierarchy, enabling TCD8+ specific to the more abundantly presented NP366–374 to dominate. By comparison, at high viral doses, TCD8+ avidity and precursor numbers appeared to be the major influences on ID hierarchy, resulting in PA224–233 TCD8+ usurping NP366–374 cells as the result of higher avidity and precursor numbers. These results demonstrate that the nature of primary TCD8+ responses to influenza A virus is highly influenced by Ag dose, which, in turn, determines the relative importance of Ag presentation, TCD8+ avidity, and precursor numbers in shaping the ID hierarchy. These findings provide valuable insights for future TCD8+-based vaccination strategies.

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CD8+ T cells (TCD8+) are a critical component of the adaptive immune response against viral infections. These cells recognize complexes of peptides and MHC class I (pMHC-I) molecules on the surface of APCs. In theory, following viral infection, TCD8+ can recognize a vast range of presented viral peptides. However, for any given virus, TCD8+ responses are, in reality, directed toward only a limited number of pMHC-I. This phenomenon, referred to as “immunodominance” (ID), is further characterized by a hierarchy of immunodominant determinants that reproducibly elicit the largest TCD8+ response and of subdominant determinants that induce relatively smaller responses (1).

Elucidating the mechanisms that contribute to ID is critical to the future development of effective TCD8+-based vaccines. Numerous factors that contribute to ID have been identified. On the one hand, multiple factors directly related to the Ag appear to be important, including the efficiency of Ag processing and presentation (2–4), the stability of pMHC-I (5, 6), the nature of the pMHC-I structure (7–9), and the total number of pMHC-I presented by APCs (10, 11). On the other hand, various properties specific to the host TCD8+ also play a role, including their relative diversity, precursor numbers, functional avidity, recruitment rate, ability to expand, and propensity to compete with one another (2, 5, 12–15). However, the relative importance of each of these factors is still uncertain.

Another issue that remains unclear is how variations in viral dose affect the TCD8+ ID hierarchy. Probst et al. (16) addressed this question by examining how the ID hierarchy changed in response to increasing doses of lymphocytic choriomeningitis virus (LCMV). Specifically, these investigators found that the ID hierarchy is directly contingent upon viral dose, with one TCD8+ response (to NP396) dominant at low viral doses but another (to GP33) dominant at high viral doses. Whether such findings extend to other viral systems is unknown, but recent mathematical modeling suggested that alterations in Ag dose should routinely affect ID hierarchies and that immunodominant epitopes will increasingly outcompete subdominant ones as Ag dose increases (17).
In the current study, we addressed the issue of how Ag dose affects the ID hierarchy to influenza A virus (IAV) by using a combination of experimental and modeling approaches. To begin, we selected an I.p. route of administration for IAV rather than using the conventional intranasal route, which supports productive infection of virus (18) and, therefore, results in a highly saturated viral dose even at very low inoculation doses (M.T. Sanders and W. Chen, unpublished observations). In contrast, the dose of viral inoculation with I.p. administration is more likely to reflect the true Ag dose available for presentation, a point that we were keen to exploit to address how changes in viral dose affect the ID hierarchy of TCD8+. We subsequently challenged mice with increasing amounts of IAV over a five-log range and observed that, as IAV dose increases, there is a generalized expansion in the total numbers of subdominant and immunodominant TCD8+, as well as a notable switch in the ID hierarchy, whereby NP366-374 (NP366) is replaced as the immunodominant epitope by PA224-233 (PA224). To better understand these results, we used a stochastic model to simulate TCD8+ responses to IAV infection under a range of scenarios. By doing so, we found that relative epitope presentation is the critical influence on the TCD8+ ID hierarchy at low viral doses, enabling the TCD8+ specific for the more abundantly presented NP366 epitope to dominate the response. However, at high viral doses, relative TCD8+ avidity (defined as the probability that a TCD8+ proliferates upon encountering its cognate Ag) and precursor numbers become important, and the PA224 TCD8+ usurp NP366 TCD8+ as a result.

Materials and Methods

**Mice, viruses, and infection**

B6 female mice were purchased from Walter and Eliza Hall Institute animal services (Kew, Australia), and animals were generally used at 8–12 wk of age. Experiments were conducted under the auspices of the Austin Health Animal Ethics Committee and conformed to the National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes. IAV (A/Puerto Rico/8/34 [H1N1], PR8) was grown in 10-d embryonic chicken eggs. The titer of infectious virus was determined by plaque formation on confluent monolayers of Madin-Darby canine kidney cells, as previously described (19). Mice were infected by injecting IAV i.p. at doses ranging from 10^3 to 10^6 PFU.

**Abs and peptides**

For flow cytometry, FITC-labeled anti-IFN-γ and Cy-Chrome-labeled anti-CD8α were purchased from Becton Dickinson (North Ryde, Australia). Peptides were procured and characterized by the Biologic Resource Branch, National Institute of Allergy and Infectious Diseases (Rockville, MD) and were kind gifts from Drs. Jonathan Yewdell and Jack Bennink (National Institute of Allergy and Infectious Diseases, National Institutes of Health). They included NP366 (ASNENMETM), PA224 (SSLEN-RTFSFQLI), and PB1F262 80 3 (PB1F262; NS2114 90 3). To establish the background level of the ICS assay. This background value was subtracted from each of the peptide-stimulated wells to obtain a peptide-specific level of response.

**Estimates and modeling of Ag-presentation levels**

Our method to assess the kinetics of Ag presentation using BFA was described previously (12, 20, 21). The resulting data from these assays were used to obtain quantitative estimates of the relative presentation levels and speed of epitope presentation (3, 12). To do so, we assumed that the proportion of T cells stimulated in each well in the Ag-presentation assay was a direct reflection of peptide density presented on the surface of the APC. Ag-presentation data from BFA-kinetics assays were used to derive the time at which 50% of the T cell activation was achieved (Km), as well as the maximum Ag presentation levels (Amax). To obtain Amax for each epitope, we assumed that the maximal presentation on an APC is proportional to the amount of epitope-specific T cells stimulated at the highest level of presentation in the BFA-kinetics assay (3, 12) (W. Chen, unpublished observations). The resultant estimates are summarized in Table I and were used to feed the two parameters of a Hill function describing Ag-presentation levels over time.

Mathematical modeling of TCD8+ ID

Two mathematical models were explored. Model 1: Handel and Antia model. We adapted the model of Handel and Antia (22) to simulate the five major TCD8+ responses following primary IAV infection. This model considers viral dose (V), pMHC-I on APCs (Pi), and two populations of TCD8+, naive epitope-specific TCD8+ (Tn) and activated (or effector) TCD8+ (T ′), where T ′ is the index representing the five epitopes. The model is given as a system of ordinary differential equations that deterministically describe the T cell dynamics as follows:

\[ V = \alpha V - \beta \sum_i \lambda_i T_i \]

\[ P_i = f_i V - d_i P_i \]

\[ T_i = -\alpha_i P_i T_i \]

\[ T_i = g_i T_i^* + \alpha_i P_i T_i \],

In this model, virus grows exponentially with rate \( \alpha \) and is eliminated via activated TCD8+, at rate \( \alpha_i \). Ag presentation for a given epitope is modeled as proportional to the amount of virus with a peptide-specific scaling factor \( f_i \), and is eliminated at a fixed rate \( d_i \). PMHC-I activate Ag-specific TCD8+ at rate \( \alpha_i \). These activated TCD8+ proliferate at rate \( \gamma_i \), via clonal expansion. Because the model describes only the expansion phase, it does not include the death of Ag-specific TCD8+. We used the same parameter values that were used by these investigators except that we set \( \alpha \) to 0 because we were modeling a nonreplicative i.p. infection, varied \( \alpha_i \) over a 3-log range (10^-2 – 10^3) to simulate the experimental increase in viral dose; and adjusted \( f_i \) to reflect observed differences in Amax between epitopes (Table I) (note that Kmax estimates cannot be used in this model because the model assumes a linear interaction between T cells and Ag-presentation level). The entire list of parameter values is provided in Table II.

Model 2: Individual-based model. We refined an individual-based model (IBM) proposed by Scherer et al. (17), which is a stochastic version of the seminal model proposed by de Boer and Perelson (23), for which a general deterministic population-based approach was used to describe the responses of Ag-specific TCD8+, following interaction with APCs. This IBM simulates the individual encounter of naïve TCD8+ with APCs over time, and each encounter—as well as its downstream sequelae, such as TCD8+-activation, proliferation, and killing—is described as a separate probabilistic event. In this way, the model simulates thousands of individual stochastic events that occur simultaneously at each time point to eventually produce an in silico version of the TCD8+ response at 7 d. The novelty of

Table I. Estimates of Ag processing and presentation using BFA-kinetics data

<table>
<thead>
<tr>
<th>Ag-Specific</th>
<th>Maximum Epitope Presentation</th>
<th>Presentation for NP366*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Cell Response</td>
<td>(as a Percentage of the Maximum)</td>
<td>Kmax (h)</td>
</tr>
<tr>
<td>NP366</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>PA224</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>PB1F262</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>NS2114</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>PB1703</td>
<td>60</td>
<td>4</td>
</tr>
</tbody>
</table>

*Based on BFA Ag-presentation experiments (3, 12) for NP366, PA224, PB1F262, NS2114, and W. Chen, unpublished observations (PB1703). See text for details.
our implementation of the original IBM (17) is that we modeled Ag presentation as a dynamic process to take into account our previous experimental data on the kinetics of influenza epitope presentation (3, 12).

Our IBM separately models three aspects of IAV infection and the subsequent immune response: infected cells, APCs, and T CD8+. The model begins with a starting number of virally infected cells. Because we are simulating what happens when different amounts of virus are inoculated via a nonproductive i.p. route, the model begins with a starting number of virally infected cells that was varied according to initiating viral dose and did not increase thereafter. APCs were then generated from the population of infected cells at a constant rate ($\sigma$) and were also assumed to die at a constant rate. Each APC was allowed to be able to bind up to 150 T CD8+ because this value was roughly in the middle of the observed experimental range. Similarly, we estimated that each of these naive T CD8+ scans 50 APCs/d, a range chosen based upon experimental evidence that T cells can engage with a single APC simultaneously (24). The number of pMHC-I for any given epitope was described by a Hill function based on estimates of $K_c$ and $A_{\text{max}}$, as detailed above. Previous evidence suggests that naive T CD8+ occur at a frequency of 10–2000, depending upon the particular T CD8+ specificity (25–29). Therefore, we selected a precursor frequency of 500 cells as our default starting value, as suggested by imaging experiments showing that between 10 and 300 T cells can engage with a single APC simultaneously (24). For each scanning event, a T CD8+ has a given probability of subsequently binding to the APC as determined by the level of Ag presentation. After binding, each T CD8+, interacted with the APC for an average of 1 h before dissociating. In reality, experimental data on the dynamics of the interactions between APCs and T CD8+ suggest that T CD8+ first engage in multiple, short encounters and then in longer interactions that last up to 30 min (31), but we condensed these multiple encounters into one long-lasting interaction for simplicity. During this interaction, each Ag-specific T CD8+ has a given probability of becoming activated, which we ascribed to a single parameter that we termed “functional avidity.” In reality, this avidity is likely to reflect multiple factors, including the nature of APC:T CD8+ pairing, the strength of costimulatory signals via CD28, as well as intrinsic properties of the T CD8+, itself (24, 32, 33). To differentiate dominant from subdominant responses, we assumed, as did Scherer et al. (17), that dominant responses have a higher functional avidity. Following successful activation, a T CD8+ dissociated from its APC and went through multiple rounds of proliferation. In our model, the first round of proliferation was slow, based on experimental evidence that T cells only start to proliferate after an initial lag phase $\sim 1$ d. Thereafter, proliferation occurred every 6 h, with the default number of total proliferation rounds set at 8, which was chosen based on experimental evidence that T CD8+ go through 5–10 rounds of programmed proliferation without the need for re-exposure to Ag (34). The model also incorporated the spontaneous death of T CD8+ at a constant rate $\delta$ that was based on previously published estimates (35). After proliferation, T CD8+ became effector cells and cleared virally infected cells at a rate $k$. In this way, the number of infected cells followed a decline curve from $t = 0$ determined by the total number of effector T CD8+ and the clearance rate $k$.

Thus, our refined IBM uses multiple parameters to simulate the T CD8+ response following i.p. IAV infection. In theory, simulations using our IBM could be performed by allowing the values of multiple parameters to run free simultaneously. However, with so many parameters to vary, the number of possible outcomes that the model could describe is very large, and the simulations become computationally intractable. For this reason, we fixed the majority of the parameters to known values based upon existing experimental or computational estimates found in the literature (Table III). Where such estimates were lacking, as was the case for the dynamics of viral-infected cells and the rate of APC generation $\gamma$, or where the literature-based estimates varied considerably, as was the case for the APC death rate or the number of cycles of programmed T CD8+ proliferation, we performed preliminary analyses to tune the model and obtain estimates that enable a realistic reproduction of the T CD8+ ID hierarchy (see Discussion).

Approximate Bayesian computation

We used approximate Bayesian computation (ABC) algorithms to estimate parameters of the IBM. These algorithms are particularly useful for obtaining estimates in situations in which standard techniques, such as those based on likelihood functions, are no longer applicable or difficult to

Table III. Parameters and their values used in the analyses based upon an individual-based model

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Parameter</th>
<th>Default Value</th>
<th>Experimental Value</th>
<th>Simulated Values</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virally infected cells</td>
<td>Maximum number of infected cells allowed in the simulations (carrying capacity)</td>
<td>$5 \times 10^5$ cells</td>
<td></td>
<td></td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>Initial viral load (infected cells)$^a$</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intrinsinc growth rate</td>
<td>$0$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Per T cell clearance rate of infected cells</td>
<td>$1.2 \times 10^{-4}$d</td>
<td>$10^{-6}$–$10^{-5}$d</td>
<td></td>
<td>(58–61)</td>
</tr>
<tr>
<td>T cells</td>
<td>Number of APCs scanned by a T cell</td>
<td>50d</td>
<td>10–100d</td>
<td>10–300d</td>
<td>(24, 30)</td>
</tr>
<tr>
<td></td>
<td>Minimal number of epitopes required to bind to an APC site</td>
<td>5 copies</td>
<td>2–10 copies</td>
<td></td>
<td>(62)</td>
</tr>
<tr>
<td></td>
<td>Probability of T cell proliferation upon dissociation of the APC:T cell conjugate (“T cell avidity”)$^a$</td>
<td>0.2/d (NP$\text{sp6}$)</td>
<td>0.1–0.25/d</td>
<td>0.01–0.3/d</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>Duration of one round of T cell division</td>
<td>6 h</td>
<td>3–10 h</td>
<td>1–10 h</td>
<td>(34)</td>
</tr>
<tr>
<td></td>
<td>Rate at which T cells start scanning for Ag after proliferation</td>
<td>$0.2/d$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T cell death rate</td>
<td>$0.2/d$</td>
<td>0.05–0.2/d</td>
<td>0.05–0.3/d</td>
<td>(35)</td>
</tr>
<tr>
<td></td>
<td>Rate of production of APCs</td>
<td>0.004/d</td>
<td></td>
<td></td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>Number of T cell binding sites/APC</td>
<td>150</td>
<td>10–300</td>
<td>10–300</td>
<td>(63)</td>
</tr>
<tr>
<td></td>
<td>Maximum epitope presentation level (copies per epitope)$^a$</td>
<td>Table IV</td>
<td>$10^2$–$10^4$</td>
<td>Table IV</td>
<td>(20, 63, 64)</td>
</tr>
<tr>
<td></td>
<td>Time at which the epitope presentation reaches 50% of the maximum</td>
<td>Table I</td>
<td>1–48 h</td>
<td>Table I</td>
<td>(2, 3, 12)</td>
</tr>
<tr>
<td></td>
<td>APC death rate</td>
<td>$0.2/d$</td>
<td>0.15–0.5/d</td>
<td>0.1–0.3/d</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td>Rate of dissociation of a T cell from an APC</td>
<td>24/d</td>
<td>1–24/d</td>
<td>24/d</td>
<td>(31, 66)</td>
</tr>
</tbody>
</table>

$^a$Parameters estimated via ABC (see also Tables IV–VI and text).
compute, as is the case for complex stochastic models, such as our IBM (36, 37). Provided that the underlying model is close enough to the real system and the experimental observations are well captured by the summary statistics used to estimate the model parameters, this approach provides reliable and satisfying results. For example, previous applications of ABC showed that ABC estimates are accurate in scenarios in which likelihood-based inference methods are also possible (38), and various groups have used ABC to estimate the evolutionary rates of human or bacterial populations (39, 40), the transmission dynamics of Mycobacteria tuberculosis (41), and the fitness cost associated with bacterial drug resistance (36). In keeping with Bayesian statistics, ABC parameter estimates are represented as a posterior distribution that provides information on the likelihood that the unknown parameter falls within a certain range of values. This range is represented as a 95% credibility interval (CI), and from this interval one can obtain an idea of the reliability of the ABC estimates.

With all of the other IBM parameters fixed (Tables II, III), we used ABC to estimate Ag-expression levels, $T_{CD8}$, precursor numbers, and relative $T_{CD8}$ avidities. The ABC algorithm used in this work was the rejection algorithm (40), where we corrected the estimates using a linear-regression method as previously suggested (42), and the algorithm was implemented using the statistical package R (43). ABC algorithms approximate the data $D$ by a set of summary statistics $S$, which can be derived from observations. The idea is then to sample parameter values from the prior distribution and compare the result of the simulations with the observed data via summary statistics. The comparison is performed by defining a distance function that reports on the similarity between simulated and observed data sets. We used as summary statistics the five Ag-specific $T_{CD8}$ counts observed within the spleens of mice 7 d after i.p. infection with different doses of IAV. For each parameter set, we simulated the primary infection six times, because this was the minimal number of mice used for our in vivo experiments, and then compared the observed and simulated mean values. As a distance function, we considered the Euclidean distance

$$d = \sqrt{\sum_{i=1}^{5} \frac{(T_{i}^{s} - T_{i}^{r})^2}{\text{var}(T_i)}}$$

where the $T_{i}$ are the absolute numbers of each of the five Ag-specific $T_{CD8}$ responses measured at day 7, $T = (T_{NP366}, T_{PB1F262}, T_{NK2114}, T_{PB1703})$; are the corresponding simulated values, and $\text{var}(T_i)$ is the variance for each response measured from the six repeated simulations with the same parameter values. The prior distribution for Ag-presentation levels and $T_{CD8}$ precursor numbers was chosen as a uniform distribution with values ranging between 0 and infinity (in reality, a very large number), whereas the prior distribution for $T_{CD8}$ avidity ranged between 0 and 1.

**Results**

**Increasing viral dose during primary IAV infection causes a generalized expansion in $T_{CD8}$ responses and changes in the ID hierarchy**

To address the role of Ag dose in the overall primary $T_{CD8}$ response, we measured Ag-specific $T_{CD8}$ numbers 7 d after i.p. infection of B6 mice with IAV in doses ranging from $10^3$ to $10^8$ PFU. Responses were measured to five well-characterized peptides presented on H-2D$^b$ or K$^b$: the two dominant $T_{CD8}$ epitopes, D$^b$/PA224 and D$^b$/NP366, and three subdominant epitopes, D$^b$/PB1F262, K$^b$/NK2114, and K$^b$/PB1703. We chose to assess these responses at 7 d based upon pilot experiments, which showed that peak $T_{CD8}$ responses occurred either on or slightly after this day for a range of IAV doses, including $10^3$, $10^4$, $10^5$, and $10^6$ PFU (data not shown). As shown in Fig. 1A, the total number of splenic $T_{CD8}$ for both immunodominant and subdominant determinants increased as viral dose increased. Specifically, only a few hundred $T_{CD8}$ were elicited for each determinant at the lowest viral dose ($10^3$ PFU), and this increased approximately two and three orders of magnitude at the highest dose ($10^8$ PFU) for the subdominant and dominant determinants, respectively. In support of these trends, there was a statistically significant dose-dependent increase in immune responses for each epitope ($p < 0.05$ for $T_{CD8}$ responses of each determinant measured at different viral doses, Wilcoxon signed-rank test), with the exception of some responses between $10^6$ and $10^7$ and $10^7$ and $10^8$ PFU, for which we observed only a modest increase with viral dose, indicating likely saturation of the response. These data suggest that, as IAV viral dose increases, there is a generalized expansion of $T_{CD8}$ responses and no apparent effect of dominant responses supplanting subdominant ones via competition, as previously postulated (17).

To specifically assess the effect of increasing viral dose on the ID hierarchy, we examined the relative contribution of each of the five Ag-specific $T_{CD8}$ populations to the total immune response in the above experiment (Fig. 1B). At the lowest viral dose ($10^3$ PFU), all five determinants elicited a similar level of response. Of note, at this dose the overall magnitude of $T_{CD8}$ responses was close to background levels, and there was large variation in the ID hierarchy between individual mice, which likely reflects the stochastic nature of the interaction between $T_{CD8}$ and APC at very low Ag levels. Beyond $10^4$ PFU, an interesting picture emerged. Notably, the relative contribution of PA224 $T_{CD8}$ progressively increased as viral dose increased; NP366 $T_{CD8}$ showed the opposite response, peaking at an IAV dose of $10^5$–$10^6$ PFU and decreasing thereafter. Meanwhile, the relative contribution of the three subdominant $T_{CD8}$ responses tended to remain constant. Overall, the effect of these changes was to produce a reversal in ID: at lower viral doses ($10^4$ PFU), the NP366-specific $T_{CD8}$ response was dominant over PA224 ($p < 0.05$, Wilcoxon signed-rank test), whereas at higher doses ($10^6$ PFU), PA224 $T_{CD8}$ were dominant over NP366 ($p < 0.05$, Wilcoxon signed-rank test). This switch in ID was even more striking among peritoneal $T_{CD8}$ (Supplemental Fig. 1), for

![Figure 1](http://www.jimmunol.org/)
which similarly statistically significant differences were observed. Together, these results indicate that the relative contribution of different Ag-specific TCD8+ to the overall response following primary IAV infection is dependent upon viral dose.

A simple mathematical model fails to explain the observed changes in ID hierarchy

Previously, we found that NP366 and PA224 were presented by APCs at different rates and efficiencies (3, 12). We hypothesized that this difference in Ag presentation might explain why an increase in IAV dose leads to a change in the primary ID hierarchy. To test this idea, we used the mathematical model proposed by Handel and Antia (22), which is predicated on the assumption that TCD8+ activation varies linearly with Ag presentation and was previously shown to successfully explain changes in TCD8+ ID hierarchy between primary and secondary IAV infection.

To begin, we obtained estimates for the maximal relative presentation level and rate of presentation for each epitope (Table I) using our published Ag-presentation data for NP366, PA224, PB1F262, and NS2114 (3, 12), as well as more recent results for PB1703 (W. Chen, unpublished observations) (Materials and Methods). We found that NP366-specific TCD8+ were activated and rapidly reached half-maximal stimulation (Km) after only ~1 h. In contrast, PA224 presentation was much slower (Km = 8 h) and less effective, stimulating only a minority of PA224-specific TCD8+, whereas the rates and relative presentation levels of the subdominant epitopes NS2114, PB1F262, and PB1703 fell in between NP366 and PA224.

Taking into account these differences in relative presentation levels, we used the maximum relative presentation level to estimate the rate constant at which Ag are presented on the surface of APC in the Handel and Antia model (Materials and Methods) (Table II). We found that the Handel and Antia model recapitulated our observation that an increase in viral dose causes an overall expansion in the absolute number of Ag-specific TCD8+ at day 7 (Fig. 2A). However, the model failed to reproduce the change in ID hierarchy that we observed experimentally; instead, it produced a scenario whereby all five Ag-specific TCD8+ under study contributed equally at the highest Ag dose (Fig. 2B). These results suggested that a simple linear dependency between Ag-presentation levels and TCD8+ responses is, by itself, insufficient to explain what happens as viral dose increases.

IBM recapitulates the Ag dose-dependent trends in ID hierarchy and highlights the importance of rapid and effective NP366 presentation in its immunodominant status at lower Ag doses

We next evaluated and adapted a previously published IBM (17) that takes into account differences in Ag presentation, as well as the dynamic and stochastic interactions between TCD8+ and APCs (Materials and Methods). In our modified IBM, Ag presentation occurs as a saturating function over time at a rate that is specific for each individual epitope.

To model the experimental increase in viral dose, we first ran a series of simulations in which the total number of infected cells (and hence the number of APCs) was allowed to increase, but the Ag-presentation level/APC constant remained constant. For these simulations, we arbitrarily assumed a value of n = 500 precursor cells for each Ag determinant, based on previous estimates of viral Ag-specific TCD8+ precursor numbers that ranged from 10 to 1200 (14, 25). Similar to our earlier modeling, this reproduced the generalized expansion in the total number of responding TCD8+ response as viral dose increased, but it failed to reproduce the observed ID changes (Supplemental Fig. 2). Therefore, we ran another series of simulations in which both the level of Ag presentation/APC and the total number of infected cells were allowed to rise with increasing viral dose. The simulations showed a generalized expansion in the absolute numbers of Ag-specific TCD8+ as Ag dose increased (Supplemental Fig. 3). Importantly, they also demonstrated dose-dependent trends in ID hierarchy that mirrored those seen experimentally (compare Fig. 3A and 3B).

Higher precursor numbers and/or higher avidity can explain the rise of PA224 TCD8+ to ID at increased Ag doses

Despite the success of the above modeling in describing the general trends observed for the IAV TCD8+ hierarchy as a function of viral dose, one aspect that the simulations failed to reproduce was the final switch in ID: instead, PA224- and NP366-specific TCD8+ remained codominant at the highest Ag levels (Fig. 3). Therefore, we under-
took an additional series of simulations to ascertain whether other immune parameters of the model might explain this shortcoming.

First, we studied the role of TCD8+ precursor numbers in the simulations. To begin, we modeled scenarios with either low (n = 50) or high (n = 1000) numbers of TCD8+ precursors for each determinant (instead of n = 500 cells, as described earlier). Fig. 4 shows the relative proportion of each Ag-specific TCD8+ response at day 7 when the precursor numbers were set either low (Fig. 4A) or high (Fig. 4B). In both scenarios, plotting the average trend across hundreds of simulation runs provided very similar results to each other, although, interestingly, the distribution of individual simulations was different. Specifically, when precursor TCD8+ numbers were lower, the simulated results showed greater variability; conversely, when precursor TCD8+ numbers were higher, the modeling followed a much tighter distribution. This makes intuitive sense because, regardless of the prevailing Ag-presentation settings, and continuous lines represent the fitted splines. The y-axis shows the proportion of Ag-specific TCD8+ of the total splenic response observed at day 7 (y-axis) is plotted as a function of viral dose (x-axis). Solid lines represent the interpolation of the mean values, and dashed lines represent the 95% CIs for the experimental data shown in Fig. 1B. (B) Simulated Ag-specific TCD8+ responses are shown. Simulations were performed assuming a fixed value of 500 precursors for each of the five TCD8+ responses and equal avidity values (0.2) for NP366 and PA224-specific TCD8+ (Tables II, III). Each dot represents the mean values of six repeated simulation runs with identical parameter settings, and continuous lines represent the fitted splines. The y-axis indicates the proportion of Ag-specific TCD8+ of the total response at day 7, and the x-axis shows the combined per-APC Ag-presentation level for the five epitopes of interest.

In any case, these simulations failed to improve upon our previous modeling with 500 precursor TCD8+/epitope (Fig. 3B); therefore, we examined how relative differences in epitope-specific precursor numbers might affect the simulated TCD8+ response. To do so, we again simulated two scenarios: one in which the ratio of PA224/NP366 precursors was 0.2 (100:500) and another in which the ratio was 2 (1000:500). Reducing the PA224 precursor numbers resulted in greater discordance between the simulations and experimental results (Fig. 4C): specifically, the simulated PA224 response decreased to the level of the three subdominant epitopes, whereas the NP366 response remained dominant throughout. In contrast, increasing the PA224 precursor number almost exactly reproduced the ID hierarchy that we observed experimentally, including the switch in ID at higher Ag doses (compare Figs. 4D and 3A), indicating that TCD8+ precursor frequencies can indeed have a decisive influence upon TCD8+, ID hierarchies. This seems logical, because raising TCD8+ precursor numbers increases the likelihood of a TCD8+:APC encounter and, hence, the probability of TCD8+ activation and subsequent proliferation.

Next, we examined how relative differences in TCD8+, avidity might affect the TCD8+ response. To do so, we allowed the avidity of PA224-specific TCD8+ to be slightly higher than that of NP366-specific TCD8+ (earlier we had assumed that the two were equivalent; Table III), while maintaining the precursor frequencies for all epitopes at the original value of n = 500 cells. As shown in Fig. 5, this minor alteration was sufficient to allow the modeled ID hierarchy to precisely recapitulate the one observed experimentally, including the ascendance of PA224-specific TCD8+ to sole ID at the highest Ag levels (compare Figs. 3B and 5). The importance of TCD8+, avidity was further highlighted by simulations in which we allowed the avidity of PB1F262, NS2114, and PB1703 TCD8+ to be equivalent to that of NP366 and PA224: in this case, these previously subdominant responses rose to codominance, reaching similar saturation levels as for NP366 and PA224 at higher Ag levels (data not shown). Taken together, these results suggested that, once Ag levels were no longer limiting, relative differences in TCD8+ avidity critically shaped ID. Again, this makes sense, because avidity directly determines the probability of TCD8+ activation and, hence, the likelihood of proliferation.

Estimates of pMHC-I numbers, TCD8+ precursor numbers, and avidities

In modeling the various scenarios above, in each case we made different assumptions about the parameter values for Ag presentation, TCD8+ precursor numbers, and avidities before the simulations were actually performed. Although useful for illustrating
the potential role of each parameter in shaping ID; such an approach did not, for example, indicate whether the ID of PA224 TCD8+ at high viral doses was most likely due to greater avidity, greater precursor numbers, or both. ABC is a statistical method that allows de novo inference of parameter estimates in situations in which standard techniques are not applicable or difficult to compute, as is the case for complex stochastic models, such as our IBM (36, 37). Therefore, we applied ABC to our IBM-based simulations to formally estimate pMHC-I levels, TCD8+ avidity, and precursor numbers (Materials and Methods), as well as to gain additional insights into the underlying basis for IAV ID.

The resultant ABC-derived estimates (Tables IV–VI) were revealing. First, the method provided clear estimates for the average number of pMHC-I likely to be found on an individual APC as...
viral dose increased. Specifically, the method estimated that the total number of pMHC-I/APC (for the five determinants under study) increases from 75 at a dose of $10^3$ PFU IAV (95% CI: 44–144) to 5303 at a dose of $10^8$ PFU IAV (95% CI: 4289–5808) (Table IV). Second, the method predicted that the median avidity of PA224 TCD8+ is $\sim40\%$ higher than that of NP366 (Table V). Third, the method estimated that the median precursor number of PA224 TCD8+ is higher than that of NP366 TCD8+ (Table VI), consistent with previous experimental estimates (14), although the difference was only $\sim25\%$, and the large CIs suggested that a wide range of possible precursor values is able to produce a good fit with the experimental data. Taken together, the latter two results are, nevertheless, in agreement with our earlier ad hoc modeling and suggest that the increase in PA224 TCD8+ to ID at high viral doses is likely due to a combination of greater avidity and higher precursor numbers.

**Discussion**

The amount of inoculating Ag is an essential variable in immunization design. Therefore, understanding how Ag dose affects subsequent TCD8+ responses is important to the rational optimization of TCD8+-based vaccines. We began this study by examining how Ag dose affects the overall TCD8+ response to IAV. Not surprisingly, other investigators examined this general question before, although, interestingly, the results have not been wholly consistent. For example, infecting BALB/c mice with 1,000, 5,000, or 25,000 live *Listeria monocytogenes* failed to alter the magnitude of the immunodominant LLO91–99 TCD8+ response in vivo (44), but other investigators found that infection with $10^7$ attenuated *L. monocytogenes* bacteria elicited a 5-fold increase in responding TCD8+ compared with $10^5$ bacteria (45). Similar to this second study, Murata et al. (46) reported that TCD8+ responses to a *Plasmodium yoelii* epitope encoded by engineered IAV or vaccinia virus were enhanced by increasing the immunizing viral dose. Wherry et al. (47) also demonstrated that the TCD8+ response size to epitopes from IAV’s nucleoprotein, as well as OVA, is proportional to epitope expression, although they varied Ag expression not by altering overall viral dose but by using different vaccinia virus–expression constructs. All three of these latter reports are consistent with the results of our current study, which demonstrates a significant increase in the total number of IAV-specific TCD8+, as viral dose increases (Fig. 1A). However, it should be noted that, beyond a certain Ag dose, significant TCD8+ expansion ceased (see especially Supplemental Fig. 1A), which is consistent with earlier findings indicating that once a certain threshold density of epitopes is reached, saturation of the TCD8+ response occurs (46–48).

Having a broad immune response against multiple TCD8+ epitopes is believed to confer better antiviral protection (49) and, therefore, is a desirable goal for TCD8+-based vaccines. Scherer et al. (17) predicted that the repertoire of TCD8+ is likely to contract as Ag levels increase: specifically, using an IBM they reported that subdominant responses will be sequentially outcompeted to the point that only one immunodominant response remains. If this is true, then increasing Ag dose in vaccines might actually sacrifice immune breadth (despite optimizing depth) and have deleterious consequences for immunity. Interestingly, our experiments did not bear out these predictions. Instead, we found that, even at the highest Ag doses where saturation of the immunodominant TCD8+, responses became evident, the total number of TCD8+ directed against subdominant epitopes continued to increase (Fig. 1A). Notably, when we ran simulations assuming very low numbers of TCD8+ binding sites per individual APC (<10), we obtained predictions similar to those of Scherer et al. (data not shown), suggesting that competition phenomena can be relevant if TCD8+ access to APCs is extremely limited. However, professional

**Table IV.** Posterior estimates of epitope density per APC

<table>
<thead>
<tr>
<th>Stimulus (Viral Titer, PFU)</th>
<th>Total Ag Level/APC</th>
<th>NP366/APC</th>
<th>PA224/APC</th>
<th>PB1F262/APC</th>
<th>NS2114/APC</th>
<th>PB1703/APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^3$</td>
<td>75 (44–144)</td>
<td>21 (5–37)</td>
<td>10 (3–19)</td>
<td>16 (4–29)</td>
<td>19 (5–32)</td>
<td>12 (3–21)</td>
</tr>
<tr>
<td>$10^5$</td>
<td>702 (568–1085)</td>
<td>151 (146–156)</td>
<td>76 (73–78)</td>
<td>121 (117–125)</td>
<td>136 (131–142)</td>
<td>91 (88–94)</td>
</tr>
<tr>
<td>$10^6$</td>
<td>1768 (1412–2689)</td>
<td>361 (328–405)</td>
<td>180 (164–202)</td>
<td>288 (263–324)</td>
<td>324 (296–364)</td>
<td>216 (197–243)</td>
</tr>
<tr>
<td>$10^7$</td>
<td>3127 (1704–5126)</td>
<td>670 (536–990)</td>
<td>335 (268–495)</td>
<td>536 (429–792)</td>
<td>603 (483–891)</td>
<td>402 (322–594)</td>
</tr>
<tr>
<td>$10^8$</td>
<td>5303 (4288–5808)</td>
<td>1371 (1004–1678)</td>
<td>686 (502–834)</td>
<td>1097 (803–1342)</td>
<td>1234 (903–1510)</td>
<td>823 (602–1007)</td>
</tr>
</tbody>
</table>

Data are median (95% Bayesian CI).
those specific for PA 224, whose presentation is much slower and demonstrated by the observation that NP 366 TCD8+ dominate over number of pMHC-I, is limited. In our case, this was most readily the primary ID hierarchy when Ag supplies and, hence, the gests that relative differences in Ag presentation powerfully shape be the exception, rather than the rule, in primary responses.

Consistent with the findings of Probst et al. (16) in relation to LCMV, we report that TCD8+ ID hierarchies following IAV infection are clearly dependent upon Ag dose. Interestingly, at low viral doses, the immunodominant TCD8+ against both LCMV and IAV are directed toward the epitopes that show the most rapid presentation kinetics (NP396 and NP366, respectively). This suggests that relative differences in Ag presentation powerfully shape the primary ID hierarchy when Ag supplies and, hence, the number of pMHC-I, is limited. In our case, this was most readily demonstrated by the observation that NP366 TCD8+ dominate over those specific for PA224, whose presentation is much slower and less abundant (Table I). Indeed, when we performed simulations in which either the $K_m$ or $A_{max}$ for NP366 presentation was reduced, the dominance of NP366 TCD8+ at low Ag levels was lost (data not shown). Moreover, when comparing the subdominant TCD8+ responses at low viral doses, we observed a similar trend: namely, the epitope with the highest and most rapid presentation (NS2114) elicited the more robust response (Fig. 3). In contrast, when Ag levels are plentiful, it appears that these relative differences in Ag presentation lose functional significance, as exemplified by the rise of PA224 to ID, despite its poor presentation. Intuitively, this makes sense because at high Ag doses where the number of APCs is in relative excess and it is the number of APCs that critically limits the TCD8+ activation, the influence of Ag presentation on ID appears to vary depending upon Ag dose. This variability might explain why relative differences in Ag presentation, in the context of the ID hierarchy following primary IAV infection at least, were shown by some investigators to have a decisive role (50), whereas others assessed the TCD8+ response at different time points.

At high viral doses, the alteration in the TCD8+ ID hierarchy to LCMV and IAV appears due to different causes. For LCMV, higher viral doses lead to chronic infection and apparent exhaustion of the NP366 response with subsequent ID by GP13 TCD8+ (16). For IAV, we found that the rise of PA224 TCD8+ at higher Ag doses could be explained by both superior functional avidity and precursor numbers. With regard to functional avidity, it was shown that inefficiencies in precursor cell recruitment, as well as their subsequent expansion, can render TCD8+ responses subdominant (14). Our simulations varying the relative avidities of PB1703, NS2114, and PB1F262 TCD8+ were consistent with this notion, because the subdominance of these responses at high Ag levels was strictly dependent upon their having a lower avidity than NP366 and PA224 (Table III). Moreover, our results indicate that the converse might also apply (i.e., that highly efficient recruitment and/or expansion can lead to ID, as evidenced by our results with PA224). Taken together, the relative functional TCD8+ avidity plays a critical role in tuning the proportional response toward any given peptide determinant either up (for dominant TCD8+) or down (for subdominant TCD8+), provided that Ag levels are not limiting. As for precursor numbers, their role in determining primary ID hierarchies has been unclear. Using double tetramer–staining techniques and TCR diversity assays to estimate Ag-specific TCD8+ numbers, multiple studies showed a positive correlation between naive precursor numbers and the resultant primary TCD8+ response following infection with IAV and LCMV (28, 49–51). However, others failed to observe such trends (14, 29). Our finding that higher precursor numbers of PA224 TCD8+ are likely to contribute to the ID of PA224 at high Ag doses, albeit in probable combination with a higher avidity, provides two insights. First, the role of precursor numbers in shaping ID hierarchies may only be apparent when Ag doses are high. Again, this makes intuitive sense because, at high Ag doses where the number of APCs is in relative excess and the number of TCD8+ precursors is a limiting factor, increasing precursor frequency should raise the likelihood that a TCD8+ will interact with an APC bearing its cognate epitope. In contrast, at low Ag doses, where the number of TCD8+ is in relative excess and it is the number of APCs that critically limits the TCD8+ response, increasing precursor frequency should have minimal effect. Second, even when Ag levels are sufficient, higher precursor numbers alone are unlikely to guarantee ID because avidity must also be considered, as elegantly highlighted by other investigators (14). Indeed, the large CIs that we obtained when estimating precursor numbers are consistent with previous findings that TCD8+ precursor numbers by themselves do not predict ID hierarchy (14, 29).

One possible explanation for the changes in ID hierarchy that we observed is that alterations in Ag dose differentially affect the kinetics of individual TCD8+ responses. For example, the apparent decrease in the NP366 TCD8+ response at higher viral doses at day 7 might simply reflect a shift in the peak of the NP366 TCD8+ response to a different time point. Indeed, the TCD8+ response to NP366 was observed to occur later than for PA224 following both intranasal and i.p IAV infection (2, 14). Similarly, the ID hierarchy to LCMV also changes over the course of an acute infection (52). Thus, there remains a possibility that our day-7 ex vivo assessment missed the peak of the NP366 TCD8+ response and that these cells, in fact, remained as dominant as PA224 TCD8+ at higher viral doses. A related argument could be made for PA224 TCD8+ at lower viral doses. To examine this issue, we experimentally assessed the TCD8+ response at different time points.

Table V. Posterior estimates of TCD8+ precursor numbers

<table>
<thead>
<tr>
<th></th>
<th>NP366</th>
<th>PA224</th>
<th>PB1F262</th>
<th>NS2114</th>
<th>PB1703</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior</td>
<td>426 (97–874)</td>
<td>516 (87–1142)</td>
<td>538 (10–1167)</td>
<td>506 (43–989)</td>
<td>481 (90–1000)</td>
</tr>
</tbody>
</table>

These estimates were based on experimental data for 10⁶ PFU of IAV, because these estimates showed the minimum error from a cross-validation test. Data represent median (95% Bayesian CI).

Table VI. Posterior estimates of T cell avidity

<table>
<thead>
<tr>
<th></th>
<th>NP366</th>
<th>PA224</th>
<th>PB1F262</th>
<th>NS2114</th>
<th>PB1703</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior</td>
<td>0.15 (0.10–0.20)</td>
<td>0.20 (0.16–0.22)</td>
<td>0.02 (0.01–0.08)</td>
<td>0.02 (0.01–0.06)</td>
<td>0.02 (0.01–0.08)</td>
</tr>
</tbody>
</table>

These estimates were based on experimental data for 10⁶ PFU of IAV, because these estimates showed the minimum error from a cross-validation test. Data represent median (95% Bayesian CI).
Importantly, we found that the peak response consistently occurs between days 7 and 9 and that the overall ID hierarchy does not change (data not shown). This was further supported by additional IBM that showed that, even though the peak of the TCD8+ response moved from day 6 to 9 at low versus high Ag levels, respectively (Supplemental Fig. 4: compare peak responses in A–D), the overall ID hierarchy remained stable across time for any given Ag dose (Supplemental Fig. 4: compare the relative TCD8+ response within each panel). Taken together, these results suggested that our ex vivo observations at day 7 truly reflected a dose-dependent change in hierarchy and not simply differential kinetics.

Given that ID reflects a complex interplay between complex factors, it is hardly surprising that mathematical modeling has been used as a means to better understand ID (17, 22, 23, 53). Indeed, it was suggested that, in the future, ID research will increasingly rely upon such modeling (3). In this study, we found that complex stochastic modeling enables the successful simulation of our experimental findings, and it provides novel insights into the underlying basis for ID hierarchy establishment at different Ag doses. Complex models are more likely to resemble reality compared with simple simulations, but they tend to have a higher number of parameters and, therefore, can be more difficult to tune to the correct region of higher-dimensional parameter space. Our use of advanced Bayesian statistics enabled the estimation of multiple immune parameters (pMHC-I densities, TCD8+, avidities, and naive precursor frequencies) for five IAV epitopes, armed only with the experimental details of relative Ag presentation and day-7 TCD8+ responses. With recent technological developments, some of these parameters can now be estimated experimentally, be it through tetramer enrichment to enumerate naive precursors (29, 54), the use of pMHC-I–specific mAb and HPLC fractionation to elucidate epitope densities (20, 55, 56), and tetramer/BrdU-based methods to analyze recruitment rates (14). Nevertheless, such approaches remain technically challenging, prone to underestimation, and not always readily applicable given the limited availability of key reagents.

Therefore, mathematical modeling offers an alternative approach to parameter estimation, and the framework used in our study should be readily adaptable to other contexts (e.g., the study of non-IAV infections or CD4+ T cell dynamics). Notably, comparison between our novel modeling-based estimates and those obtained experimentally reveals broad agreement. Specifically, our estimates of between ~5000 and 6000 pMHC-I/APC for the five major IAV epitopes at the highest viral dose is very similar to the HPLC-derived estimate of ~7000 pMHC-I previously reported for the five major IAV epitopes in the BALB/c mouse system (20). Similarly, our estimated median precursor frequencies were consistent with the published tetramer-based estimates of La Gruta et al. (14), which also indicated that there are more naive PA224 TCD8+ than NPS66 TCD8+, although our estimates were higher and had broad Bayesian CIs. Finally, our estimates of functional avidity, although not directly comparable with any existing experimental data, are in keeping with evidence that PA224 TCD8+ display a broader diversity of TCRs (14, 26, 32, 33) and, thus, may have a greater probability of proliferating upon Ag encounter.

Taken together, our work suggests that mathematical modeling is an important adjunct to the experimental dissection of ID. Of course, it is important to emphasize that mathematical modeling of ID is still in its relative infancy and, just like many of the cutting-edge experimental techniques mentioned earlier, it has its own set of restrictions and caveats. Looking ahead, there is great scope to build upon the approaches that we developed in this study, and this is discussed below.

First, it will be interesting to see how well our modeling translates to the context of a productive infection. For this study, we specifically chose the nonproductive i.p. route of infection, because we reasoned that the dose of viral inoculation would be more likely to reflect the true Ag dose available for presentation, and it would be more directly relevant to standard influenza immunization practices that rely upon nonproductive i.m. injection. However, because much of the research literature on ID uses productive-infection models, testing and validating our IBM and ABC methods in these other systems will be important.

Second, there is undoubtedly scope to further improve the IBM. Indeed, despite its apparent success in recapitulating the observed experimental outcomes, the IBM in its current form has several key limitations. Chief among these is the need to preassign values to some of the various model parameters (Table III). In many cases, one can assign these values with some confidence, given existing estimates in the experimental literature. However, in other cases, such estimates either vary considerably (e.g., APC death rates and number of programmed TCD8+ proliferation rounds) or simply do not exist (e.g., APC recruitment rates). To overcome this challenge, we ran preliminary tuning analyses to choose “best guess” parameter values that enabled the simulations to resemble the major features of the observed experimental responses (Materials and Methods). For example, our tuning analyses indicated that both the timing of the TCD8+ response peak and the overall number of responding TCD8+ (but not the ID hierarchy itself) were sensitive to changes in either the number of rounds of programmed TCD8+, proliferation or the APC recruitment rate (data not shown); we eventually chose values whose simulations best approximated our experimental findings. In the future, as experimental innovations occur, estimating parameters such as these will hopefully become more accurate and less dependent upon guesswork. Another key limitation of the IBM is the need to break down what is an inherently complex process into a simplified set of defined parameters. Although our IBM took into consideration almost 30 parameters, it failed to capture many aspects of the immune response that one would ideally like to include in future modeling efforts. For instance, the model does not take into direct consideration the role of inflammation, which might be expected to impact upon the TCD8+ response in multiple ways, especially as the viral dose is altered. Specifically, inflammation, through the action of cytokines, such as IFNs and TNF-α, is likely to enhance MHC-I and costimulatory molecule expression on APCs, as well as to increase TCD8+ survival (57), through the upregulation of molecules such as 4-1BB. In this way, one can envisage that, as viral dose increases, increased levels of inflammation will dynamically impact upon various parameters that already exist within our IBM, including pMHC-I levels, TCD8+, death rates, and TCD8+ avidities (by affecting the likelihood of costimulation and, therefore, the probability of activation). Therefore, efforts to incorporate the dynamic impact of inflammation into future models appear warranted.

Third, finding ways to improve the ABC-based methods should enable more reliable estimates of various immune parameters. As is evident from the breadth of the 95% CIs shown in Tables IV through VI, our current estimates vary considerably in their reliability. Specifically, epitope density estimates appear quite reliable, TCD8+ avidity estimates are moderately reliable, and TCD8+ precursor numbers appear much less reliable. There are at least three possible explanations for the latter. First, it could simply be that the summary statistics that we used, the TCD8+ responses at day 7, are not sufficient to properly capture the entire dynamics of the TCD8+ response. However, given the apparent success in using these same parameters to estimate epitope density, this seems
unlikely, as one uses ABC to estimate more and more parameters, the accuracy of these estimates may be reduced (42), but again this did not seem to prevent us from obtaining reliable estimates for epitope density. The third and most likely possibility is that the actual number of T<sub>CD8+</sub> precursors does not greatly influence the T<sub>CD8+</sub> response (i.e., we obtained a large CI because a wide range of values successfully simulates the observed T<sub>CD8+</sub> counts). Nevertheless, looking ahead, it would be interesting to see whether the reliability of our parameter estimates improves if we use additional summary statistics. For example, if we had included a longitudinal component to our initial experimental setup by assessing T<sub>CD8+</sub> responses daily from days 5 to 10 postinfection (rather than just day 7), it might be possible that the parameter estimates improve. Similarly, because the performance of the ABC approach also depends upon the fidelity of the model to real-life, future efforts to refine our underlying IBM might also enable improved ABC-based estimates.

In conclusion, we presented a computational modeling approach to better understand the complex dynamics that underlie the generation of primary T<sub>CD8+</sub> immune responses. Although this approach has various restrictions and caveats, it has nonetheless enabled us to draw useful insights into the basis for understanding how the T<sub>CD8+</sub> ID hierarchy is established after infection influenza. Just as importantly, our model also provides a foundation upon which future refinements can be based and will hopefully facilitate the design of in silico methods that can assist in the development of safe and effective vaccines.

Acknowledgments
We thank Almut Scherer and Marcel Salathé for sharing the code of their model from which the model proposed in this article was developed.

Disclosures
The authors have no financial conflicts of interest.

References


**Figure S1.** Primary peritoneal T<sub>CD8+</sub> responses vary according to the dose of inoculating virus. Ag-specific T<sub>CD8+</sub> responses from peritoneal washout cells were assessed following infection with increasing doses of IAV. Ag-specific T<sub>CD8+</sub> were identified at day 7 *ex vivo* by ICS after stimulation with the indicated peptides. Panel A depicts the Ag-specific T cell counts measured as the absolute number of CD8<sup>+</sup> IFN<sub>γ</sub> T cells in the peritoneal washout. Panel B shows the proportion of each Ag-specific response among the total T<sub>CD8+</sub> response. Data correspond to the average results from 8-15 individual mice per viral dose, and error bars represent SEM.
**Supplementary Figure 2**

**A**

![Graphs showing simulations of Ag-specific TCD8+ responses](image)

**B**

![Graphs showing proportions of Ag-specific TCD8+ responses](image)

**Figure S2.** Simulations fail to reproduce the observed dose-dependent changes in TCD8+ hierarchy when increasing viral load is assumed to affect only the number of infected cells. Simulated Ag-specific TCD8+ responses are shown. Simulations were performed with all modeling parameters set to the default values shown in Table II with the exception that the combined per-APC antigen presentation level for the five epitopes of interest was held constant at 380. **First row (A):** Simulated Ag-specific TCD8+ numbers at day 7 after primary IAV infection (y-axis) are shown as a function of the number of infected cells (x-axis) starting the infection. Each dot represents the mean values of 6 repeated simulation runs with identical parameter settings, and continuous lines represent the fitted splines. **Second row (B):** Same data as in A but here plotted to show the relative proportions of the five Ag-specific TCD8+ responses.
**Supplementary Figure 3**

**Figure S3. Comparison of observed and IBM-simulated Ag-specific T_{CD8+} numbers.** *First row (A):* Experimentally observed Ag-specific T_{CD8+} responses are shown. The total number of Ag-specific T_{CD8+} observed in the spleen at day 7 (y-axis) is plotted as a function of viral load (x-axis). Solid lines represent the interpolation of the mean values (black) and 95% confidence intervals (red) for the experimental data shown earlier in Fig. 1A. *Second row (B):* Simulated Ag-specific T_{CD8+} responses are shown. Simulations were performed assuming a fixed value of 500 precursors for each of the five T_{CD8+} responses, and equal avidity values (0.2) for NP_{366}⁻ and PA_{224}⁻ specific T_{CD8+} (Table II). Each dot represents the mean values of 6 repeated simulation runs with identical parameter settings, and continuous lines represent the fitted splines. Y-axis indicates the total number of Ag-specific T_{CD8+} at day 7, and the x-axis shows the combined per-APC antigen presentation level for the five epitopes of interest.
Supplementary Figure 4

**Figure S4. Simulated time course of the primary T\textsubscript{CD8+} response.** Displayed are plots of the simulated time course for the five Ag-specific T\textsubscript{CD8+} responses at increasing Ag presentation levels. **Panels A to D:** simulations were performed assuming a combined per-APC antigen presentation level for the five epitopes of interest of 400 (A), 800 (B), 3000 (C), and 5400 (D) pMHC-I complexes. All other modeling parameters were set to the default values shown in Table II.