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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 T CD8+. 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 T CD8+ as well as a notable switch in the ID hierarchy, whereby NP 366–374 (NP 366) is replaced as the immunodominant epitope by PA224–233 (PA224). To better understand these results, we used a stochastic model to simulate T CD8+ responses to IAV infection under a range of scenarios. By doing so, we found that relative epitope presentation is the critical influence on the T CD8+ ID hierarchy at low viral doses, enabling the T CD8+ specific for the more abundantly presented NP 366 epitope to dominate the response. However, at high viral doses, relative T CD8+ avidity (defined as the probability that a T CD8+ proliferates upon encountering its cognate Ag) and precursor numbers become important, and the PA224 T CD8+ usurps NP 366 T CD8+ 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 103 to 108 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 NP 366 (ASNSNEMTM), PA224 (SSLENFRAYV), PB1F262–70 (PB1F262; LSLRNPLLIV), NS2114–121 (NS2114; RTTFSFQKL), and PB1203–711 (PB1203; SYYRPRPGVI).

Intracellular cytokine staining

For intracellular cytokine staining (ICS), splenic and peritoneal cells from infected animals were suspended in 200 μl RPMI 1640 with 10% FCS at 1.5–2 × 106 cells/well in round-bottom 96-well plates. Peptides were added to cells at a final concentration of 1 μM, and cells were incubated with peptides for 2 h at 37°C and then for 4 h with brefeldin A (BFA; Sigma-Aldrich, St. Louis, MO) at 10 μg/ml. Cells were stained with Cy-Chrome–labeled anti-CD8α mAb at 4°C for 30 min, washed, and fixed with 1% paraformaldehyde in PBS at room temperature for 20 min, and then further stained with fluorescein-anti-IFN-γ in PBS containing 0.4% saponin (Sigma-Aldrich). Stained cells were acquired on a FACSCalibur (Becton Dickinson) and analyzed using FlowJo software (TreeStar, Ashland, OR). As a negative control, cells were not exposed to peptide 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 T CD8+ 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 T CD8+ responses following primary IAV infection. This model considers viral dose (V), pMHC-I on APCs (Pi), and two populations of T CD8+, naive epitope-specific T CD8+ (Ti) and activated (or effector) T CD8+ (Te), where i 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:

\[
\frac{dV}{dt} = -rV - \sum_{i} k_i T_i^e
\]

\[
\frac{dP_i}{dt} = f_i V - d_i P_i
\]

\[
T_i^e = -a_i P_i T_i
\]

\[
T_i^e = g_i T_i^e + a_i P_i T_i
\]

In this model, virus grows exponentially with rate r and is eliminated via activated T CD8+, at rate g_i. Ag presentation for a given epitope is modeled as proportional to the amount of virus with a epitope-specific scaling factor f_i and is eliminated at a fixed rate d_i, pMHC-I activates Ag-specific T CD8+ at rate a_i. These activated T CD8+ proliferate at rate g_i via clonal expansion. Because the model describes only the expansion phase, it does not include the death of Ag-specific T CD8+. We used the same parameter values that were used by these investigators except that we set r to 0 because we were modeling a nonreplicative i.p. infection, varied f_i over a 3-log range (10^-2 to 10^-5) to simulate the experimental increase in viral dose; and adjusted f_i to reflect observed differences in Amax between epitopes (Table I) (note that K_m 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 T CD8+ following interaction with APCs. This IBM simulates the individual encounter of naive T CD8+ with APCs over time, and each encounter—as well as its downstream sequelae, such as T CD8+ 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 T CD8+ response at 7 d. The novelty of

<table>
<thead>
<tr>
<th>Table I. Estimates of Ag processing and presentation using BFA-kinetics data</th>
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<tbody>
<tr>
<td><strong>Ag-Specific</strong></td>
</tr>
<tr>
<td><strong>T Cell Response</strong></td>
</tr>
<tr>
<td><strong>K_m</strong></td>
</tr>
<tr>
<td>NP 366</td>
</tr>
<tr>
<td>PA224</td>
</tr>
<tr>
<td>PB1F262</td>
</tr>
<tr>
<td>NS2114</td>
</tr>
<tr>
<td>PB1203</td>
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</table>

*Based on BFA Ag-presentation experiments (3, 12) for NP 366, PA224, PB1F262, NS2114, and W. Chen, unpublished observations (PB1203). See text for details.
For each scanning event, a TCD8+ has a given probability of subsequently 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:TCD8+ signaling (e.g., costimulatory signals via CD28), as well as intrinsic properties of the TCD8+, 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 TCD8+, 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 ∼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 TCD8+ 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 TCD8+ at a constant rate δ that was based on previously published estimates (35). After proliferation, TCD8+, 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 TCD8+ and the clearance rate k.

Thus, our refined IBM uses multiple parameters to simulate the TCD8+ 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 or, where the literature-based estimates varied considerably, as was the case for the APC death rate or the number of cycles of programmed TCD8+ proliferation, we performed preliminary analyses to tune the model and obtain estimates that enable a realistic reproduction of the TCD8+ 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 II. Parameters and their values used in the analyses based upon the Handel and Antia model (22) |
|-----------------------------------------------|----------------|----------------|----------------|
| Parameter | Explanation | Valuea | Reference |
| r | Viral growth rate | 0 | |
| d | Deactivation rate of pMHC-I | 1 | |
| s | Rate of Ag presentation on APC | Varied (see Table I) | |
| α | Activation rate of TCD8+ | 5 × 10⁻⁴ | |
| g | Expansion rate of TCD8+ | 0.45 | |
| k | Virus killing rate | 3 × 10⁻³ | |

aFrom Handel and Antia (22). All parameters are in units of 1/d.

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 × 10⁵ cells</td>
<td>10⁻¹⁰⁸</td>
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<td></td>
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<tr>
<td>T cells</td>
<td>Intrinsic growth rate</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Per T cell clearance rate of infected cells</td>
<td>1.2 × 10⁻⁴/d</td>
<td>10⁻⁴⁻¹⁰⁶</td>
<td></td>
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<td></td>
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<tr>
<td>Number of APCs scanned by a T cell</td>
<td>50/d</td>
<td>10–1000</td>
<td>10–300/d</td>
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<td></td>
</tr>
<tr>
<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></td>
<td></td>
</tr>
<tr>
<td>Probability of T cell proliferation upon dissociation of the APC:T cell conjugate (“T cell avidity alpha”)</td>
<td>0.2/d (NP54)</td>
<td>0.1–0.25/d</td>
<td>0.01–0.3/d</td>
<td></td>
<td></td>
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<tr>
<td>APCs</td>
<td>Duration of one round of T cell division</td>
<td>6 h</td>
<td>6 h</td>
<td>4–10 h</td>
<td></td>
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<tr>
<td>Rate at which APCs start scanning for Ag after proliferation</td>
<td>2/h</td>
<td>2/h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<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></td>
<td></td>
</tr>
<tr>
<td>T cell precursor numbers</td>
<td>500</td>
<td>10–2000</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rate of production of APCs</td>
<td>0.004/d</td>
<td>2/h</td>
<td></td>
<td></td>
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<tr>
<td>Number of T cell binding sites/APC</td>
<td>150</td>
<td>10–300</td>
<td>10–300</td>
<td></td>
<td></td>
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<tr>
<td>Maximum epitope presentation level (copies per epitope)</td>
<td>Table IV</td>
<td>10⁻²⁻¹⁰⁴</td>
<td>Table IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time at which the epitope presentation reaches 50% of the maximum APC death rate</td>
<td>0.2/d</td>
<td>0.15–0.5/d</td>
<td>0.1–0.3/d</td>
<td></td>
<td></td>
</tr>
<tr>
<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></td>
<td></td>
</tr>
</tbody>
</table>
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

\[ d = \sqrt{\sum_{i=1}^{5} \frac{(T_{oi} - T_{si})^2}{\text{var}(T_{i})}} \]

where the $T_{oi}$ are the absolute numbers of each of the five Ag-specific $T_{CD8}$ responses measured at day 7, $T = (T_{NP368}, T_{PA224}, T_{PB1F262}, T_{NS2114}, T_{PB1703})$, 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-expression 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$^b$/NP368, and three subdominant epitopes, D$^b$/PB1F2, K$^b$/NS2114, 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 $10^4$, $10^5$, and $10^8$ 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; NP368 $T_{CD8}$ 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 NP368-specific $T_{CD8}$ response was dominant over PA224 ($p$ value < 0.05, Wilcoxon signed-rank test), whereas at higher doses ($10^5$ PFU), PA224 $T_{CD8}$ were dominant over NP368 ($p$ < 0.05, Wilcoxon signed-rank test). This switch in ID was even more striking among peritoneal $T_{CD8}$ (Supplemental Fig. 1), for
which similarly statistically significant differences were observed. Together, these results indicate that the relative contribution of different Ag-specific $T_{CD8+}$ 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 NP$_{366}$ and PA$_{224}$ 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 $T_{CD8+}$ activation varies linearly with Ag presentation and was previously shown to successfully explain changes in $T_{CD8+}$ 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 NP$_{366}$, PA$_{224}$, PB1F262, and NS2$_{114}$ (3, 12), as well as more recent results for PB1$_{703}$ (W. Chen, unpublished observations) (Materials and Methods). We found that NP$_{366}$-specific $T_{CD8+}$ were activated and rapidly reached half-maximal stimulation ($K_m$) after only $\sim$1 h. In contrast, PA$_{224}$ presentation was much slower ($K_m = 8$ h) and less effective, stimulating only a minority of PA$_{224}$-specific $T_{CD8+}$, whereas the rates and relative presentation levels of the subdominant epitopes NS2$_{114}$, PB1F262, and PB1$_{703}$ fell in between NP$_{366}$ and PA$_{224}$.

Taking into account these differences in relative presentation levels, we used the maximum relative presentation level to estimate the rate $f$ 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 $T_{CD8+}$ 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 $T_{CD8+}$ under study contributed equally at the highest Ag dose (Fig. 2B). These results suggested that a simple linear dependency between Ag-presentation levels and $T_{CD8+}$ 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 NP$_{366}$ 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 $T_{CD8+}$ 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 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 $T_{CD8+}$ 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 $T_{CD8+}$ 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 $T_{CD8+}$ 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). Namely, at very low Ag levels, all five determinants showed a similar response; as Ag levels increased, NP$_{366}$-specific $T_{CD8+}$ rose to dominance and then diminished, the proportion of PA$_{224}$-specific $T_{CD8+}$ gradually increased, and the contribution of the three subdominant $T_{CD8+}$ decreased. Notably, the dominant status of NP$_{366}$-specific $T_{CD8+}$ at low Ag levels was contingent upon the rapid and highly effective presentation of NP$_{366}$ (Table I), because any downregulation of this presentation in the modeling failed to replicate the experimental response for NP$_{366}$.

Higher precursor numbers and/or higher avidity can explain the rise of PA$_{224}$ $T_{CD8+}$ to ID at increased Ag doses

Despite the success of the above modeling in describing the general trends observed for the IAV $T_{CD8+}$ hierarchy as a function of viral dose, one aspect that the simulations failed to reproduce was the final switch in ID: instead, PA$_{224}$- and NP$_{366}$-specific $T_{CD8+}$ 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 T<sub>CD8+</sub> precursor numbers in the simulations. To begin, we modeled scenarios with either low (n = 50) or high (n = 1000) numbers of T<sub>CD8+</sub> precursors for each determinant (instead of n = 500 cells, as described earlier). Fig. 4 shows the relative proportion of each Ag-specific T<sub>CD8+</sub> 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 T<sub>CD8+</sub> numbers were lower, the simulated results showed great variability; conversely, when precursor T<sub>CD8+</sub> numbers were higher, the modeling followed a much tighter distribution. This makes intuitive sense because, regardless of the prevailing Ag-presentation levels, any reduction in the number of precursor T<sub>CD8+</sub> will lower the probability that a T<sub>CD8+</sub> encounters an APC and, hence, the likelihood of proliferation.

In any case, these simulations failed to improve upon our previous modeling with 500 precursor T<sub>CD8+</sub>/epitope (Fig. 3B); therefore, we examined how relative differences in epitope-specific precursor numbers might affect the simulated T<sub>CD8+</sub> response. To do so, we again simulated two scenarios: one in which the ratio of PA<sub>224</sub>/NP<sub>366</sub> precursors was 0.2 (100:500) and another in which the ratio was 2 (1000:500). Reducing the PA<sub>224</sub> precursor numbers resulted in greater discordance between the simulations and experimental results (Fig. 4C): specifically, the simulated PA<sub>224</sub> response decreased to the level of the three subdominant epitopes, whereas the NP<sub>366</sub> response remained dominant throughout. In contrast, increasing the PA<sub>224</sub> 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 T<sub>CD8+</sub> precursor frequencies can indeed have a decisive influence upon T<sub>CD8+</sub>-ID hierarchies. This seems logical, because raising T<sub>CD8+</sub> precursor numbers increases the likelihood of a T<sub>CD8+</sub>:APC encounter and, hence, the probability of T<sub>CD8+</sub> activation and subsequent proliferation.

Next, we examined how relative differences in T<sub>CD8+</sub>-avidity might affect the T<sub>CD8+</sub> response. To do so, we allowed the avidity of PA<sub>224</sub>-specific T<sub>CD8+</sub> to be slightly higher than that of NP<sub>366</sub>-specific T<sub>CD8+</sub> (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 PA<sub>224</sub>-specific T<sub>CD8+</sub> to sole ID at the highest Ag levels (compare Figs. 3B and 5). The importance of T<sub>CD8+</sub>-avidity was further highlighted by simulations in which we allowed the avidity of PB1<sub>703</sub>, NS2<sub>114</sub>, and PB1F2<sub>62</sub> T<sub>CD8+</sub> to be equivalent to that of NP<sub>366</sub> and PA<sub>224</sub>; in this case, these previously subdominant responses rose to codominance, reaching similar saturation levels as for NP<sub>366</sub> and PA<sub>224</sub> at higher Ag levels (data not shown). Taken together, these results suggested that, once Ag levels were no longer limiting, relative differences in T<sub>CD8+</sub>-avidity critically shaped ID. Again, this makes sense, because avidity directly determines the probability of T<sub>CD8+</sub> activation and, hence, the likelihood of proliferation.

**Estimates of pMHC-I numbers, T<sub>CD8+</sub>-precursor numbers, and avidities**

In modeling the various scenarios above, in each case we made different assumptions about the parameter values for Ag presentation, T<sub>CD8+</sub>-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

FIGURE 4. Effect of TCD8+ precursor numbers on the simulated ID hierarchies. Simulated Ag-specific TCD8+ responses under different TCD8+ precursor scenarios are shown. The format is the same as used for Fig. 3B, and all modeling parameters were set to the default values shown in Tables II and III, unless otherwise indicated. (A) Simulations were performed assuming 50 precursors for each of the five TCD8+ responses. (B) Simulations were performed with 1000 precursors for each of the five TCD8+ responses. (C) Simulations were performed assuming 500 and 100 precursors for NP366- and PA224-specific TCD8+, respectively. (D) Simulations were performed assuming 500 and 1000 precursors for NP366- and PA224-specific TCD8+, respectively.
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 ~40% 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 ~25%, 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>
</tbody>
</table>

Data are median (95% Bayesian CI).
APCs contact as many as 50–300 T cells at any one time (24, 30), suggesting that the number of T cell binding sites/APC is in reality much $>10$ and that T cell competition, therefore, is more likely to 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 $T_{CD8^+}$ ID hierarchies following IAV infection are clearly dependent upon Ag dose. Interestingly, at low viral doses, the immunodominant $T_{CD8^+}$ against both LCMV and IAV are directed toward the epitopes that show the most rapid presentation kinetics (NP$_{396}$ and NP$_{366}$, 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 NP$_{366}$ $T_{CD8^+}$ dominate over those specific for PA$_{224}$, 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 NP$_{366}$ presentation was reduced, the dominance of NP$_{366}$ $T_{CD8^+}$, at low Ag levels was lost (data not shown). Moreover, when comparing the subdominant $T_{CD8^+}$ responses at low viral doses, we observed a similar trend: namely, the epitope with the highest and most rapid presentation (NS2$_{114}$) 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 PA$_{224}$ to ID, despite its poor presentation. Intuitively, this makes sense because one would expect that as both the number of pMHC-I and the total number of APCs increase, the overall level of pMHC-I for all five epitopes will be in relative excess and no aTCD8+ 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 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. It is the number of TCD8+ 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 NP$_{366}$ $T_{CD8^+}$ response at higher viral doses at day 7 might simply reflect a shift in the peak of the NP$_{366}$ $T_{CD8^+}$ response at a different time point. Indeed, the $T_{CD8^+}$ response to NP$_{366}$ was observed to occur later than for PA$_{224}$ 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 NP$_{366}$ $T_{CD8^+}$ response and that these cells, in fact, remained as dominant as PA$_{224}$ $T_{CD8^+}$ at higher viral doses. A related argument could be made for PA$_{224}$ $T_{CD8^+}$ at lower viral doses. To examine this issue, we experimentally assessed the $T_{CD8^+}$ response at different time points 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 PA$_{224}$ $T_{CD8^+}$ are likely to contribute to the ID of PA$_{224}$ 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.

Table V. Posterior estimates of TCD8+ precursor numbers

<table>
<thead>
<tr>
<th>NP$_{366}$</th>
<th>PA$_{224}$</th>
<th>PB1F2$_{62}$</th>
<th>NS2$_{114}$</th>
<th>PB1$_{703}$</th>
</tr>
</thead>
<tbody>
<tr>
<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 $10^8$ 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>NP$_{366}$</th>
<th>PA$_{224}$</th>
<th>PB1F2$_{62}$</th>
<th>NS2$_{114}$</th>
<th>PB1$_{703}$</th>
</tr>
</thead>
<tbody>
<tr>
<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 $10^8$ 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 $T_{CD8+}$ 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 $T_{CD8+}$ 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, $T_{CD8+}$ avidities, and naive precursor frequencies) for five IAV epitopes, armed only with the experimental details of relative Ag presentation and day-7 $T_{CD8+}$ responses. With recent technological developments, some of these parameters can now be estimated experimentally, but 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 $\sim$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 $\sim$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 $T_{CD8+}$ than NP566 $T_{CD8+}$, 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 $T_{CD8+}$ 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 $T_{CD8+}$ 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 $T_{CD8+}$ response peak and the overall number of responding $T_{CD8+}$, but not the ID hierarchy itself, were sensitive to changes in either the number of rounds of programmed $T_{CD8+}$ 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. In fact, the model does not take into direct consideration the role of inflammation, which might be expected to impact upon the $T_{CD8+}$ 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 $T_{CD8+}$ 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, $T_{CD8+}$ death rates, and $T_{CD8+}$ 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, $T_{CD8+}$ avidity estimates are moderately reliable, and $T_{CD8+}$ 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 $T_{CD8+}$ responses at day 7, are not sufficient to properly capture the entire dynamics of the $T_{CD8+}$ response. However, given the apparent success in using these same parameters to estimate epitope density, this seems
unlikely. Second, 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 TCD8+ precursors does not greatly influence the TCD8+ response (i.e., we obtained a large CI because a wide range of values successfully simulates the observed TCD8+ 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 TCD8+ 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 TCD8+ 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 TCD8+ ID hierarchy is established after influenza infection. Just as importantly, our model also provides a foundation upon which future refinements can be based and will hopefully facilitate the design of in vivo methods that can assist in the development of safe and effective vaccines.

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Disclosures
The authors have no financial conflicts of interest.

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