Quantitative Analysis of Long-Term Virus-Specific CD8\(^+\)-T-Cell Memory in Mice Challenged with Unrelated Pathogens

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The consequences for the long-term maintenance of virus-specific CD8\(^+\)-T-cell memory have been analyzed experimentally for sequential respiratory infections with readily eliminated (influenza virus) and persistent (gammaherpesvirus 68 [\(\gamma\)HV68]) pathogens. Sampling a broad range of tissue sites established that the numbers of CD8\(^+\) T cells specific for the prominent influenza virus D\(^{\text{aNP}}\)\(_{366}\) epitope were reduced by about half in mice that had been challenged 100 days previously with \(\gamma\)HV68, though the prior presence of a large CD8\(^+\) D\(^{\text{aNP}}\)\(_{366}\) population caused no selective defect in the \(\gamma\)HV68-specific CD8\(^+\) R\(^{\text{aP79}}\)\(^+\) response. Conversely, mice that had been primed and boosted to generate substantial \(\gamma\)HV68-specific CD8\(^+\) D\(^{\text{p56}}\) populations did not show any decrease in prevalence for this set of CD8\(^+\) memory cytotoxic T lymphocytes (CTL) at 200 days after respiratory exposure to an influenza A virus. However, in both experiments, the total magnitude of the CD8\(^+\)-T-cell pool was significantly diminished in those that had been infected with \(\gamma\)HV68 and the influenza A virus. The broader implications of these findings, especially under conditions of repeated exposure to unrelated pathogens, are explored with a mathematical model which emphasizes that the immune effector and memory “phenome” is a function of the overall infection experience of the individual.

Our understanding of virus-specific CD8\(^+\)-T-cell-mediated immunity has been revolutionized over the past 5 years by the availability of tetrameric complexes of major histocompatibility complex class I glycoprotein plus peptide (tetramers) for the flow cytometric analysis of responder lymphocyte populations (2, 9). Prior to the advent of the tetramer technology, the flow cytometric analysis of responder lymphocyte populations did not reflect the “bystander” activation of memory CD8\(^+\) T cells specific for other antigens (29). This perception was based on estimates of virus-specific CD8\(^+\)-T-cell frequency determined by limiting-dilution analysis in 6-day microcultures (10). These limiting-dilution analysis values turned out, on average, to be at least 10-fold lower than the epitope-specific CD8\(^+\)-T-cell counts found with the tetramers (13, 20).

The extent of proliferation of tetramer\(^+\) CD8\(^+\) T cells can now be measured directly by giving infected mice the thymidine analogue bromodeoxyuridine in drinking water and then staining for the high-bromodeoxyuridine-expressing tetramer\(^+\) CD8\(^+\) set (14). With this approach, it can be formally shown that intercurrent infection with an unrelated virus does indeed induce some apparently unrelated CD8\(^+\) memory T cells to cycle (3, 7). The extent of proliferation may, however, be threefold to fourfold less than that observed for naïve or memory T cells responding to the inducing pathogen. This “bystander” effect has been variously attributed to unpredicted cross-reactivities (22) at the level of the clonotypic T-cell receptor and to induction by cytokines that operate via a final pathway involving interleukin-15 (16, 35).

The questions raised immediately by the very high CD8\(^+\)-T-cell frequencies (20) generated in, for example, mice infected with lymphocytic choriomeningitis virus included how there can be sufficient space within the immune system to accommodate the need to respond to a variety of pathogens and what the consequences are for immune homeostasis. One possibility that has been looked at is that bystander activation of established memory CD8\(^+\)-T-cell populations leads to the deletion of suboptimally activated lymphocytes (19, 22, 31). Another consideration is that there is some form of biological “sensor” that determines the overall magnitude of the CD8\(^+\)-T-cell pool. However, the size of the total CD8\(^+\) set within a mouse can, at least for a time, be greatly expanded. This was shown very clearly for the murine gammaherpesvirus 68 (\(\gamma\)HV68), which causes massive CD8\(^+\)-T-cell proliferation following respiratory challenge (11, 28).

The present experiments analyzed the extent to which established virus-specific CD8\(^+\)-T-cell memory to a prominent (13, 27) influenza A virus epitope (D\(^{\text{aNP}}\)\(_{366}\)) is modified by subsequent exposure to \(\gamma\)HV68 and vice versa. There is no known cross-reactivity between these two viruses. Unlike previous studies that have addressed this question, the focus of our study was on the long-term consequences for immune memory.

MATERIALS AND METHODS

Mice and viral infection. Female, 8-week-old C57BL/6J (B6) mice (The Jackson Laboratory, Bar Harbor, Maine) were primed intraperitoneally with \(10^{10}\) 50% egg infectious doses of the A/PR/8/34 (PR8, H1N1) influenza A virus and

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then challenged intranasally with $10^6$ 50% egg infectious doses of the HKx31 (H3N2) virus (13) and later given $10^4$ PFU of /H9253HV68 intranasally (19). In a further study, naïve B6 mice were infected intranasally with /H9253HV68, boosted intraperitoneally with $5 \times 10^7$ PFU of a recombinant vaccinia virus (Vacc-p56) incorporating the AGPHNDMEI p56 peptide (25), and then challenged intranasally with the HKx31 virus. The interval between sequential infections was 6 weeks in every case. In all experiments, naïve, age-matched controls were challenged with the final virus used.

FIG. 1. Changes in the distribution of CD8$^+$ D$^\alpha$NP$_{366}$ T cells at days 35, 60, and 100 after intranasal challenge of influenza virus-primed mice (PR8-X31) with murine γHV68 (PR8-X31-γHV). B6 mice were primed intraperitoneally with the PR8 influenza A virus (H1N1), boosted intranasally with the HKx31 (H3N2) virus, and then challenged intranasally with γHV68 (PR8-X31-γHV) or left unchallenged (PR8-X31) as described in the text. The interval between each virus treatment was 6 weeks, and the 35-, 60-, and 100-day time points are after the final γHV68 challenge. The bronchoalveolar lavage (BAL), peripheral blood lymphocyte (PBL), and mediastinal lymph node (MLN) populations from day 100 from two mice were pooled, and each data point shows the mean ± standard deviation for four samples (eight mice), while the spleens, lungs, livers, bone marrow (BM), and cervical lymph node (CLN) were analyzed from individual mice and each data point shows mean ± standard deviation for five mice. Otherwise, the results from day 35 and day 60 that show error bars are for five individuals, while the absence of error bars (day 35) indicates that the samples were pooled. The asterisk designates groups that were significantly different ($P < 0.05$).

FIG. 2. Numbers of D$^\alpha$NP$_{366}$ CD8$^+$ T cells per organ were calculated from the total CD8$^+$-T-cell counts and the values shown in Fig. 1. The asterisk designates groups that were significantly different ($P < 0.05$). See Fig. 1 legend for abbreviations.
populations were enriched for the CD8+Kb79+, CD8+Dn77+ (A and B), CD8+Kb79+CD8+ (C), and all CD8+ T cells (panels D and E) were calculated for the day 100 (after γHV68) time point for the experiments shown in Fig. 1 to 4. The counts shown here were derived by summing the numbers per mouse for all the organs sampled. The spleens, lungs, livers, bone marrow, and cervical lymph node were analyzed from mice 1 to 5 of the eight sampled. The bronchoalveolar lavage, peripheral blood lymphocyte, and mediastinal lymph node samples were pooled for two mice (1 and 2, 3 and 4, 5 and 6, and 7 and 8). The numbers for the first three pools were halved and then added to the data for the other organs. The asterisk designates groups that were significantly different (P < 0.04).

**Tissue sampling.** The mice were anesthetized with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich, St. Louis, Mo.) and bled from the right axillary artery (17). The blood was collected into heparinized phosphate-buffered saline (1,000 U/ml; Elkins-Sinn Inc., Cherry Hill, N.J.), and the inflammatory population was recovered from the pulmonary lung by bronchoalveolar lavage. Each mouse was then perfused with 40 to 60 ml of heparinized phosphate-buffered saline, and the lungs, mediastinal lymph nodes, cervical lymph nodes, livers, and spleens were recovered from the pneumonic lung by bronchoalveolar lavage. Each mouse was then perfused with 40 to 60 ml of heparinized phosphate-buffered saline, and the lungs, mediastinal lymph nodes, cervical lymph nodes, livers, and spleens were analyzed from individual mice. While the spleens, lungs, livers, bone marrow, and cervical lymph node populations were pooled from two mice (1 and 2, 3 and 4, 5 and 6, and 7 and 8). The numbers for the first three pools were halved and then added to the data for the other organs.

**Flow cytometry.** Spleen, cervical lymph node, and mediastinal lymph node populations were enriched for the CD8+ set by in vitro depletion with monoclonal antibodies to La1 (Becton Dickinson, Mountain View, Calif.). The numbers presented in some of the figures were calculated from the total cell counts, the percent CD8+, and the percent CD8+ tetramer.

**Mathematical modeling.** The two basic variables in the mathematical model are the virus populations and memory CTL specific for each virus. It is assumed that a host can become infected with n different viruses. Virus of type i is denoted by vi, and the memory CTL response specific to that virus is denoted by zi, where i = 1, 2, . . . , n. The model is given by the following set of differential equations:

\[
\frac{dvi}{dt} = p_v (1 - \frac{v_i}{k_v}) - p_v z_i
\]

\[
\frac{dz_i}{dt} = c_v z_i - b_z z_i - z_i \sum_{j=1}^{n} \alpha_j v_j
\]

The virus replicates at a rate \(r_v\). Replication is limited by the availability of susceptible host cells, which is captured in the parameter \(k_v\). Virus replication is inhibited by the specific CTL response at a rate \(p_v\). The memory CTL response activates and proliferates in response to its specific antigen at a rate \(c_v\). In the absence of antigen, the CTL response has an average life span of \(1/\beta_v\). In addition, the model assumes that heterologous antigenic stimuli, \(v_j\), reduce the memory CTL response to virus i (\(z_i\)) at a rate \(g_i\). Thus, the model assumes the following mechanism for the reduction of memory CTL upon heterologous infection: heterologous antigen induces activation of the memory CTL through low-level cross-reactivity or bystander effects. Since the activation is induced by noncognate stimulation, these memory CTL do not go on to expand and increase significantly, but die (22, 31). Hence, the presence of the heterologous antigen is the force driving the reduction of CTL memory. An alternative idea would be that the CTL response to the heterologous infection competes with, and reduces, previously established memory. Such an assumption would, however, lead to the prediction that this new CTL response would also be selectively reduced if the existing memory pool is large.

When plotting the results of the model, it was assumed for simplicity that all infections have identical parameters. The qualitative outcome of the model does...
not, however, depend on this simplification. For a mathematically detailed analysis of the equations, see reference 32. The use of parameter values specific for the infections in question will require further experiments which would allow us to estimate the relevant parameters (presently unknown).

RESULTS

Challenging influenza virus-primed mice with γHV68. The C57BL/6J (B6) mice were first primed and boosted (13) with two different influenza A viruses (H3N2 and then H1N1) to give large numbers of CD8$^+$ D$^{\text{NP}}_{366}$ $^+$ T cells, rested for 6 weeks, and then challenged intranasally with γHV68 (6). The influenza viruses cause a transient, lytic infection that is substantially confined to the superficial epithelium of the trachea and lung and is generally resolved within 8 to 12 days (1, 8). There is no cross-neutralization (with antibody) between the H1N1 and H3N2 viruses, though both carry the NP$_{366-374}$ peptide that dominates the CD8$^+$-T-cell response (15). Respiratory exposure to γHV68 also induces a transient, lytic infection in lung epithelial cells (21, 23). However, this virus establishes latency in B cells and macrophages, and there is indirect evidence of sporadic reactivation to lytic phase for months after the initial encounter with the virus (3, 12, 34). In this respect it resembles other gammaherpesviruses, like Epstein-Barr virus (EBV), which can be detected routinely by PCR in the oropharynx of >30% of the human population (16).

Groups of at least five γHV68-challenged, H3N2- and H1N1-primed B6 mice (together with the appropriate controls) were sampled at 35, 60, and 100 days after the γHV68 challenge. At every time point tested, the CD8$^+$ D$^{\text{NP}}_{366}$ $^+$ T-cell frequencies were significantly reduced for the spleen, peripheral blood lymphocytes, and bone marrow recovered from those that had also been exposed to γHV68 (Fig. 1). This was also true for the lymphocytes that were isolated from the perfused, digested pneumonic lung (17) and for the inflammatory population that had first been removed by bronchoalveolar lavage. This pattern held up for the spleen, lung, and bone marrow when we calculated the numbers of CD8$^+$ D$^{\text{NP}}_{366}$ $^+$ T cells in each site (Fig. 2). Though the difference was not apparent for the draining mediastinal lymph node, cervical lymph node, bronchoalveolar lavage, and liver populations from those sampled on day 100 (Fig. 2), the total CD8$^+$ D$^{\text{NP}}_{366}$ $^+$ T-cell counts per mouse were approximately halved in the γHV68-infected, influenza virus-primed mice (Fig. 3A and B). The effect was selective, as estimates of the total CD8$^+$ lymphocyte count showed that there was no significant difference between the groups of influenza virus-immune mice that were and were not given γHV68 (Fig. 3D and E).

We also asked whether the presence of large, influenza virus-specific memory T-cell populations at the time of γHV68 challenge would modify the γHV68-specific response. Though the γHV68-specific CD8$^+$ D$^{\text{p56}}$ $^+$ set expands first after intranasal exposure to γHV68, this quickly gives way to CD8$^+$ K$^{\text{p79}}$ $^+$ T cells, which predominate in the long term (24). The only significant difference in CD8$^+$ K$^{\text{p79}}$ $^+$ T-cell numbers was found for the liver on day 100, when the counts were decreased for the mice that had first been exposed to the influenza A viruses (Fig. 4). Calculating the totals (per mouse) for CD8$^+$ K$^{\text{p79}}$ $^+$ T cells also showed that the value overall was significantly lower for the mice that had first been primed and boosted with the influenza A viruses (Fig. 3C). However, unlike the effect for the CD8$^+$ D$^{\text{NP}}_{366}$ $^+$ T cells (Fig. 3A, B, C, and D), this difference (Fig. 3C) was not specific for the CD8$^+$ K$^{\text{p79}}$ set, as the total counts for all CD8$^+$ T cells were...
significantly lower in the mice that were given both influenza virus and γHV68 (Fig. 3D).

Over the first 20 days or so after intranasal challenge with γHV68, the proliferation rate (measured by 6-day bromodeoxyuridine incorporation) of the CD8$^{+}$ D$p56^{+}$ and CD8$^{+}$ K$p79^{+}$ sets reached peak values of $>85\%$, with evidence of cycling falling off more rapidly for the CD8$^{+}$ D$p56^{+}$ population (3). The CD8$^{+}$ K$p79^{+}$ T cells also turned over more slowly with elapsed time, supporting other indications that the lytic-phase K$p79$ epitope is present at higher levels in the long term. In fact, the CD8$^{+}$ K$p79^{+}$ T cells cycled at levels substantially above background for several months. During the acute phase of γHV68 infection (until day 16), an established CD8$^{+}$ D$p56^{+}$ T-cell population was also found to cycle at levels substantially above background (4 to 6%). The present findings establish beyond any doubt that this increased replication does not lead to augmentation of the CD8$^{+}$ D$p56^{+}$ T-cell population and are in accord with findings from other experimental systems that the consequence of such bystander activation may be partial depletion of the memory T-cell pool (22).

**Influenza virus pneumonia in mice with large numbers of CD8$^{+}$ D$p56^{+}$ T cells.** Respiratory exposure to the HKx31 influenza A virus causes a substantial, nonfatal pneumonia, which generally starts to resolve within 14 days of infection (1). Memory T cells specific for unrelated antigens are readily detected in the population recovered by bronchoalveolar lavage (7, 29), and it is not clear whether such lymphocytes return to the recirculating pool or are lost to the host. Some increase in cycling is found for these “irrelevant” T cells (7).

Influenza virus challenge of mice that had been infected with γHV68 and then boosted with Vacc-p56 (4, 25) did not significantly modify the numbers of CD8$^{+}$ D$p56^{+}$ T cells recovered from the spleen on day 14 (Fig. 5A). Significantly more CD8$^{+}$ D$^{3}$NP$_{366}^{+}$ T cells were detected in the spleens of the γHV68-Vacc-p56 mice that were sampled on day 14, but the converse was true on day 200 (Fig. 5B). However, the total numbers of CD8$^{+}$ T cells in the spleen were also significantly reduced on day 200, though not on day 14 (Fig. 5C). In the present experiments, this did not lead to any decrease in the size of the established CD8$^{+}$ D$p56^{+}$ T-cell population in the spleen, though there is the caveat that the sporadic reactivation of γHV68 to lytic phase may have been associated with some degree of further antigenic stimulation. The effect of persistent if low-level γHV68 infection on T-cell turnover rates may also have had some influence on the significantly decreased numbers of both CD8$^{+}$ and CD8$^{+}$ D$^{3}$NP$_{366}^{+}$ T cells on day 200 (Fig. 5).

**DISCUSSION**

The preceding experimental analysis (Fig. 1 to 5) shows what happens when there are two sequential infections. In nature,
however, people are subject to many such events. We thus turned to a mathematical modeling approach for insight into how repeated exposure to pathogens might impact the establishment and maintenance of memory in the CD8^+ T-cell compartment (32). The model assumes that the mechanism of CTL reduction is the activation of the memory CTL through low-level cross-reactivity or bystander effects induced by the heterologous antigen. The first step was to model the two-virus situation (Fig. 6A) that we analyzed experimentally (Fig. 1 to 4) in order to show that the mechanism underlying the model is consistent with observations. This simulation (Fig. 6A) does not, for simplicity, include any consideration of latency.

The model (Fig. 6A) starts with one infection which is resolved by a specific CTL response, corresponding to influenza virus in the experiment. After the virus is cleared, the memory CTL are long lived and decay only at a very slow rate. During this memory phase, a second virus (γHV68) is added to the system. The maximum virus load phase of lytic replication is resolved by the effector CTL response, leading to long-term CTL memory and control. During the time that the γHV68
load increases, the CTL memory response to influenza virus is reduced (Fig. 6A). As the γHV68 load is diminished, the CTL memory response against influenza virus is no longer negatively affected. The experimental data show that the CTL response against γHV68 is not selectively impaired (Fig. 3) despite the presence of high initial numbers of influenza virus-specific T cells. This is in accord with the model simulation, assuming that the heterologous antigen itself, and not CTL competition, is the reason for the reduction in CTL memory (Fig. 6A).

The two-virus situation (Fig. 6A) can then be extrapolated (Fig. 6B) to illustrate how such dynamics play out in the long term when a host is infected with a variety of pathogens over time. An obvious consequence is that established CTL memory to any given antigen will be successively diminished as more infections are experienced. Hence, the level of protection against secondary challenge will be concomitantly decreased. The consequences can, however, be more complicated. Some viruses, such as γHV68 and EBV, persist at low levels and are never eradicated. In addition, if several infections occur simultaneously or at close intervals, the model (Fig. 6B) suggests that a pathogen that might normally be cleared could become persistent, though held at low levels by a suboptimal response. At first, infections are readily controlled, and the total size of the antigen-specific CTL memory pool is relatively small. With the addition of further infection experiences, the overall pathogen load increases slightly and the total pool of memory CTL rises. If the number of infection events crosses a threshold, an accelerated loss of virus control will be seen with each sequential event (Fig. 6B). When the number of infections increases further, the overall CTL memory pool will in fact decrease with every additional event. This decline can potentially lead to the collapse of all memory CTL. The net consequence would be that the host becomes overwhelmed with pathogens.

The experimental analysis thus illustrates what could occur following a de novo encounter (Fig. 1 to 4) with EBV (5) or when people who are carriers of EBV are infected with other pathogens (Fig. 5). This describes the situation for the majority of humanity, as almost every adult is an EBV carrier (33). The initial encounter with γHV68 causes massive CD8+ T-cell proliferation (11, 28) that is in some senses similar to the infectious mononucleosis syndrome characteristic of primary EBV infection in adolescents (5). It is reassuring that the reduction in T-cell memory is, in both cases, only about twofold or less. This relatively subtle effect is not likely to have much impact on the capacity to develop an effective recall response.

However, extrapolating these effects with the mathematical model (Fig. 6) illustrates how the lifetime immune effector and memory “phenome” will be shaped by the overall antigenic (particularly the infection) history of the individual. The consequences for those in high-infection-load environments are potentially disastrous. Obvious examples are people living in the developing world and those involved in high-risk behavior such as intravenous drug use. Such interactive effects need to be kept in mind as we attempt to develop effective vaccination strategies for these vulnerable populations. Furthermore, the model (Fig. 6) has important implications for antigenically variable pathogens (such as human immunodeficiency virus and hepatitis C virus) that expose the immune system to a wide variety of different antigenic stimuli over a relatively short time. This could result in the persistence of the virus variants, with overall virus load increasing as more antigenic strains are produced (32). When the number of antigenic strains crosses a threshold, CTL memory will decline and eventually collapse.

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