Memory Inflation: Continuous Accumulation of Antiviral CD8+ T Cells Over Time

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CD8+ T lymphocytes play an important role in the control of intracellular pathogens during both acute and persistent infections. This is particularly true in the case of persistent herpesviruses such as human CMV, which are typified by large virus-specific CD8+ T cell populations during viral latency. To understand the origin of these populations and the factors shaping them over time, we investigated the CD8+ T cell response after murine CMV (MCMV) infection. The kinetics of the acute response were characterized by rapid expansion of activated T cells, followed by a contraction phase. Thereafter, we observed a striking pattern, where MCMV-specific memory CD8+ T cells steadily accumulated over time, with 20% of all CD8+ T cells at 1 year specific for one MCMV epitope. Accumulation of MCMV-specific CD8+ T lymphocytes was seen in all organs tested and was associated with continuous activation of specific CD8+ T lymphocytes, primarily within lymph nodes. The pattern of accumulation was observed in only two of five epitopes tested, and was accompanied by a gradual restriction in usage of the variable region of the TCR β-chain over time. This novel pattern of a virus-specific CD8+ T cell response suggests that continuous or repetitive exposure to Ag can slowly mold memory T cell populations over time. This may be relevant for understanding the evolution of the large human CMV-specific CD8+ T cell populations seen in humans. The Journal of Immunology, 2003, 170: 2022–2029.

Materials and Methods

Mice and animal experiments

Mice were purchased from Biomedical Services Unit, John Radcliffe Hospital (Oxford, U.K.). Animals were bred under specific pathogen-free conditions and housed in a controlled conventional unit during the experiment. All animal experiments were performed with age-matched female BALB/c...
mice with permission of the home office according to the Animals (Scientific Procedures) Act of 1986, requiring the use of minimal numbers of animals. Mice were infected i.v. with 10⁶ PFU MCMV or 5 × 10⁶ PFU Vac89 in 200 μl of PBS. Organs were prepared after i.v. perfusion with 15–20 ml of PBS (4°C), followed by preparation of single-cell suspensions by homogenization over a fine mesh grid within 1 h of sacrifice. To separate lymphocytes, liver preparations were then centrifuged over Percoll (Sigma-Aldrich, Poole, U.K.) before staining. Other organ suspensions were centrifuged once to remove adherent debris and used directly for staining and flow cytometric analysis. Bronchoalveolar lavage (BAL) was performed by insertion of a fine cannula into the trachea after perfusion, followed by direct lavage of PBS into the lungs. Cells were centrifuged and used directly for staining and flow cytometric analysis.

### Viruses

MCMV (Smith strain, ATCC VR-194; American Type Culture Collection, Manassas, VA) and Vac89 (15) were provided by Prof. U. Koszinowski (Department of Virology, Max von Pettenkofer Institute). MCMV was grown on mouse embryonic fibroblasts and purified by sucrose gradient centrifugation according to established protocols (16). MCMV titer of virus stocks and organ homogenates were determined by virus plaque assay on mouse embryonic fibroblasts as described using centrifugal enhancement of infectivity (16). Titters are expressed as log_{10} (PFU MCMV/organ).

The detection limits of the assays are indicated in Fig. 1. Vac89 was grown and plaque on thymidine kinase (TK)-deficient (TK-) cells. Different lytic states of TK- cells were used for infection. A single stock was used for the majority of experiments, particularly for those following the development of immunity over time.

### Assessment of IFN-γ-secreting peptide-specific CD8⁺ T lymphocyte frequencies

Frequencies of IFN-γ-producing peptide-specific T cells in the spleen were quantified by intracellular IFN-γ staining after stimulation with specific peptide, according to the protocols of the supplier of reagents and Abs (BD Biosciences, San Jose, CA). After 6-h peptide (10⁻⁶ M) stimulation, spleen cells were stained with allophycocyanin- or PerCP-labeled rat anti-mouse CD8 (clone 53-6.7), fixed and permeabilized, and then stained with FITC-labeled rat anti-mouse IFN-γ (clone XMG1.2) or with an FITC-labeled rat IgG1-isotype control Ab. Samples were analyzed with a FACS calibur (BD Biosciences) using CellQuest software (BD Biosciences). Gates were set according to the isotype control and frequencies are given as percentage of IFN-γ-secreting cells of total CD8⁺ T cells.

The following peptides defining MCMV-derived CD8⁺ T cell epitopes in the MHC haplotype H₂⁻ were from different MCMV proteins were used: m123/pp89 (pp89, H₂-Ld-restricted, 168AGPPRYSRI 265 (21)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)), M84/p65 (M84, H₂-Kd-restricted, 243YGPSLYRRF 251 (18)). As a negative control, a peptide derived from an peptide from an epitope following two different infections. We next measured the frequency of Ld-restricted, pp89-specific CD8⁺ T cells by tetramer staining (pp89-tet) or intracellular cytokine staining for IFN-γ (pp89-IFN-γ) after infection of BALB/c mice with MCMV or Vac89. Vaccinia virus does not persist or reactivate after resolution of primary infection in immunocompetent mice (24), whereas MCMV establishes a latent infection in many sites with a continuous potential for reactivation (1). Thus, we compared the frequencies of CD8⁺ T cells directed against the same epitope following two different infections.

In the blood, an initial peak of 5.9% of pp89-specific CD8⁺ T cells was reached 8 days after MCMV infection. A contraction phase then ensued and frequencies declined to 3.4% at day 15 and were maintained at this level until day 40 (3.7%). Remarkably, thereafter pp89-specific CD8⁺ T cell frequencies began to rise again at a time when replicating MCMV was no longer detectable. More than 20% of CD8⁺ T cells were specific for this single viral epitope 400 days after MCMV infection (Fig. 1B).

In the spleen, a similar biphasic pattern was observed. Initial expansion of pp89-specific CD8⁺ T cells peaking at day 8–10 (5.9%) was followed by a contraction phase and, from day 40 onward, a gradual increase in pp89-specific T cells. By day 400, 15.3% of CD8⁺ T cells were specific for pp89 by tetramer staining, and at least 80% of these cells produced IFN-γ within 6 h of Ag recognition (Fig. 1C). In terms of total lymphocyte numbers, after an initial expansion and limited contraction phase, an accumulation of the total number of pp89-specific cells per spleen was also observed (2- to 3-fold between days 40 and 400) (Fig. 1D).

In contrast, after infection with Vac89, the peak of the pp89-specific CD8⁺ T cell response was reached between days 6 and 9.
(2% of CD8\(^+\) T cells were tetramer positive). By day 28, the frequencies had halved and were maintained at a constant level thereafter (~0.7% of CD8\(^+\) T cells). Similar results were obtained using both tetramer and intracellular cytokine staining (Fig. I, B–E).

This accumulation of virus-specific CD8\(^+\) T cells after MCMV infection was not restricted to the blood and spleen, as a similar pattern was observed in other organs. We analyzed the frequency of pp89-specific CD8\(^+\) T cells in LN (cervical, mesenteric, and axillary) and in organ infiltrates from liver, lung (BAL), and salivary gland over time with an increase in frequency during viral latency seen in all cases (Fig. 2A). Additional data from other organs, including ovaries, adrenal glands, and bone marrow, confirmed this general pattern (data not shown). Thus, during latency, there is an accumulation in multiple body compartments of pp89-specific CD8\(^+\) T lymphocytes in both the spleen, and at indicated time points, MCMV titers were determined in spleen (○) and salivary gland (□) by virus plaque assay (lung and liver not shown). MCMV titers represent log_{10} PFU (PFU/organ). Each line represents the mean of three to four mice per time point. Error bars indicate the SD. Detection limits of the assays were as follows: spleen, 1.6 (40 PFU/organ), and salivary gland, 2.0 (100 PFU/organ). B, pp89-specific CD8\(^+\) T cells in blood: mice were infected with 10\(^{6}\) PFU MCMV (●) or 5 × 10\(^{5}\) PFU Vac89 (○) i.v. At the indicated time points, blood was taken and PBL were stained with pp89 tetramer and anti-CD8. The percentage of CD8\(^+\) T cells staining with pp89 tetramer is plotted over time. Each line represents the mean of three to four mice per experimental group. Error bars indicate the SD within experimental groups. C and D, pp89-specific CD8\(^+\) T cells in the spleen: at indicated time points, spleen cells were harvested and the frequency of pp89-specific T cells was determined by pp89 tetramer staining (MCMV (●) and Vac89 (○)) and by pp89-IFN-γ (MCMV (○) and Vac89 (□)). In C, the percentage of CD8\(^+\) T cells positive for pp89 tetramer or pp89-IFN-γ is plotted over time, and in D, the total number of pp89-specific CD8\(^+\) T cells per spleen are depicted (number × 10\(^5\)). Each line represents the mean of three to four mice per experimental group. Error bars indicate the SD within an experimental group. E, FACs dot plots from mice 10 and 400 days after MCMV infection, from mice 6 and 300 days after Vac89 infection, and from naive mice. Panels were gated on live lymphocytes without B cells (excluded by anti-CD45R staining). Numbers indicate the mean percentage of pp89 tetramer- or IFN-γ-producing CD8\(^+\) T cells of three to four mice per group. Day 300 and day 400 are data from a single experiment of four mice per group. Data were confirmed by two similar experiments extended until day 250 after infection.

**FIGURE 1.** Accumulation of pp89-specific CD8\(^+\) T cells after infection with MCMV. A, MCMV titer: mice were infected with 10\(^6\) PFU MCMV i.v., and at indicated time points, MCMV titers were determined in spleen (○) and salivary gland (□) by virus plaque assay (lung and liver not shown). MCMV titers represent log_{10} PFU (PFU/organ). Each line represents the mean of three to four mice per time point. Error bars indicate the SD. Detection limits of the assays were as follows: spleen, 1.6 (40 PFU/organ), and salivary gland, 2.0 (100 PFU/organ). B, pp89-specific CD8\(^+\) T cells in blood: mice were infected with 10\(^6\) PFU MCMV (●) or 5 × 10\(^{5}\) PFU Vac89 (○) i.v. At the indicated time points, blood was taken and PBL were stained with pp89 tetramer and anti-CD8. The percentage of CD8\(^+\) T cells staining with pp89 tetramer is plotted over time. Each line represents the mean of three to four mice per experimental group. Error bars indicate the SD within experimental groups. C and D, pp89-specific CD8\(^+\) T cells in the spleen: at indicated time points, spleen cells were harvested and the frequency of pp89-specific T cells was determined by pp89 tetramer staining (MCMV (●) and Vac89 (○)) and by pp89-IFN-γ (MCMV (○) and Vac89 (□)). In C, the percentage of CD8\(^+\) T cells positive for pp89 tetramer or pp89-IFN-γ is plotted over time, and in D, the total number of pp89-specific CD8\(^+\) T cells per spleen are depicted (number × 10\(^5\)). Each line represents the mean of three to four mice per experimental group. Error bars indicate the SD within an experimental group. E, FACs dot plots from mice 10 and 400 days after MCMV infection, from mice 6 and 300 days after Vac89 infection, and from naive mice. Panels were gated on live lymphocytes without B cells (excluded by anti-CD45R staining). Numbers indicate the mean percentage of pp89 tetramer- or IFN-γ-producing CD8\(^+\) T cells of three to four mice per group. Day 300 and day 400 are data from a single experiment of four mice per group. Data were confirmed by two similar experiments extended until day 250 after infection.

**Oligoclonality of pp89-specific CD8\(^+\) T lymphocyte population**

We next addressed the issue of whether these expanding populations represented the accumulation of polyclonal CD8\(^+\) T lymphocyte populations, or oligoclonal populations with restricted TCR usage (as has been observed in HCMV (11, 25)). To study this directly ex vivo, we identified pp89-specific T cells using the tetramer and costained with a panel of Vβ-specific TCR Abs. We analyzed the proportions of pp89-specific cells that expressed TCRs of different Vβ types over time. This experiment (Fig. 2B) shows at later time points an increase in the proportion of the tetramer-positive population that use the Vβ 8.1 or Vβ 8.2 TCR chain. Early after MCMV infection (days 8–12), pp89-specific T cells used a diverse set of Vβ TCR chains. The Vβ 8.1/8.2 usage among the pp89-specific T cell population then increased over time: about half the tetramer-positive population expressed Vβ 8.1 or 8.2 by day 270. As a negative control, Vβ-specific TCRs (e.g., Vβ3) which are not expressed in the BALB/c mouse were <0.1% (data not shown), and the background expression of Vβ 8.1/8.2 in tetramer-negative CD8\(^+\) T cells did not increase significantly over time. Thus, we have shown a restriction of TCR Vβ chain usage among the tetramer-positive population, which parallels the continuous accumulation of Ag-specific T cells during viral latency.

**Phenotype of pp89-specific T cells early and late after infection with MCMV and Vac89**

The phenotype of pp89-specific CD8\(^+\) T cells was analyzed early and late after infection with MCMV and Vac89. A panel of phenotypic markers was used to assess the activation and maturation state of pp89-specific CD8\(^+\) T cells. Ten days after MCMV infection, pp89-specific T cells showed an activated phenotype in the spleen (mainly CD43^high and CD62L^low). Only 9% of pp89-specific CD8\(^+\) T cells were positive for the early activation marker
CD69, and the percentage of CD25^{high}/CD8^{+} T cells was negligible (Fig. 3). The vast majority expressed the proinflammatory adhesion molecules CD44 and CD18.

Six days after infection with Vac89, specific CD8^{+} T cells showed slightly lower expression levels of CD43^{1B11} and CD69, while the expression level of CD44, CD18, and CD62L was comparable to those after MCMV infection. During the memory phase of MCMV and Vac89 infection, pp89-specific CD8^{+} T cells were CD43^{1B11-low}, CD44^{high}, and CD18^{high}. However, there was a clear difference in the expression of CD62L: the majority of pp89-specific CD8^{+} T cells remained CD62L^{low} late after MCMV infection, whereas after Vac89 infection, only a minority were CD62L^{low}. In addition, as late as 400 days after MCMV infection, a distinct population (11%) of pp89-specific cells expressed CD69, slightly more than during acute infection.

We went on to analyze CD69 expression on tetramer-positive CD8^{+} T cells in different organs. Strikingly, while moderate amounts of CD69^{high} pp89-specific T cells could be seen in the spleen, blood, liver, and lung, the frequency was significantly higher in the LN, often reaching 30% (Fig. 4A). This pattern was reproducible across LN from the mesenteric, cervical, and axillary groups. As controls, tetramer-negative CD8^{+} T cells from LN in MCMV- and Vac89-infected mice and naive mice all showed much lower levels of CD69 expression (percentage of CD69^{high} was <10%; data not shown). The relationship between the cells in the different compartments over time is complex (26), but a reasonable conclusion from these experiments would be that Ag-specific activation of pp89-specific CD8^{+} T cells is occurring within the LN throughout MCMV latency.

Table 1. **Total counts of virus-specific CD8^{+} T cells in spleen over time**

| Virus | Day Postinfection | Total Number of Epitope-Specific CD8^{+} T Cells/Spleen (×10^{6}) |
|-------|-------------------|---------------------------------------------------------------|
|       |                   | E1E/pp89 m04 M83 M84                                         |
| MCMV  | 8                 | 143 ± 36 16 ± 6 6 ± 1.7 ± 3 38 ± 3.8                         |
|       | 32                | 82.5 ± 2 2.5 ± 0.8 2 ± 0.8 1.5 ± 0.5                           |
|       | 400               | 150 ± 21 1.1 ± 0.2 1.8 ± 0.9 1.2 ± 0.2                         |
| Vac89 | 6                 | 103 ± 38 ND ND ND                                             |
|       | 28                | 16 ± 4 ND ND ND                                               |
|       | 300               | 71.9 ± 3.4 ND ND ND                                            |

Data are derived from experiments as shown in Figs. 1 and 5 (six to eight mice per group). Total counts of splenocytes were made and virus-specific T cell frequencies were calculated. The frequencies for m64-specific cells (which were analyzed in a separate set of experiments) showed frequencies similar to those of pp89-specific cells (see Fig. 5, A and B).
Proliferation of pp89-specific memory CD8+ T cells late after MCMV infection

To examine the proliferation of tetramer-positive CD8+ T lymphocytes in vivo, we performed BrdU-labeling experiments. After 10 days of labeling, the incorporation of BrdU into tetramer-positive and tetramer-negative CD8+ T lymphocytes was analyzed in MCMV-infected mice. An example of such an experiment is shown in Fig. 4B. As expected, a very high proportion of tetramer-positive cells have incorporated BrdU during the acute phase of infection (~90%). The rate of incorporation of BrdU in tetramer-positive cells during the latent or memory phase—250 days later—was approximately one-fifth of this. A small but consistent difference in the incorporation of BrdU into tetramer-positive cells and total CD8+ T cells was seen (mean 16.7% vs 12.7%), as has been noted in similar experiments after infection with murine γ-herpesvirus-68, where similar incorporation rates were obtained (27). The lower frequencies of pp89-specific cells late afterVac89 infection limit the information that can be obtained from such analyses during the memory phase. Therefore, overall, the rate of proliferation of tetramer-positive cells, as judged using this assay, was sustained during MCMV latency at a level substantially lower than that seen acutely but on a par with other persistent infections.

Long-term accumulation of memory cells is not uniform and not restricted to pp89-specific CD8+ T cells

To evaluate whether accumulation of MCMV-specific CD8+ T cells was restricted to the IE1/pp89-specific response, we measured specific CD8+ T cell frequencies for epitopes derived from five different viral proteins up to 200 days after MCMV infection. Of these, only pp89 is generated during the IE phase of MCMV replication (17), gp34 (m04), p65 (M84), and probably the m164 gene product are expressed during the early phase and the structural virion protein pp105 (M83) is expressed during the late phase (18–21). The MCMV-specific CD8+ T cell response, as measured by intracellular cytokine staining for IFN-γ, was clearly dominated by pp89-specific and m164-specific cells during productive infection and particularly during latency. Ten days after infection, 3–4% of CD8+ T cells were specific for pp89 and m164-derived epitopes (Fig. 5A), while only 0.2–0.5% were specific for the other three epitopes (Fig. 5B). Thereafter, CD8+ T cell responses for subdominant epitopes all declined to 0.07% (close to the limit of detection of the assay) and were maintained at such low levels without evidence for accumulation. This was in stark contrast to pp89- and m164-specific cells, which increased in frequency over the period of latency (Figs. 1 and 5, A and B; Table I).

Discussion

CMV is a major pathogen that infects most of the world’s population. It causes little disease normally because it is well controlled by the host’s immune system. A number of mechanisms are important, but a key element is the CD8+ T lymphocyte response. However, if these mechanisms fail, as a result of immunosuppression (e.g., during AIDS or after transplantation), the disease caused by CMV is very significant. Similarly, it may also be a significant pathogen in the fetus and newborn. Understanding the immune response against CMV is of relevance for two main reasons: 1) because of its pathogenic potential and 2) in a normal human immune system, a significant effort is expended daily in simply suppressing CMV reactivation.

Of all the CD8+ T cell responses against human virus infections uncovered with recently developed technologies, those against herpesviruses like EBV and CMV are by far the most striking (28). After acute EBV infection, massive CD8+ T cell responses directed against lytic viral epitopes are culled substantially with resolution of lytic infection while other responses become more prominent during latent infection (29–32). Because acute HCMV infection is usually clinically silent, very little is known about how antiviral CD8+ T cells evolve from primary infection into the large populations often seen in asymptomatic seropositive individuals (up to 10% of CD8+ T cells), particularly in the elderly (8–13). This observation prompts the immediate question of how such responses emerge and what their evolution is in the long term. We have addressed these questions using the murine model of CMV infection. This allowed us to experimentally address the mechanisms behind the development of such populations and the factors behind their maintenance.

Most strikingly, after the initial expansion and contraction phases, the frequency of CD8+ T cells specific for some MCMV-
and the mean percentage of IFN-γ production. Permeabilized cells were stained with anti-IFN-γ and anti-CD8 Abs, and the mean percentage of IFN-γ-producing CD8+ T cells of three to four mice per group is plotted. Error bars indicate the SD within an experimental group. The following MCMV-derived peptides from five different viral proteins were used (MHC class I presenting molecule): pp89 (H2-Ld), m04/pp34 (H2-Dd), M84/pp65 (H2-Kd), M83/pp105 (H2-Ld), and m164 (H2-Dd). A, Frequency of IFN-γ-producing T cells specific for the immunodominant epitopes pp89 and m164. B, Frequency of IFN-γ-producing T cells specific for subdominant epitopes derived from m04, M83, and M84. Note the 10-fold difference in scale between A and B. One of three comparable experiments is shown.

FIGURE 5. Long-term accumulation of memory cells is not restricted to pp89-specific CD8+ T cells. BALB/c mice were infected with 10⁶ PFU MCMV i.v. Spleen cells were taken at different time points thereafter and were tested ex vivo by intracellular IFN-γ staining after peptide stimulation. Permeabilized cells were stained with anti-IFN-γ and anti-CD8 Abs, and the mean percentage of IFN-γ-producing CD8+ T cells of three to four mice per group is plotted. Error bars indicate the SD within an experimental group. The following MCMV-derived peptides from five different viral proteins were used (MHC class I presenting molecule): pp89 (H2-Ld), m04/pp34 (H2-Dd), M84/pp65 (H2-Kd), M83/pp105 (H2-Ld), and m164 (H2-Dd). A, Frequency of IFN-γ-producing T cells specific for the immunodominant epitopes pp89 and m164. B, Frequency of IFN-γ-producing T cells specific for subdominant epitopes derived from m04, M83, and M84. Note the 10-fold difference in scale between A and B. One of three comparable experiments is shown.

derived epitopes continues to increase long after the resolution of primary infection. This accumulation, which we have termed memory “inflation,” was not uniform. CD8+ T cells specific for IE1/pp89 and for m164 clearly showed this phenomenon, but frequencies of cells specific for other viral epitopes did not increase. Both epitopes that showed increases in population size are already relatively dominant in acute disease, but the pattern is not restricted to IE proteins nor to specific MHC class I molecules. From the experiments presented in this study, we cannot conclude that immunodominance during early productive infection is necessary for later accumulation, because other factors within the virus-host balance might influence the long term kinetics of the CD8+ T cell response considerably. This issue is currently under investigation in C57BL/6 mice using rMCMVs, which express foreign CD8+ T cell epitopes (U. Karrer, S. Sierro, P. Kleenerman, R. E. Phillips, M. Wagner, H. Hengel, and U. H. Koszinowski, unpublished data). The initial infectious dose used, although relatively high in this study, was not important in determining the later accumulation of cells, as similar results were obtained using infectious doses three orders of magnitude lower (data not shown).

One reasonable explanation for the accumulation of IE1/pp89-specific CD8+ T cells would be that this protein is expressed very early at each site where reactivation is initiated from latent MCMV genomes. Indeed, mRNA for IE1/pp89 and IE2 has been found during MCMV latency, but usually transcription does not proceed to those early and late genes studied to date, unless MCMV reactivation is promoted by immunosuppression or allogenic transplantation (5–7, 33, 34). Thus IE1-specific CD8+ T lymphocytes active in such a site might provide an early checkpoint preventing progression of the virus to full reactivation. In addition, presentation of IE-derived CD8+ T cell epitopes by macrophages, which are an important source of latent virus (35), is efficient despite MHC class I down-regulation by MCMV, which strongly interferes with presentation in general of early- and late-derived CD8+ T cell epitopes (36, 37).

M164 is not expressed under the same promoter as IE1/pp89, and its biological function is not yet clear. Although it is probably expressed during the early phase, the epitope is efficiently presented despite MCMV interference with MHC class I presentation (38). Whether this then provides a second checkpoint is currently a matter for speculation. However, the crucial role of CD8+ T cells overall in the maintenance of MCMV latency and prevention of reactivation has been clearly established, because after depletion of critical immunological mediators in combination, viral recrudescence is extremely rapid (4). Therefore, the observed phenomenon may arise from the unique biology of CMVs, where a large depot of virus exists in multiple sites, which is readily reactivated and which demands a rapid response from effector memory T cells.

These patterns—inflammatory responses to some epitopes but not others—may well be mirrored in HCMV, although there are important differences. Seropositive individuals often have persistently high frequencies of HCMV-specific CD8+ T cells with a narrow clonality suggesting Ag-driven expansion (11, 25). These responses are readily detected against the HCMV-togument protein UL83/pp65, a response that has no apparent parallel in MCMV. As shown in Fig. 5C, the positional and functional MCMV homologs of UL83/pp65, namely M83/pp105 and M84/pp65, elicit only weak CD8+ T cell responses without evidence for accumulation. More recently, strong CD8+ T cell responses specific for HCMV IE1/UL123, the human equivalent of pp89, have been described, suggesting a coinmunodominance for pp65 and IE1 (12, 39–42). It has also been shown that MHC class I loading by UL83/pp65-derived peptides occurs efficiently by an alternative pathway which is not affected by interference by HCMV with Ag presentation to CD8+ T cells (43). Although by a different mechanism, this is reminiscent of m164 presentation in MCMV-infected mice (38). To our knowledge, no systematic study has described the long-term evolution of HCMV-specific CD8+ T lymphocyte responses and their relative specificity, but our findings suggest that such a specific study would be of considerable interest.

Accumulation of pp89-specific CD8+ T cells was observed only when mice were infected with MCMV and not after infection with Vac89, indicating a crucial role of the viral biology in driving these accumulating populations, and excluding an artifact due to cross-reactivity between pp89 and some other Ag. Multiple patterns of memory kinetics have been studied in murine systems, and to date, these have roughly fallen into two patterns. Several viruses show a large initial expansion followed by contraction, with a stable memory pool (the burst size model), where the initial expansion of specific cells is followed by 90–95% contraction to stable memory T cell frequencies. Thus, the level of memory is determined by the peak of the acute response (44). Vac89, which does not persist, may well evoke such a pattern. In contrast, for persistent infections, this concept has already been adapted, because the frequency of memory CD8+ T cells stabilizes at higher levels than anticipated by the burst size model (45). However, in none of the cases studied to date has this inflammatory pattern been observed, so this might represent yet another potential pattern for viruses where persistence is an important part of their biology.
The issue of different patterns is raised after infection with EBV, another persistent herpesvirus, where there seem to be at least two different sets of CD8+ T cell responses (30–32). Frequencies of cells specific for lytic viral proteins usually showed the kinetic of a burst size response, with rapid but rather asymmetric contraction after the initial expansion. In contrast, the frequency of T cells specific for latent viral proteins was less dominant during acute infection and sometimes gradually increased, dominating the EBV-specific memory pool. Although some evidence is accumulating for programmed contraction of CD8+ T cell pools after infection (46), in some situations where virus persists and where it represents a continuous threat to the host, this contraction may be less apparent. Thus, the exact viral biology becomes crucial in determining the shape of the immune response in vivo.

We have addressed additional questions about the mechanism behind this phenomenon. During primary infection with MCMV, pp89-specific T cells have an activated phenotype (Fig. 2) but this is also the case in Vac89 infection. However, there was a clear difference in CD62L expression of pp89-specific CD8+ T cells late after infection (days 100–400): >80% of pp89-specific CD8+ T cells were CD62Llow after persistent MCMV infection but CD62Lhigh after transient Vac89 infection. In mice, Ag persistence has been correlated with long-term maintenance of CD62Llow effector memory cells (47) and their preferential localization outside of lymphoid tissue (48). More recently, protection against bacterial challenge with Listeria monocytogenes was shown to be mediated exclusively by CD62LlowCD8+ T cells (49). However, ex vivo, we did not find a significant functional difference between pp89-specific CD62Llow effector memory cells after MCMV infection and CD62Lhigh central memory cells after Vac89 infection, as assessed by IFN-γ production. Nevertheless, the finding of accumulating effector memory cells capable of patrolling solid organs like lung, liver, and salivary gland, supports the concept that latent virus is constantly initiating reactivation which is prevented by circulating MCMV-specific CD8+ effector memory cells.

Consistent with this phenotype, we observed striking activation of pp89-specific CD8+ T lymphocytes during viral latency. This was most marked within LN, where, as in other model systems, the frequency of tetramer-positive cells is not especially high (48), but nearly one-third of these showed signs of recent activation 1 year after infection. Interestingly, prolonged maintenance of CD69highCD8+ T cells particularly within draining LN has also been observed after infection with influenza A virus, which does not establish a persistent infection, although very late time points have not been similarly studied (26). It is likely that the localization of CD69high MCMV-specific cells is related to the distribution of peptide-loaded APCs, which may be concentrated in LN. In experiments with HCMV, cross-presentation of IE1-derived epitopes by dendritic cells has been observed, so the localization of APC and virus-infected cells may be distinct (50).

The fate of tetramer-positive cells after activation is not yet clear, because some may divide and many may disperse into tissues thereafter or potentially die in situ. The data from the BrdU experiments gives some indication of the rate of proliferation of the pool, which appears marginally higher than that of tetramer-negative cells. Although this assessment cannot account for redistribution of cells and death in tissues, it does indicate that a difference exists between proliferation rates of the pp89-specific cells and other heterogeneous CD8+ T cells. Even if this difference is small, it could account on its own for the slow accumulation seen after MCMV infection. A difference in proliferation rate of 0.03% per day, over a 200-day period, would account for a relative doubling in size of the tetramer-positive population in comparison to the rest of the CD8+ T cell pool. However, 90 days after infection with persistent murine γ-herpesvirus-68, a similar picture of proliferation among tetramer-positive and total CD8+ T cells has been described (27), but no such accumulation of tetramer-positive cells has been documented. Therefore, further analysis of the rate and site of death of specific cells across the entire organism is required to understand the gradual accumulation that appears unique to this system. Also, even longer experiments will be required to assess the long-term fate of these populations over the lifetime of the animal.

Recent reports on the phenotype of human CD8+ T cells after infection with several persistent viruses revealed a substantial heterogeneity dependent on the infecting agent, the time after infection, and the particular epitope studied (32, 51, 52). In addition, they are also heterogeneous between individuals. The experiments shown in this study represent a controlled attempt to understand one particular outcome of a host-virus interaction but one which is still clearly relevant to man, as strongly emphasized by recent reports (53). The specific phenotype observed is likely to be dependent on the peculiar biology of CMVs and the particular host studied. Nevertheless, the virus-specific CD8+ T cell responses described in this report followed unique kinetics during latency that are unprecedented. These results are highly relevant to persistent human infections and present a potential new paradigm for understanding certain specific host-virus or host-vaccine relationships. Because infection with HCMV is widespread, understanding this phenomenon has potentially significant implications for the development over time of the normal human immune system.

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