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Memory Inflation: Continuous Accumulation of Antiviral CD8$^+$ T Cells Over Time[1]

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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.**

Many persistent viruses have coevolved with their hosts for millions of years and have reached a remarkably successful state of coexistence. Sixty to 90% of adult persons are seropositive for the cytopathic β-herpesvirus human CMV (HCMV)[4]. After primary infection, HCMV persists lifelong, usually without clinical sequelae, but bears the potential to reactivate. Thus, during immunosuppression, following HIV infection or medical intervention (e.g., transplantation or tumor therapy), HCMV infection and reactivation cause substantial morbidity and mortality (1).

Infections with herpesviruses are usually controlled by a broad array of immune effector mechanisms. Of these, the antiviral CD8$^+$ T cell response has been shown to be important to limit acute CMV disease and reactivation of latent infection in both humans and mice (2–4). It has been demonstrated for murine CMV (MCMV) that viral reactivation events are frequently initiated from the immediate-early (*IE*) gene complex during latent infection. However, the full viral replication cycle is usually not completed unless the host is immunocompromised suggesting an efficient immunological checkpoint, possibly involving CD8$^+$ T cells specific for *IE* gene products (5–7).

Recent studies have shown that very large numbers of functional HCMV-specific CD8$^+$ T cells are present in seropositive individuals long after resolution of primary infection (8–12). Indeed, such responses in the elderly may comprise a significant proportion of the total lymphocyte pool (13, 14). However, because primary infection is usually clinically silent, we know little about the longitudinal evolution of these large HCMV-specific CD8$^+$ T cell pools over time.

To address this, we have characterized the CD8$^+$ T cell response after infection with MCMV using a combination of phenotypic and functional assays. We focused on the immunodominant CD8$^+$ T cell response directed against the IE1 protein m123/phosphoprotein (pp)89 (pp89) after infection with MCMV and compared it to the same response generated after infection with a nonpersistent recombinant vaccinia virus expressing pp89 (Vac89) (15)). We have also analyzed responses directed against other recently described CD8$^+$ T cell epitopes that are not derived from IE1. The results presented in this study suggest that apparently latent MCMV has a continuous and profound influence on the frequency, phenotype, and distribution of MCMV-specific CD8$^+$ T cells, and give an insight into the possible evolution of CMV-specific T cell responses in humans.

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

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[4] Abbreviations used in this paper: HCMV, human CMV; MCMV, murine CMV; IE, immediate early; pp, phosphoprotein; Vac89, vaccinia virus recombinant for pp89; BAL, bronchoalveolar lavage; TK, thymidine kinase; Vβ, variable region of the TCR β-chain; CD62L, CD62 L-selectin; BrdU, 5-bromo-2′-deoxyuridine; LN, lymph node.

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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 10^6 PFU MCMV or 5×10^6 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 disruption over a fine metal 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 directly used for staining and flow cytometric analysis. Bronchoalveolar lavage (BAL) was performed by insertion of a fine canula 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 titers of virus stocks and organ homogenates were determined by virus plaque assays on mouse embryonic fibroblasts as described using centrifugal enhancement of infectivity (16). Titers are expressed as $\log_{10}$ (PFU MCMV/organ). The detection limits of the assays are indicated in Fig. 1. Vac89 was grown and plaqued on thymidine kinase (TK)-deficient (TK^-) cells. Diluted and sonicated lysates 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 FACSCalibur (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 $H2^d$ from five different MCMV proteins were used: m123/pp89 (pp89, H2-L^d-restricted, ^{168}YPHFMPTNL^{176} (17)), m04/gp34 (m04, H2-D^d-restricted, ^{243}YGPSLYRRF^{251} (18)), M84/p65 (M84, H2-K^d-restricted, ^{297}AYAGLFTPL^{305} (19)), M83/pp105 (M83, H2-L^d-restricted, ^{761}YPSKEPFNF^{769} (20)), and m164 (m164, H2-D^d-restricted, ^{168}AGPPRYSRI^{265} (21)). As a negative control, a peptide derived from an endogenous retrovirus of the murine CT26 colon carcinoma cell line was used (CTpep, H2-L^d-restricted, SPSYVYHQF) (22). Background IFN-γ staining after stimulation with the control CTpep was $<0.1\%$ in all experiments. Peptides were synthesized at a purity of $>70\%$ (Research Genetics, Huntsville, AL), diluted, and used at a concentration of 10^{-6} M.

Ex vivo detection and phenotypic characterization of Ag-specific T cells with MHC class I tetrameric complexes and flow cytometry

The MHC class I tetrameric complexes (tetramers) used in this study were produced as described (23). Briefly, recombinant H2-L^d-H chain was expressed and produced in *Escherichia coli* (strain BL21), purified from inclusion bodies, biotinylated enzymatically, and refolded with human β_2-microglobulin and peptide. Refolded complexes were purified by HPLC and tetramerized using PE-labeled extravidin (Sigma-Aldrich) at a molar ratio of 4:1. The tetramers were produced with the immunodominant peptide from MCMV-IE1 m123/pp89 (pp89 tetramer) or with the control CTpep (CT tetramer). The control CT tetramer was generously provided by Dr. A. Gallimore (John Radcliffe Hospital, Oxford, U.K.). In all experiments, background staining with CT tetramer was $<0.1\%$ of $CD8^+$ T cells (not shown).

Fifty microliters of peripheral blood or 2×10^5–10^6 nucleated cells from spleen, liver, lymph node (LN), salivary gland, and lung BAL were prepared in cold PBS containing 2% FCS, 0.2% NaN_3, and 10 mM EDTA. Cells were stained for 20 min at 37°C with pp89 or CT tetramer. Surface markers were quantified by staining for 30 min at 4°C with the following Abs (purchased from BD Biosciences): allophycocyanin- or PerCP-labeled rat anti-mouse CD8 (clone 53-6.7), PerCP- or FITC-labeled rat anti-mouse CD45R/B220 (clone RA3-6B2), FITC-labeled rat anti-mouse CD18 (clone C71/16), FITC-labeled rat anti-mouse CD25 (clone 7D4), FITC-labeled rat anti-mouse $CD43^{1B11}$ (clone 1B11), FITC-labeled rat anti-mouse CD44 (PgP-1, clone IM7), allophycocyanin- or FITC-labeled rat anti-mouse CD62 L-selectin (CD62L; MEL-1), and a panel of FITC-labeled Abs specific for the variable region of TCR β-chains (Vβ). FITC-labeled hamster anti-mouse CD69 (clone H1.2F3) was purchased from Serotec (Oxford, U.K.) FITC-labeled rat IgG2a (BD Biosciences) and hamster IgG (Serotec) isotype controls were used for gate settings. Analysis was performed as described above.

5-Bromo-2′-deoxyuridine (BrdU) labeling and calculation of cycling cell populations

Mice were treated with 0.8 mg/ml BrdU in the drinking water and injected daily with 1 mg of BrdU i.p. for 10 days. After 10 days, the spleens were removed, and tetramer staining was performed as above. BrdU staining was performed after permeabilization of cells using a BrdU-labeling kit according to the manufacturer's instructions (BD Biosciences).

Results

Accumulation of functional pp89-specific $CD8^+$ T cells after MCMV infection

Initial experiments were performed to correlate the immune response against MCMV with the kinetics of viral production. The titer of replicating MCMV was determined by virus plaque assay (Fig. 1*A* for spleen and salivary gland; liver and lung not shown). In the spleen and liver, the peak MCMV titer was reached 3 days after infection with titers falling below the limit of detection by day 12. In the lungs and salivary glands, the peak was ~1 wk later (day 10–15), and the decline was slightly slower. By day 40, replicating virus was detectable only in salivary gland tissue in 33% of the infected mice. At all time points thereafter (days 75–400), productive MCMV replication was below the detection limit for all organs tested. Thus, while the kinetics of viral growth and decline differed in different organs, after ~8–10 wk replicating virus was no longer detectable.

We next measured the frequency of L^d-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. 1*B*).

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. 1*C*). 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. 1*D*).

In contrast, after infection with Vac89, the peak of the pp89-specific $CD8^+$ T cell response was reached between days 6 and 9

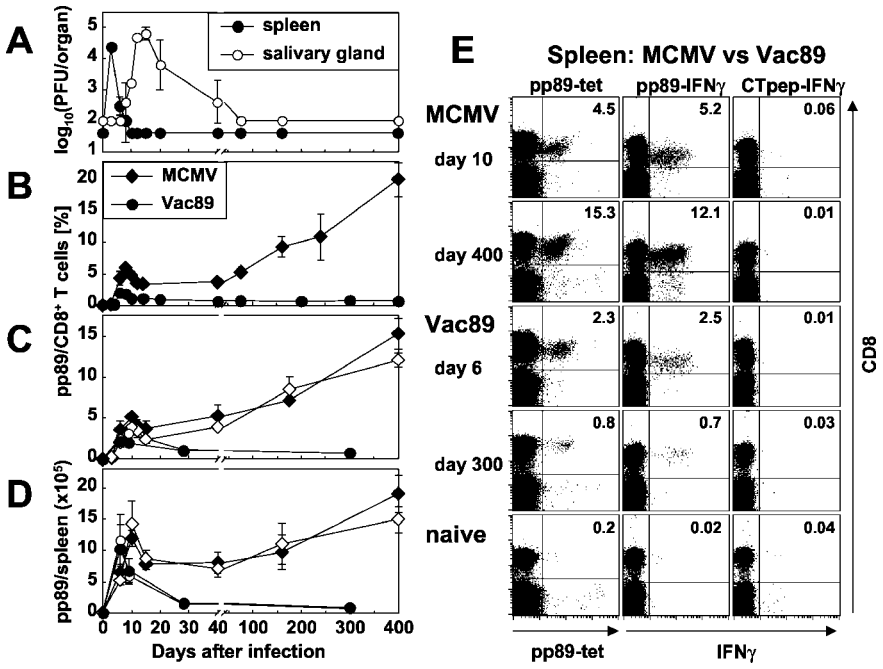

FIGURE 1. Accumulation of pp89-specific CD8$^+$ T cells after infection with MCMV. *A*, MCMV titer: mice were infected with 10^6 PFU MCMV i.v., and at indicated time points, MCMV titers were determined in spleen (●) and salivary gland (○) by virus plaque assay (lung and liver not shown). MCMV titers represent $\log_{10}$ (PFU/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 5×10^6 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 $\times 10^5$). 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.

(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. 1, *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. 2*A*). 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 terms of both frequency among CD8$^+$ T lymphocytes and absolute numbers (Table I, Fig. 1*D*).

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. 2*B*) 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$^{1B11\text{-high}}$ and CD62L^{low}). Only 9% of pp89-specific CD8$^+$ T cells were positive for the early activation marker

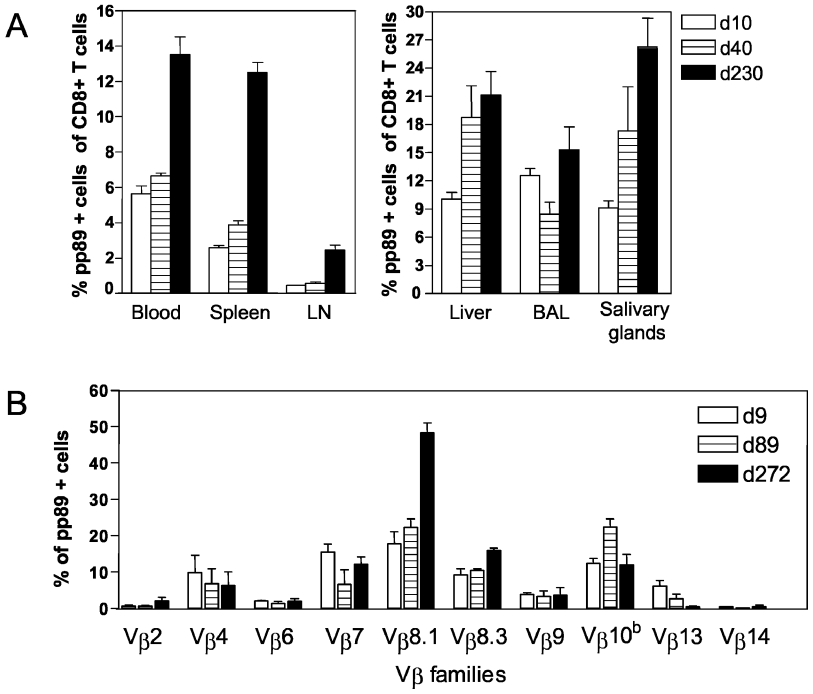

FIGURE 2. *A*, Evolution and distribution of pp89-specific T cells in different organs. BALB/c mice were infected with MCMV (1×10^6 PFU i.v.). Ten, 40, or 230 days after MCMV infection, lymphocytes were isolated from different organs and stained with the pp89 tetramer and anti-CD8. The percentage of tetramer-positive $CD8^+$ T cells is shown for each organ. The *left panel* shows data from lymphoid organs and blood, and the *right panel* shows data from nonlymphoid organs. Note the difference in scale. Columns represent the mean of three mice per group; error bars indicate the SD within an experimental group. One of three similar experiments is shown. *B*, Increasing oligoclonality of pp89-specific $CD8^+$ T lymphocytes over time. A panel of TCR Vβ-reactive Abs were used to identify Vβ usage by pp89-specific T cells over time in the spleen (days 9, 89, and 272 postinfection). The columns show the percentage of pp89-specific T cells positive for the indicated Vβ family. Three to four mice were used per experimental group. Error bars indicate the SD within an experimental group. One of two representative experiments is shown. Abs to Vβ 8.1 also detect Vβ 8.2-positive cells.

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 $CD43^{1B11\text{-}low}$, $CD44^{high}$, 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. 4*A*). 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 I. *Total counts of virus-specific $CD8^+$ T cells in spleen over time*[a]

Virus	Day Postinfection	Total Number of Epitope-Specific $CD8^+$ T Cells/Spleen ($\times 10^4$)			
		1E1/pp89	m04	M83	M84
MCMV	8	143 ± 36	16 ± 6	6 ± 1.7	6 ± 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			
	300	7.1 ± 0.3			

[a] 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 ml64-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*).

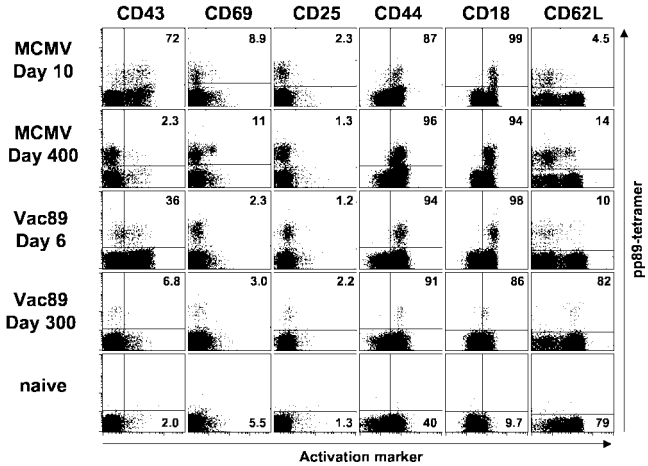

FIGURE 3. Phenotypic characterization of pp89-specific $CD8^+$ T cells after infection with MCMV or Vac89. Mice were infected with 10^6 PFU MCMV, with 5×10^6 PFU Vac89 i.v., or left uninfected as a naive control. Spleen cells were harvested at the indicated time points and stained with pp89 tetramer, anti-CD8, anti-CD45R, and the indicated phenotypic marker. Panels were gated on live $CD8^+$ lymphocytes. Numbers indicate the mean of three to four mice per group and represent the mean percentage of pp89 $tetramer^+CD8^+$ T cells (rows 1–4) or of naive $CD8^+$ T cells (row 5) expressing the respective marker. One of four (days 6 and 10) similar experiments is shown. Results from day 300 and day 400 were confirmed by two comparable experiments from 160 and 230 days after infection.

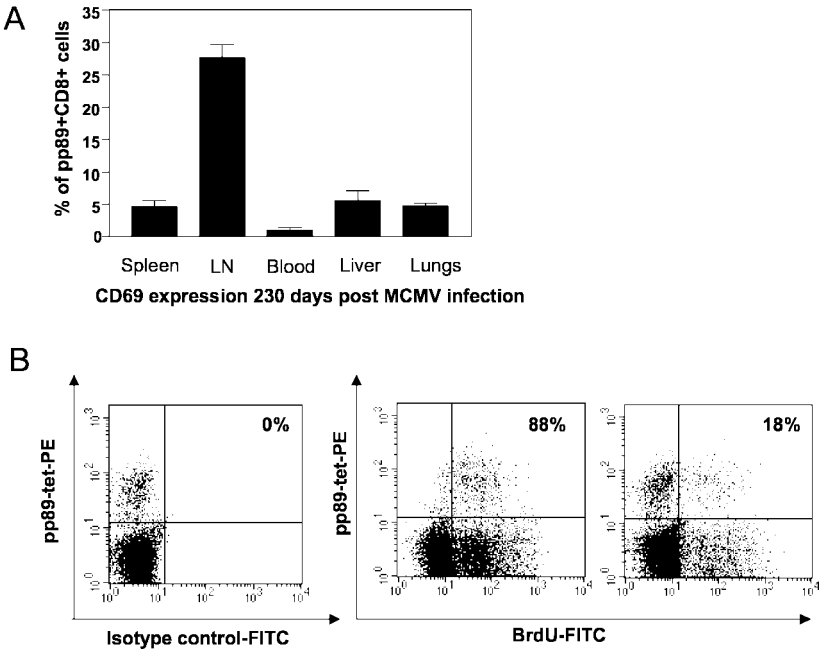

FIGURE 4. *A*, CD69 expression by pp89-specific T cell during MCMV latency in different organs. Lymphocytes of mice infected with MCMV 230 days previously were isolated from different organs and stained with pp89 tetramer, anti-CD8, and anti-CD69. The percentage of pp89 tetramer-positive $CD8^+$ T cells expressing CD69 in each organ is plotted. Columns represent the mean of three mice, and error bars indicate the SD. One of two comparable experiments is shown. *B*, In vivo proliferation of pp89-specific $CD8^+$ T cells after MCMV infection. MCMV-infected mice (day 0 and day 220) were treated with BrdU for 10 days. Splenocytes were then isolated and stained with pp89 tetramer, anti-CD8 and anti-BrdU Abs, or an isotype control. Panels are gated on $CD8^+$ T cells. Numbers indicate the percentage of tetramer-positive cells that are BrdU positive. Staining with an isotype control Ab is shown on the *left*. In the *middle*, BrdU incorporation is shown 10 days after infection, and on the *right*, 230 days after infection. Data from one of three mice are shown. The experiment was repeated three times with similar results.

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. 4*B*. 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 after Vac89 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. 5*A*), while only 0.2–0.5% were specific for the other three epitopes (Fig. 5*B*). 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-

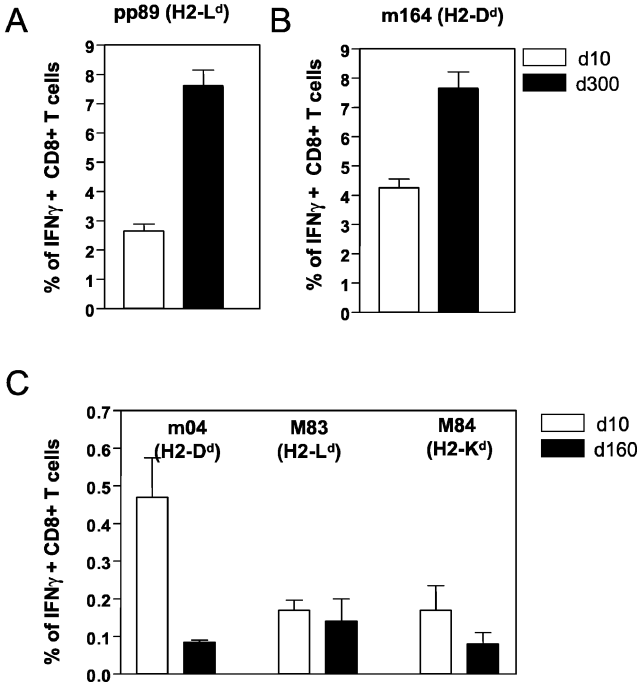

FIGURE 5. Long-term accumulation of memory cells is not restricted to pp89-specific CD8$^+$ T cells. BALB/c mice were infected with 10^6 PFU MCMV i.v. Spleen cells were taken at different time points thereafter and were tested ex vivo by intracellular IFN-γ staining after peptide stimulation. Permeabilized 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-L^d), m04/gp34 (H2-D^d), M84/p65 (H2-K^d), M83/pp105 (H2-L^d), and m164 (H2-D^d). *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. Klenerman, 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—inflationary 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-tegument protein UL83/pp65, a response that has no apparent parallel in MCMV. As shown in Fig. 5*C*, the positional and functional MCMV homologs of UL83/pp65, namely M83/pp105 and M84/p65, 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 coimmunodominance 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 cells (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 inflationary 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 CD62L^{low} after persistent MCMV infection but CD62L^{high} after transient Vac89 infection. In mice, Ag persistence has been correlated with long-term maintenance of CD62L^{low} 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 CD62L^{low}CD8$^+$ T cells (49). However, ex vivo, we did not find a significant functional difference between pp89-specific CD62L^{low} effector memory cells after MCMV infection and CD62L^{high} 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 CD69^{high}CD8$^+$ 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 CD69^{high} 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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