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# Viral Interference with Antigen Presentation Does Not Alter Acute or Chronic CD8 T Cell Immunodominance in Murine Cytomegalovirus Infection<sup>1</sup>

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Both human CMV and murine CMV (MCMV) elicit large CD8 T cell responses, despite the potent effects of viral genes that interfere with the MHC class I (MHC I) pathway of Ag presentation. To investigate the impact of immune evasion on CD8 T cell priming, we infected mice with wild-type (wt) MCMV or a mutant lacking its MHC I immune evasion genes,  $\Delta m4+m6+m152$  MCMV. In acute infection, the two viruses elicited a CD8 T cell response to 26 peptide epitopes that was virtually identical in total size, kinetics, and immunodominance hierarchy. This occurred despite results demonstrating that primary DCs are susceptible to the effects of MCMV's MHC I immune evasion genes. Eight months later, responses to both wt and mutant MCMV displayed the same CD8 T cell "memory inflation" and altered immunodominance that characterize the transition to chronic MCMV infection in C57BL/6 mice. Taken together, these findings suggest either that cross-priming dominates over direct CD8 T cell priming in both acute and chronic MCMV infection, or else that the MHC I immune evasion genes of MCMV are unable to alter direct CD8 T cell priming *in vivo*. At 2 years postinfection, differences in CD8 T cell immunodominance emerged between individual mice, but on average there were only slight differences between wt and mutant virus infections. Overall, the data indicate that the presence or absence of MHC I immune evasion genes has remarkably little impact on the size or specificity of the MCMV-specific CD8 T cell response over an entire lifetime of infection. *The Journal of Immunology*, 2007, 178: 7235–7241.

Many viruses contain genes that interfere with Ag presentation to CD8 T cells, with members of the herpesvirus family being the clear masters of this form of immune evasion. Herpesviruses use a surprising diversity of genetic mechanisms to attack the MHC class I (MHC I)<sup>3</sup> pathway of Ag presentation (1, 2). These include rendering viral proteins resistant to proteasomal degradation, interfering with the peptide transporter TAP, destroying nascent MHC molecules shortly after synthesis, interfering with the peptide loading complex, retaining nascent MHC I molecules in the endoplasmic reticulum (ER), diverting them to lysosomes, or attacking them at the cell surface. Both the ubiquity of this form of immune evasion among herpesviruses and the variety of mechanisms used suggest that there is strong evolutionary pressure for herpesviruses to interfere with the MHC I pathway.

CMVs are members of the  $\beta$  subfamily of herpesviruses and establish lifelong infection of the majority of mammals in a species-specific manner. In immunocompetent hosts, these infections are usually asymptomatic, but CMV elicits an extraordinary T cell

response, larger than to any other virus in the absence of overt disease (3). CMV-specific CD8 T cell numbers "inflate" throughout life (4–7). In elderly humans, CMV is associated with massive clonal expansions of CD8 T cells and with an impaired ability to respond to other agents (8, 9). In common with other herpesviruses, CMVs interfere with MHC I, often expressing multiple genes that have this function. The role that these genes play in maintaining lifelong infection is currently unclear. Furthermore, the enormous CD8 T cell response elicited seems paradoxical given the apparent ability of these viruses to abrogate MHC class I-restricted Ag presentation.

Murine CMV (MCMV) is a natural pathogen of the laboratory mouse (*Mus musculus*). It contains three known MHC I immune evasion genes, *m04*, *m06*, and *m152*. *m152/gp40* retains MHC I in the ER-Golgi intermediate compartment (10) and *m06/gp48* diverts class I to the lysosome for destruction (11). *m4/gp34* is largely ER resident, but is also associated with MHC I at the cell surface. It inhibits Ag presentation by an unknown mechanism, without reducing cell surface class I levels (12). Each of these genes contributes to the inability of CD8 T cells to lyse cells infected with MCMV (13).

We have recently performed an extensive characterization of the CD8 T cell response to MCMV in C57BL/6 mice, which undergoes a dramatic alteration between acute and chronic infection. The response to acute infection is broadly focused, dominated by the response to M45, but followed by 5 other epitopes that also elicit strong responses (14). Altogether, responses to 24 epitopes were detected in acute infection. As in infection of BALB/c mice, where MCMV-specific CD8 T cell numbers inflate throughout life (4, 5), we found that C57BL/6 responses to some epitopes contract sharply after the acute response and develop a typical CD62L<sup>high</sup> central-memory phenotype (15). Responses to other epitopes contract less severely or not at all, instead inflating in numbers with chronic infection, and display an effector-memory phenotype (15).

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<sup>3</sup> Abbreviations used in this paper: MHC I, MHC class I; ER, endoplasmic reticulum; MCMV, murine CMV; wt, wild type; BM, bone marrow; DC, dendritic cell; ICS, intracellular cytokine staining; DRiP, defective ribosomal product.

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The immune evasion genes of MCMV work together with striking efficiency to inhibit CTLs specific for both “contracting” and “inflating” epitopes. We tested the ability of polyclonal CTL lines specific for 16 epitopes to lyse macrophage targets infected with either wild-type (wt) MCMV or a mutant virus lacking its MHC I immune evasion genes ( $\Delta m4+m6+m152$ ) (13). In every case, the T cells readily lysed cells infected with  $\Delta m4+m6+m152$ , but failed to lyse cells infected with wt virus. We have also observed that the MHC I evasion genes of MCMV completely abrogate lysis by H-2<sup>b</sup>-restricted CTL in fibroblasts, in both the presence and absence of IFN- $\gamma$ , and in primary bone marrow (BM)-derived macrophages (13, 16).

Somewhat surprisingly, removing the MHC I immune evasion genes of MCMV did not have a dramatic impact on the course of acute infection in immunocompetent adult C57BL/6 mice and did not prevent the establishment of latency (17). However, in circumstances where CD8 T cell control plays a more critical role, the MHC I immune evasion genes of MCMV have been shown to impact CTL function in vivo (18–20). This was most dramatically demonstrated in a model of acute infection in the context of BM transplantation (19). Adoptively transferred CD8 T cells specific for the H-2<sup>b</sup>-restricted M45 epitope had no impact on lung viral titers of wt MCMV, but they were able to control virus lacking *m152*. This differential impact on control of the two viruses was not reflected in T cell priming. wt and  $\Delta m152$  MCMV both primed a robust M45-specific CD8 T cell response (19, 21). This observation led us to propose that the M45-specific response was primed by cross-presented Ag rather than by directly infected APCs.

The role of cross-presented Ag in priming CD8 T cell response to viruses is contentious. Most evidence implicates dendritic cells (DCs) as responsible for priming the vast majority of T cell responses, although a role for other cell types is not completely excluded. DCs can prime the CD8 T cell response in two ways. Directly infected DCs can present Ags from viral proteins synthesized by the DC (direct or endogenous presentation). Alternately, they can acquire Ag from other virus-infected cells and process and present it on their own MHC I molecules (cross-presentation). The two routes of Ag presentation result in a different spectrum of viral peptides presented by the APC. The endogenous pathway performs a kinetic sampling of viral proteins synthesized over the entire course of virus infection of the cell. The availability of given viral Ag for processing and presentation by the endogenous pathway depends on both the amount of protein synthesized and its rate of degradation, either of the protein itself or of its defective ribosomal products (DRiPs). In contrast, the pathway of cross-presentation performs a static sampling of the viral proteins that are present in a virus-infected cell at the time of its death and uptake by the DC. In consequence, direct presentation favors rapidly degraded proteins such as DRiPs (22), whereas cross-presentation has been shown in several different systems to favor stable proteins rather than peptides (23–25).

Based on these considerations, we realized that the broad spectrum of epitopes recognized in MCMV infection of H-2<sup>b</sup> mice could provide a useful tool to probe the pathways of Ag presentation in MCMV infection. The presentation of at least some epitopes should differ markedly between directly infected DCs and those cross-presenting Ag from dying cells. We therefore hypothesized that a virus whose Ags could only be cross-presented, wt MCMV, would elicit a different CD8 T immunodominance hierarchy than  $\Delta m4+m6+m152$  MCMV, a virus in which direct presentation was possible. We also wanted to assess the impact of MHC I immune evasion on the CD8 T cell response to chronic MCMV infection, which in wt infection is characterized by an altered pattern of immunodominance where a small subset of

epitopes undergo memory inflation. Surprisingly, we found that despite having a profound impact on the ability of CTL to lyse infected cells, the MHC I immune evasion genes of MCMV had remarkably little impact on the kinetics or specificity of the CD8 T cell response to MCMV over a lifetime of infection.

## Materials and Methods

### Mice

C57BL/6 and 129/SvJ mice were purchased from The Jackson Laboratory and infected at 6–12 wk of age. Virus infections were performed i.p. with  $2 \times 10^6$  PFU for C57BL/6 mice or  $5 \times 10^4$  PFU for 129/SvJ mice. All mice were housed at Oregon Health and Science University and all studies were approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee.

### Viruses

Strain MW97.01 (26), a bacterial artificial chromosome-derived virus from Smith strain, was used as wt MCMV. The MCMV mutant  $\Delta m4+m6+m152$  MCMV, derived from MW97.01, has been previously described (27). Both viruses were grown on C57BL/6 mouse embryonic fibroblasts. Sonicated cell lysates were used for mouse infections. For cell-mediated cytotoxicity assays, viruses were concentrated and purified by pelleting over a 15% sucrose cushion. Virus stocks were titered without centrifugal enhancement on BALB-3T3 cells.

### Peptides

All 8-, 9-, and 10-mer peptides were synthesized as crude peptides (65–95% pure by HPLC) by Genemed Synthesis or Jerini Peptide Technologies (JPT) and confirmed by mass spectrometry. 15-mer peptides were synthesized by JPT at 50 nM scale.

### FACS

For intracellular cytokine staining, single-cell suspensions of splenocytes were incubated at 37°C for 6–7 h in the presence of brefeldin A and peptide, at a concentration of 10  $\mu$ M for 15-mer and 1  $\mu$ g/ml for all other peptides. Splenocytes were then surface-stained for CD8 $\alpha$  (53-6.7). Intracellular staining for IFN- $\gamma$  (XMG1.2) was with the Cytotfix/Cytoperm kit (BD Pharmingen). Samples were acquired on a FACSCalibur (BD Pharmingen) with CellQuest software and analyzed with FlowJo software (Tree Star).

### Short-term T cell lines

Spleens were harvested from C57BL/6 mice that had been infected with MCMV for at least 11 wk. As a source of DC-enriched splenocytes to stimulate T cell lines, we used spleens from mice that had been injected 10–14 days previously with B16-FL which secretes Flt3-L. B16-FL were a gift from G. Dranoff (Harvard Medical School, Boston, MA). Splenocytes from B16-FL-injected mice were pulsed with 10 nM peptide, gamma-irradiated, then cultured with splenocytes from MCMV-infected mice in RPMI 1640 supplemented with 10% FBS, 200 mM L-glutamine, 50  $\mu$ M 2-ME, 100 U/ml penicillin, and 100 mg/ml streptomycin. After 3 days, 10 U/ml rIL-2 (eBioscience) was added. After 10–14 days, the percentage of CD8 T cells responding to the stimulating peptide was assessed by intracellular cytokine staining (ICS) to ensure that the T cell lines were functional.

### BMDCs

Primary BMDCs were generated by culturing single-cell suspensions of BM on non-tissue culture-treated petri dishes in DMEM supplemented with 10% FBS, 200 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 20 ng/ml IL-4 (eBioscience), and 20 ng/ml GM-CSF. GM-CSF was generated from supernatant of a GM-CSF-transfected cell line, a gift from D. Gray (University of Edinburgh, Edinburgh, U.K.) (28). Cultures were grown for 10–12 days, replacing the culture medium every 3 days. These cultures typically yielded a population that was 75% CD11c<sup>+</sup>. Of these, 66% were CD11b<sup>+</sup>CD8 $\alpha$ <sup>-</sup>, 7% were CD11b<sup>+</sup>CD8 $\alpha$ <sup>+</sup>, and 27% were CD11b<sup>-</sup>CD8 $\alpha$ <sup>-</sup>.

### Assay for cell-mediated cytotoxicity

A total of 10<sup>4</sup> BMDC target cells per well were plated in 96-well plates. Cells were infected at a multiplicity of infection of 20 with the indicated viruses in the presence of 0.3 mg/ml phosphonoacetic acid (Sigma-Aldrich) to prevent L gene expression and were labeled with 100  $\mu$ Ci <sup>51</sup>Cr (NEN) for 12 h. For peptide-pulsed targets, <sup>51</sup>Cr-labeled cells were incubated with 1  $\mu$ M peptide for 1 h at 37°C and then washed three times. T cells were

then added at the indicated E:T ratios, incubated for 6 h, and supernatants were harvested and assayed with a Topcount scintillation counter (Packard Instrument). Background Cr release was determined by incubating targets with medium alone and total  $^{51}\text{Cr}$  release was determined by lysing targets with medium containing 1% Nonidet P40 (USB). Percent-specific lysis was calculated as follows: (experimental cpm – background cpm)/(total cpm – background cpm).

### Statistics

Student's *t* tests were performed with Excel (Microsoft), using a two-tailed analysis with equal variance.

## Results

### MCMV MHC I immune evasion does not alter the acute CD8 T cell response in vivo

To assess the impact of MHC I immune evasion on the fine specificity of the CD8 T cell response, C57BL/6 mice were infected with wt MCMV or  $\Delta m4+m6+m152$  MCMV. When the response to 26 defined CD8 T cell epitopes was measured by IFN- $\gamma$  ICS 7 days later, at the height of the response, we found that the immunodominance hierarchy was remarkably similar in both infections (Fig. 1A). The small differences that exist appear to be due to random variation, because we have not consistently seen similar differences in other experiments. In addition to having a similar repertoire, the sum of the response to all 26 epitopes tested was also nearly identical (Fig. 1A, left inset graph,  $p = 0.51$ ).

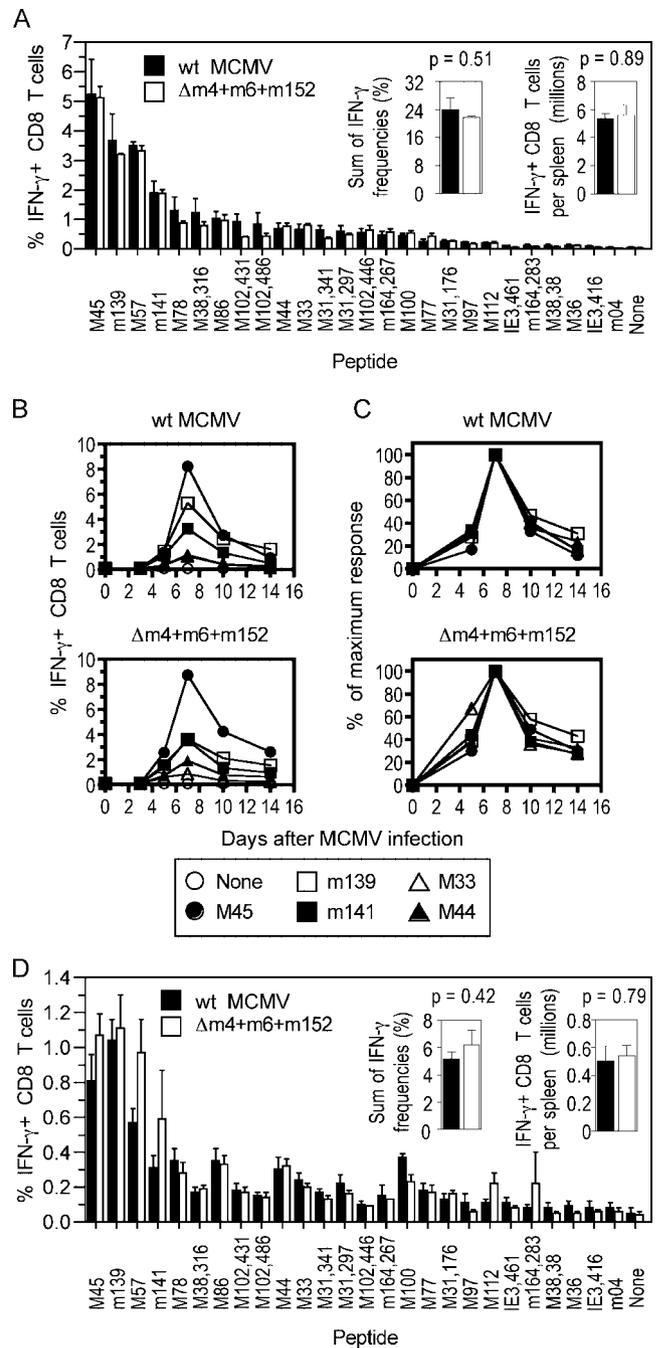
In case the immune evasion genes of MCMV could affect the total size of the virus-specific CD8 T cell response, without altering the relative contribution of each specificity, we also determined the total number of IFN- $\gamma^+$  CD8 T cells per spleen by adding the responses to the individual epitopes. The total size of the CD8 T cell response was virtually identical for both infections (Fig. 1A, right inset graph,  $p = 0.89$ ).

We have previously observed that the absence of *m152* did not affect the kinetics of the response to M45. In both wt and  $\Delta m152$  MCMV, the response peaked at day 7 postinfection (21). We now extended that finding by comparing the kinetics of the response to five epitopes in mice infected with wt or  $\Delta m4+m6+m152$  MCMV. Mice were infected for 0, 3, 5, 7, 10, or 14 days with the two viruses and the response to five epitopes was measured by ICS. Overall, the kinetics of the responses to these two viruses were very similar (Fig. 1, B and C). The only substantial difference, a decrease in the CD8 T cell response to m139 at day 7, was not observed in other experiments (e.g., Fig. 1A, also performed at day 7). We thus found no evidence that the MHC I immune evasion genes affected the magnitude, repertoire, or early kinetics of the CD8 T cell response to MCMV infection in C57BL/6 mice.

### Mice expressing nonfunctional Ly49H have equivalent CD8 T cell responses to wt and mutant MCMV

Expression of Ly49H, an activating NKR engaged by the gene product of MCMV *m157* (29–31), has a marked impact on DC subsets during acute MCMV infection (32). C57BL/6 mice express functional Ly49H, but most other laboratory strains of mice do not. In BALB/c mice (Ly49H<sup>-</sup>), the CD8 $\alpha^+$  subset of DCs that is responsible for cross-presentation (33) was largely lost from the spleen during acute MCMV infection, whereas this subset was preserved during acute infection of C57BL/6 mice (32). Thus, we considered the possibility that CD8 T cell priming would be similar between wt MCMV and  $\Delta m4+m6+m152$  MCMV only in C57BL/6 mice.

To test this possibility, we infected 129/SvJ mice, which are also H-2<sup>b</sup> but lack Ly49H expression, with wt MCMV or  $\Delta m4+m6+m152$  MCMV at the same time as the C57BL/6 mice described above. We then measured the CD8 T cell



**FIGURE 1.** Impact of MCMV MHC I immune evasion on CD8 T cell priming. *A*, C57BL/6 mice ( $n = 3$ ) were infected with wt MCMV (■) or  $\Delta m4+m6+m152$  MCMV (□) for 7 days, the height of the CD8 T cell response. The response to 26 epitopes was determined by ICS for IFN- $\gamma$  after restimulation of splenocytes with peptide *in vitro* for 7 h in the presence of brefeldin A. *Left inset graph*, The sum of the IFN- $\gamma$  responses to all peptide epitopes. *Right inset graph*, The total number of IFN- $\gamma$  + CD8 T cells per mouse spleen. Results are graphed as the average  $\pm$  SEM, and are typical of at least three experiments. *B*, The C57BL/6 CD8 T cell response to five epitopes was measured by IFN- $\gamma$  ICS at days 0, 5, 7, 10, and 14 after infection with wt MCMV (*top*) or  $\Delta m4+m6+m152$  MCMV (*bottom*) ( $n = 3$  for all groups). *C*, The data from *B* was replotted so that the response to each peptide at each time point was normalized to the maximum response for that peptide at day 7. *D*, In the same experiment as *A*, 129/SvJ mice ( $n = 3$ ) were infected with wt MCMV (■) or  $\Delta m4+m6+m152$  MCMV (□) for 7 days. The data were analyzed and plotted identically to *A*. Results are typical of two experiments.

responses to both viruses side-by-side (Fig. 1D). We confirmed our previous finding that the immunodominance hierarchy in 129/SvJ mice differs from that of C57BL/6 mice, with m139 being the dominant epitope rather than M45 (14). However, in 129/SvJ mice, we again found little difference in the CD8 T cell immunodominance hierarchy, the sum of the frequencies to all epitopes, or the total number of virus-specific CD8 T cells responding to wt MCMV or  $\Delta m4+m6+m152$  MCMV. This result ruled out the possibility that the lack of impact of MHC I immune evasion on T cell priming requires Ly49H expression, or was limited to a single strain of mice.

The fact that the immunodominance hierarchies were identical for wt and  $\Delta m4+m6+m152$  MCMV infection strongly implies that the mode of priming of CD8 T cells was the same for both infections. As discussed above, cross-presentation and direct presentation differentially affect different epitopes (24). Although it was possible that a single epitope (M45) could be equally primed by both direct and cross-presentation (21), it seems implausible that this would be true for 26 separate epitopes. We thus conclude that either cross-presentation drives the response to both infections, or that priming by directly infected APCs is possible despite the presence of the MHC I immune evasion genes of MCMV.

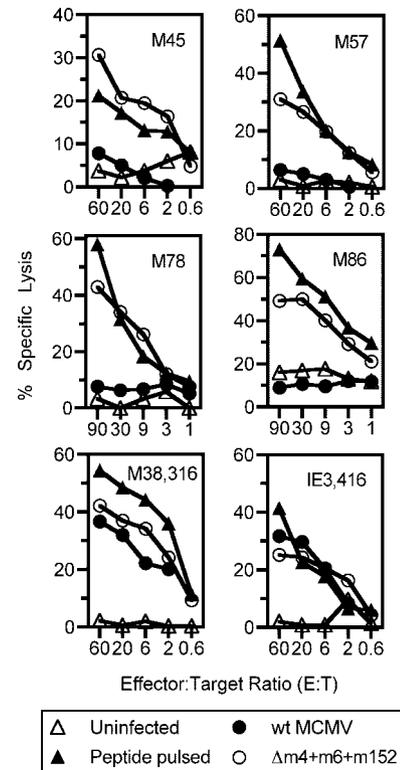
#### *DCs are not impervious to the effects of the MHC I immune evasion genes of MCMV*

MCMV infects professional APCs, including macrophages and DCs, both in vitro and in vivo (32, 34–37). Thus, it is unlikely that obligatory cross-priming is occurring simply because DCs are not infected in vivo. The MHC I immune evasion genes of MCMV profoundly inhibited CTL lysis of infected macrophages by H-2<sup>b</sup>-restricted CTL (13, 16). However, it remained possible that the immune evasion genes of MCMV do not function in primary DCs. To test this, we generated primary BM-derived DCs by culture in GM-CSF, and tested their ability to present MCMV CD8 T cell epitopes in the presence or absence of *m4*, *m6*, and *m152*. Short-term CD8 T cell lines specific for four epitopes, M45, M57, M78, and M86, lysed DCs infected with  $\Delta m4+m6+m152$  but not wt MCMV (Fig. 2), exactly as we have repeatedly observed with other cell types (12, 13, 16, 21). Thus, the MHC I immune evasion genes of MCMV do indeed function in DCs. However, these assays also revealed the first instance of susceptibility of wt MCMV-infected cells to lysis by H-2<sup>b</sup>-restricted CTL. CD8 T cells specific for two epitopes, M38 316 and IE3 416, killed DCs infected with wt MCMV as efficiently as  $\Delta m4+m6+m152$  (Fig. 2). This appeared to be DC specific, since CTL of these specificities do not lyse wt-infected macrophages (13).

If only a subset of epitopes is able to be presented by wt-infected DCs, one might predict that those epitopes would become more immunodominant in wt than  $\Delta m4+m6+m152$  MCMV infection. However, a comparison of the CD8 T cell response to M38 316 and IE3 416, compared with M45, M57, M78, and M86 priming (Fig. 1, A and D) reveals that this was clearly not the case.

#### *CD8 T cell memory inflation and the altered immunodominance hierarchy during chronic MCMV infection is not affected by MHC I immune evasion genes*

The clear dichotomy of the in vitro killing assay results was intriguing, given that CD8 T cell responses to M38 316 and IE3 416 are known to undergo “memory inflation” during the chronic phase of MCMV infection, while responses to M45, M57, M78, and M86 do not (15). In both lymphocytic choriomeningitis virus and *Listeria monocytogenes* infection, DCs are required for secondary CD8 T cell proliferation following Ag re-encounter (38). We

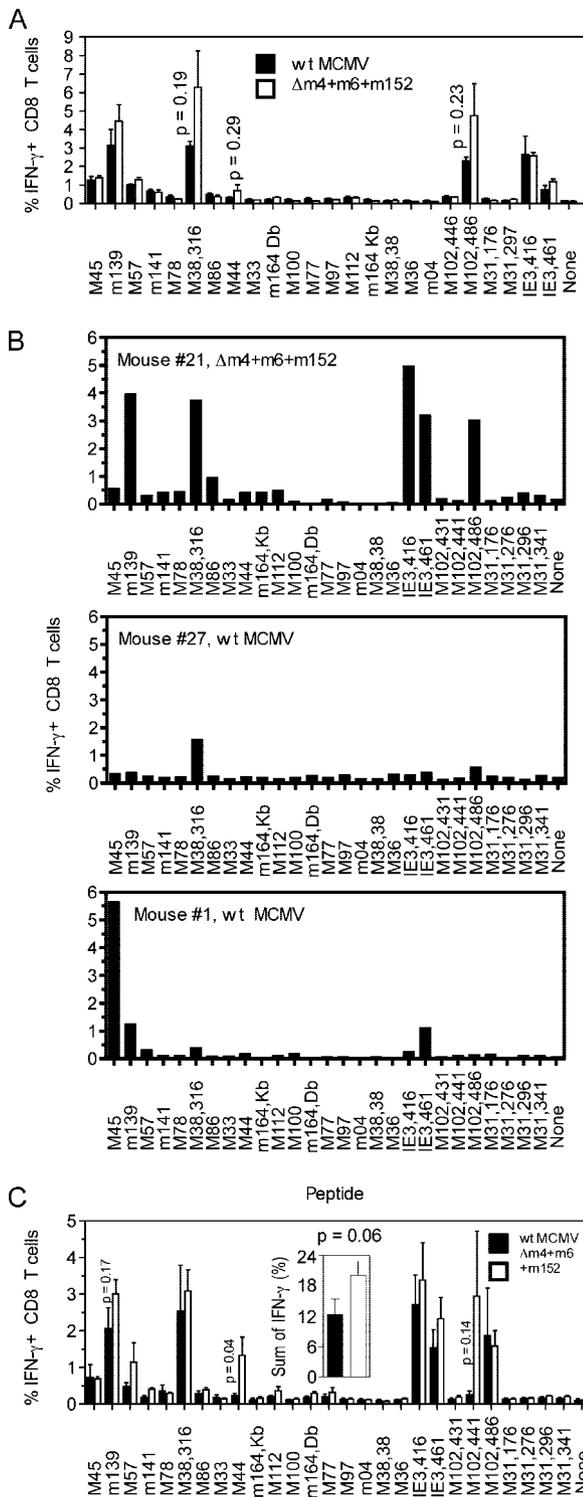


**FIGURE 2.** MCMV’s MHC I immune evasion genes are effective in BM-derived DCs. Short-term peptide-driven CTL lines were derived from MCMV-infected mice. BMDCs were grown in GM-CSF for 10–14 days, infected with virus, or pulsed with peptide as indicated. The ability of CTL lines to lyse BMDC targets was tested in a 6-h <sup>51</sup>Cr-release assay.

therefore speculated that memory inflation occurs in a subset of CD8 T cell specificities, including M38 316 and IE3 416, precisely because these epitopes can be directly presented by wt MCMV-infected DCs in vivo. In other words, we hypothesized that CD8 T cell cross-presentation dominates in acute MCMV infection, but direct presentation is more important in the chronic phase of MCMV infection.

A prediction of this hypothesis is that in chronic infection of mice with  $\Delta m4+m6+m152$  MCMV, the CD8 T cell response should not be restricted to those epitopes that can be directly presented by wt MCMV-infected DCs, and should lead to memory inflation of CD8 T cells specific for “conventional” MCMV epitopes (i.e., those that do not undergo inflation during chronic wt MCMV infection). We tested this hypothesis by examining the CD8 T cell repertoire in mice that had been infected with wt or  $\Delta m4+m6+m152$  MCMV for 8 mo (Fig. 3A). Contrary to this prediction, the CD8 T cell response in both infections showed the same altered immunodominance hierarchy, with memory inflation occurring to m139, M38 316, M102 486 and the two IE3 epitopes. The size of the response to some epitopes, notably M38 316, M44, and M102 486, was actually greater in  $\Delta m4+m6+m152$ -infected mice, but this did not reach statistical significance.

This again leads us to conclude that the mechanism of memory inflation during chronic infection must be the same for wt and  $\Delta m4+m6+m152$  MCMV infection. In addition, this altered immunodominance hierarchy and memory inflation in  $\Delta m4+m6+m152$ -infected mice provides further evidence that MCMV lacking its MHC I immune evasion genes is unimpaired in its ability to establish and maintain chronic/latent infection.



**FIGURE 3.** Impact of MHC I immune evasion on the chronic CD8 T cell response. C57BL/6 mice were infected with wt MCMV (■) or  $\Delta$ m4+m6+m152 MCMV (□). *A*, Eight months after infection, the response to 24 epitopes was determined by IFN- $\gamma$  ICS. All results are graphed as the average  $\pm$  SEM ( $n = 3$ ). IFN- $\gamma$  responses to each peptide was compared by a two-tailed  $t$  test, but none differed significantly ( $p < 0.05$ ) between groups. Results are typical of at least three similar experiments. *B* and *C*, Two years after infection, IFN- $\gamma$  ICS responses to all 27 known MCMV CD8 T cell epitopes were analyzed. Mice were 26 mo of age at the time of the study. *B*, Immunodominance hierarchies from three individual mice. Mouse 21 is the most representative of the group; mouse 27 and mouse 1 are graphed to illustrate the variety of responses seen. *C*, IFN- $\gamma$  results from all mice, graphed as the mean  $\pm$  SEM ( $n = 15$  mice/group).

### The impact of the MHC I immune evasion genes of MCMV on the CD8 T cell response in very old mice

Finally, to assess the impact of the MHC I immune evasion genes on MCMV infection over an entire lifetime, we examined two groups of mice that had been infected with wt or  $\Delta$ m4+m6+m152 MCMV for 2 years. In contrast to the similar responses between individual mice observed at earlier time points (Figs. 1 and 3*A*), at 2 years postinfection there was a marked degree of variation between animals. The responses of three individuals, chosen to exemplify the range of responses observed, are shown in Fig. 3*B*. Most commonly, mice responded to the five inflationary epitopes: m139, M38 316, IE3 416, IE3 461, and M102 486 (e.g., Fig. 3*B*, mouse 21). However, some mice had much narrower responses. For example, mouse 27 made a low overall response, focused primarily on a single inflationary epitope (M38 316), whereas mouse 1's largest response was to a noninflationary epitope, M45.

Although there was a nonsignificant tendency for  $\Delta$ m4+m6+m152-infected mice to have a higher frequency of MCMV-specific CD8 T cells (Fig. 3*C*, inset graph), the average response to wt and  $\Delta$ m4+m6+m152 MCMV was again very similar (Fig. 3*C*). Notably, the response to M102 441 was 10 times larger in  $\Delta$ m4+m6+m152-infected mice than wt MCMV-infected mice, but this was due primarily to three mice that made very large responses to that epitope, and the difference in the average response did not reach statistical significance ( $p = 0.14$ ).

### Discussion

These results demonstrate that the MHC I immune evasion genes of MCMV have little impact on the antiviral CD8 T cell response over an entire lifetime of infection. We previously observed that *m152* does not impact the acute CD8 T cell response to one epitope, M45 (21). We also previously observed that the size and phenotype of the total MCMV-specific response, assessed by ICS in response to infected APCs, was very similar in both wt and  $\Delta$ m4+m6+m152 infections (17). The results presented here extend those findings by considering the fine specificity of the response to 26 epitopes. We found that wt MCMV and  $\Delta$ m4+m6+m152 MCMV elicited a virtually identical CD8 T cell response over the course of 2 years of viral infection. The inability of MCMV interference with MHC I Ag presentation to perturb the acute immunodominance hierarchy was seen not only in MCMV-resistant C57BL/6 mice, but also in the susceptible 129/SvJ strain. A similar lack of impact of MHC I immune evasion on the CD8 T cell response has been observed in BALB/c mice (39). Because direct presentation favors proteins with short half-lives (22) while cross-presentation favors long-lived proteins (23–25), direct and cross-priming are expected to favor different epitopes. It is possible that CD8 T cell responses to one or even several epitopes might be equally primed by both pathways, but it seems implausible that direct and cross-presentation would lead to nearly identical CD8 T cell priming against 26 epitopes. We thus conclude that the mechanism of T cell priming is likely to be identical for both MCMV infections.

However, the mechanism by which these responses are primed *in vivo* remains unclear. Could there be an obligatory dependence on cross-priming in both infections, despite the ready presentation of CD8 T cell epitopes by  $\Delta$ m4+m6+m152-infected cells? This is certainly possible. MCMV infection of DCs interferes with expression of other molecules important in T cell priming, including CD80, CD86 and CD40 (35, 40). Thus, the presence of peptide-MHC complexes on the cell surface may not be sufficient to enable infected DCs to directly prime a CD8 T cell response. In addition, recent data suggest that cross-priming may play a more important

role in virus infections than previously believed. Although it has generally been thought that cross-priming is an alternative priming pathway used when viruses either fail to infect DCs or actively subvert T cell priming, a recent study suggests that cross-presentation may dominate CD8 T cell priming to influenza and HSV (41), viruses which would not have been thought to impair direct CD8 T cell priming in mice. It seems teleologically counterintuitive to consider that cross-priming is the main mode of CD8 T cell priming for most virus infections. However, if that proves to be the case, then it would explain why wt and  $\Delta m4+m6+m152$  MCMV elicit an identical immunodominance hierarchy.

Another interpretation of our results would be that responses to MCMV are primed directly by infected cells in both infections. To consider this possibility, we need to re-evaluate our extrapolation from the *in vitro*  $^{51}\text{Cr}$ -release assays to our presumptions about the impact of MHC I immune evasion on infected APCs *in vivo*. The line of reasoning that leads us to conclude that immune evasion should impact CD8 T cell priming involves two assumptions: 1) that the *in vitro* assays of Ag presentation accurately reflect Ag presentation *in vivo* and 2) that a degree of impairment of Ag presentation sufficient to impede CTL lysis would also impair T cell priming. With respect to 1), viral inhibition of MHC I Ag presentation cannot be simply an *in vitro* artifact, because several groups have found clear evidence that under certain experimental situations, the immune evasion genes of MCMV mediate escape from CD8 T cell control *in vivo* (18–20, 42). There is strong evidence that DCs are required for T cell priming *in vivo* and the Cr release results presented here suggest that the immune evasion genes of MCMV at least quantitatively impact presentation of the majority of MCMV epitopes in DCs. However, it remains possible that DCs cultured from BM do not accurately represent the DC population responsible for CD8 T cell priming in MCMV-infected mice. With respect to 2), it is not possible to know definitively how many peptide MHC complexes on the surface of a DC are required for CD8 T cell priming *in vivo*. *In vitro*, CTL are triggered to lyse infected cells by a much lower density of peptide-MHC than is needed for T cell activation or cytokine secretion (43–45). We have therefore cautiously concluded that the fact that the MHC I immune evasion genes of MCMV impair CTL lysis should indicate that T cell priming would also be impaired, since priming requires a higher density of peptide MHC. However, although our assumptions are based on the best current knowledge, the current data cannot exclude the possibility that directly infected DCs are able to prime CD8 T cell responses despite the presence of the MHC I immune evasion genes of MCMV. The question of whether the CD8 T cell response to MCMV is primed by direct or cross-presented Ag requires further investigation and is being actively pursued in our laboratory.

The results shown in Fig. 2 provide the first example we have ever observed of wt MCMV-infected cells being susceptible to lysis by H-2<sup>b</sup>-restricted CTL. This susceptibility appears (so far) to be DC specific. However, wt MCMV-infected DCs were not lysed by CTL of all specificities. To date, CTL specific for two inflationary epitopes were able to lyse wt-infected DCs, whereas CTL specific for four noninflationary epitopes were not. One potential explanation for this dichotomy is that M38 316 and IE3 416 elicit inflating responses precisely because they are “better” at eliciting higher affinity CD8 T cells. However, peptide titration experiments have not supported the notion that these are high-avidity responses (13, 14). A second possibility arises from the observation that CD8 T cells specific for inflationary epitopes have an effector memory phenotype, whereas those specific for noninflationary epitopes have a central memory phenotype (15, 46). Perhaps the dichotomy in DC lysis does not reflect a difference in the presentation of

inflationary vs noninflationary epitopes, but rather depends on the phenotype of CD8 T cells from which the short-term T cell lines were derived (15, 46). Hence, the basis of the discordance between different T cell specificities is currently unclear and is under investigation.

All the considerations above relate to the lack of impact of immune evasion on CD8 T cell priming. However, as discussed above, MHC I immune evasion is known to impact the ability of CD8 T cells to control MCMV infection, at least in infected epithelial cells in the lung and salivary gland (18–20, 42). We know that  $\Delta m4+m6+m152$  establishes latent infection (17). After resolution of acute MCMV infection, it is not possible to detect infectious virus, and even the latent viral DNA is usually below the threshold of detection. The inflating effector-memory CD8 T cell phenotype is the most direct indication of ongoing virus activity. Our previous study of the CD8 T cell response in chronic infection suggested that this latent pool continued to stimulate T cells to the same extent as wt MCMV for at least 6 mo postinfection (17).

The current results provide two additions to this story. First, we show that the CD8 T cell response in  $\Delta m4+m6+m152$  infection is not only maintained in size and phenotype, but also undergoes the same dramatic change in immunodominance as does wt infection. This lends strong support to the notion that these memory cells are not just being maintained, but are actively being stimulated by ongoing virus activity. Second, we show that this process continues for the life of the animal. These results suggest that MHC I immune evasion has an impact on CD8 T cell control of virus replicating in epithelial cells in peripheral tissues, but does not impact CD8 T cell control of the latent/persistent virus pool, at least in whichever site is most responsible for provoking the ongoing CD8 T cell response.

In very old mice that had been infected with MCMV for 2 years, the total frequency of MCMV-specific IFN- $\gamma$ -producing CD8 T cells was slightly higher in mice infected with  $\Delta m4+m6+m152$  than with wt MCMV, although this was not statistically significant. At this age, considerable differences emerged between individual animals. Although the average of the responses showed the same immunodominance hierarchy as had been seen at 8 mo, at the 2-year time point, individual animals made markedly different responses to different epitopes, evidenced by the large error bars in Fig. 3C. Stochastic variation in T cell populations between individual animals at this age is likely a consequence of the aging of the immune response. However, even at this time point, the overall similarity between the average response to wt and  $\Delta m4+m6+m152$  viruses was striking.

The current study shows that MCMV continues to elicit a strong CD8 T cell response over an entire lifetime of infection in resistant C57BL/6 mice. Surprisingly, the MHC I immune evasion genes of MCMV had little or no impact on this dynamic. This raises important questions about the nature of virus activity, and of Ag presentation, that drives this remarkable response. Studies of laboratory infections are always subject to the caveat that neither the mice, nor the dose and route of infection, faithfully mimic infection conditions in the wild. The evolutionary conservation of MHC I immune evasion indicates that these genes do serve a function for the virus. It is possible that more stringent efforts to mimic natural infection conditions may reveal an impact on virus control, and perhaps on T cell priming as well. Such experiments are currently underway. However, one implication of the results reported here concerns the exploitation of the remarkable immunogenicity of CMV for use as a vaccine vector (47), where an unnatural route and dose of infection would also be used. The current results suggest that there may be no advantage to removing CMV immune

evasion genes for such a purpose, and that a vaccine vector based on wild-type CMV should be equally immunogenic.

## Disclosures

The authors have no financial conflict of interest.

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