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Murine Cytomegalovirus Interference with Antigen Presentation Has Little Effect on the Size or the Effector Memory Phenotype of the CD8 T Cell Response¹

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As with most herpesviruses, CMVs encode viral genes that inhibit Ag presentation to CD8 T cells (VIPRs). VIPR function has been assumed to be essential for CMV to establish its characteristic lifetime infection of its host. We compared infection of C57BL/6 mice with wild-type murine CMV (MCMV) and a virus lacking each of MCMV's three known VIPRs: *m4*, *m6*, and *m152*. During acute infection, there was very little difference between the two viruses with respect to the kinetics of viral replication and clearance, or in the size and kinetics of the virus-specific CD8 T cell response. During chronic infection, a large, effector memory, virus-specific CD8 T cell population (CD8^{low}CD62L⁻CD11c⁺NKG2A⁺) was maintained in both infections; the size and phenotype of the CD8 T cell response to both viruses was remarkably similar. The characteristic effector memory phenotype of the CD8 T cells suggested that both wild-type and $\Delta m4+m6+m152$ virus continued to present Ag to CD8 T cells during the chronic phase of infection. During the chronic phase of infection, MCMV cannot be isolated from immunocompetent mice. However, upon immunosuppression, both $\Delta m4+m6+m152$ and wild-type virus could be reactivated from mice infected for 6 wk. Thus, restoring the ability of CD8 T cells to detect MCMV had little apparent effect on the course of MCMV infection and on the CD8 T cell response to it. These results challenge the notion that VIPR function is necessary for CMV persistence in the host. *The Journal of Immunology*, 2004, 172: 6944–6953.

Viral immune evasion has been an area of intensive investigation for the past decade. Viruses have been found to interfere with the functions of cytokines, chemokines, APCs, apoptotic responses, Abs, complement, NK cells, and Ag presentation to both CD4 and CD8 T cells (1–3). The MHC class I pathway of Ag presentation is a frequent target of virus-encoded immune evasion genes. Genes mediating this type of immune evasion have been called immunoevasins (4) or viral genes that inhibit Ag presentation (VIPRs)³ (5). Herpesviruses in particular all seem to encode VIPRs. Most reports have used isolated VIPR genes to characterize them, few have looked at VIPR function in the context

of virus-infected cells, and even fewer have attempted to assess the impact of VIPR function on the immunobiology of virus infection. We use the natural mouse pathogen murine CMV (MCMV) to study VIPR function, and report here a comparison of wild-type MCMV with a virus that lacks MCMV's three known VIPRs: *m4*, *m6*, and *m152* (6). Because the other immune evasion functions of MCMV are intact in both viruses, our study isolates the impact of these VIPRs on MCMV infection and the immune response to it.

MCMV is an excellent model in which to dissect the real evolutionary role of immune evasion. It is fully sequenced (7), and the expression of its genome as a bacterial artificial chromosome (BAC) has revolutionized functional genetic analysis (8). Since its isolation in 1954 (9), its immunobiology has been extensively studied, revealing complex and redundant immunological control mechanisms (4, 10, 11). Any immunological investigation of MCMV needs to take into account the marked strain difference in susceptibility to MCMV, which is mediated by NK cells. The most important resistance locus (*Cmvr-1*) has been recently mapped to the *Ly49H* gene (12–14), which C57BL/6 mice express and BALB/c mice do not. *Ly49H* is an activating NK cell receptor that recognizes the product of the MCMV *m157* gene (15, 16). In consequence, MCMV replicates to much higher titers in BALB/c than B6 mice, especially in the spleen. Most of the previous work on MCMV T cell immunology has been conducted in the BALB/c strain, and much less is known about the immunobiology of this virus in B6 mice.

MCMV encodes three known VIPRS, all of which are glycoproteins expressed in the early (E) phase of viral gene expression. *m152/gp40* causes class I MHC to be retained in the endoplasmic reticulum/Golgi intermediate compartment (ERGIC) (17). *m6/gp48* binds to class I and directs it to the lysosome for destruction

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³ Abbreviations used in this paper: VIPR, viral gene that inhibits Ag presentation; MCMV, murine CMV; BAC, bacterial artificial chromosome; LCMV, lymphocytic choriomeningitis virus; IE, immediate early; MEF, mouse embryo fibroblast; vv-ova, recombinant vaccinia virus expressing chicken OVA; ICS, intracellular cytokine staining; BrdU, 5-bromo-2'-deoxyuridine.

(18). *m4/gp34* is primarily endoplasmic reticulum resident and associates with class I there; a small amount of *m4/gp34* complexed with class I travels to the cell surface where they remain stably associated (19). Each of these three VIPRs has been shown to inhibit the ability of MCMV-specific CD8 CTLs to lyse infected cells expressing H-2^b MHC (20, 21); gp40 has also been shown to inhibit H-2^d-restricted Ag presentation (22). The relative potency of the individual VIPRs varies for different allelic variants of MHC class I (6, 20). Mutagenesis of an MCMV BAC to delete these genes alone and in combination indicated that there are no other MCMV genes that have a significant impact on cell surface MHC class I (6).

The impact of VIPR function on MCMV infection remains largely unexplored, and is not readily predicted from the literature on the role of CD8 T cells in controlling MCMV infection. For instance, even when VIPRs are functional, CD8 T cells can impair wild-type MCMV replication in vivo. Adoptively transferred CD8 T cells reduce virus titers in the lungs, spleen, liver, and adrenal glands of acutely infected, irradiated BALB/c mice (23, 24). Also, prior immunization with CD8 T cell epitopes protects BALB/c mice against a lethal dose of virus (25, 26). In contrast, animals can be completely depleted of CD8 T cells in acute (27) or chronic (28) infection without impacting virus control, because of redundant mechanisms contributed by CD4 T cells and NK cells. We predicted that removing VIPRs would enable CD8 T cells to play a much more dominant and effective role in controlling virus.

There have been three reports of the effect of *m152* on acute infection in vivo with MCMV. First, Jonjic and colleagues (22) showed that *m152* affects the ability of CD8 T cells to control acute infection, and that this led to ~1 log lower virus titers from days 8 to 10 postinfection. This effect was seen in both BALB/c and B6 mice. To increase the dependence of host defense on CD8 T cell-mediated control, all of the experiments reported in that paper used immunocompromised mice that were either neonatal, B cell deficient, or irradiated. Even so, the effect of *m152* removal was modest, with at best a 1.5 log reduction in titer. In view of the recent discovery that *m152* can also affect NK function, it should be noted that this paper clearly showed that *m152* affects the function of CD8 T cells: removing *m152* from the virus reduced virus titers at day 10 postinfection in normal B6 mice but had no effect on virus titers on day 10 postinfection in mice lacking β_2 -microglobulin or CD8 (22).

Second, it has recently been discovered that, in addition to inhibiting MHC class I-restricted Ag presentation, *m152* also inhibits the expression of the RAE-1 family of ligands for the NK cell-activating receptor NKG2D (29, 30). Jonjic and colleagues (29) found that, in BALB/c mice, this inhibition of NK function resulted in higher virus titers on day 3 postinfection. Thus, in addition to being a VIPR, *m152* can mediate anti-NK cell immune evasion.

Third, we have studied the effect of *m152* on the acute CD8 T cell response to MCMV in B6 mice, looking at its impact on the Ag specificity of the immune response. We found that *m152* did not affect the immunodominance of the D^b-restricted M45 HGIRNASFI epitope in acute infection (31). *M45* is an early gene, expressed at the same time in the infectious cycle as the immune modulators. Because *m152* profoundly inhibits the ability of M45-specific CD8 T cells to detect infected cells, including macrophages and DCs, we interpreted this result to suggest that CD8 T cells in acute MCMV infection are primed by cross-priming rather than by infected APCs.

Each of these three studies on the effect of *m152* in vivo was limited to acute infection. Because all herpesviruses appear to encode VIPRs, and the hallmark of the herpesvirus lifestyle is life-

long survival in the infected host, it is generally assumed that VIPRs will be necessary for this lifelong survival. In other words, the main phenotype of a VIPR-deficient virus would be predicted to be in the chronic phase of infection. Indeed, VIPR-deficient mutants of the gamma-2-herpesvirus murine gammaherpesvirus-68 showed little defect in the acute phase of infection in the lungs, but were markedly impaired in their ability to establish splenic latency (32). After acute infection, MCMV establishes a true latent infection that is characterized by periodic reactivation, sometimes aborted at the immediate-early (IE) phase of gene expression, and at other times presumably progressing to replication (33, 34). However, unless mice are immunosuppressed, replicating virus is not detectable, and even the latent virus DNA load is near or below the threshold of detection, especially in B6 mice. Virus activity is believed to be kept to this very low level by effective immune surveillance.

The concept of active immune surveillance in the chronic phase of MCMV infection is supported by studies of the T cell response in BALB/c mice. Memory CD8 T cell numbers remain high, their antigenic specificity narrows with the duration of infection, and they express an effector memory phenotype, remaining CD62L⁻ (26, 35, 36). Interestingly, a remarkably similar picture is emerging in studies of human CMV infection. In healthy CMV-seropositive adults, ~2% of CD4 T cells (37) and 6% of CD8 T cells (11% of memory CD8 T cells) (L. Picker, unpublished observations) are specific for CMV for the life of the infected individual. In some healthy individuals, >30% of CD8 T cells are CMV specific (38, 39). Many human CMV-specific CD8 T cells have an effector memory phenotype (CD57⁺CD45RA^{bright}CD28⁻CD27⁻) (40–42). Thus, in the chronic phase of CMV infection in immunocompetent humans and mice, the size and phenotype of the CD8 T cell response may be the best indicator of virus activity.

In this study, we report a series of experiments designed to assess the effect of VIPRs on the course of MCMV infection in B6 mice, and on the CD8 T cell response to it. We compared wild-type infection with a virus lacking all three known VIPRs: *m4*, *m6*, and *m152*. Our expectation was that a virus lacking VIPRs would establish acute infection, and would probably be able to establish some latent pool during the 5-day window before effective CD8 T cell control develops. However, after that, we expected that the VIPR-deficient virus would be rapidly controlled, and as latent virus reactivated, it would also be recognized and eradicated. Over time, we expected that this would lead to a reduced or eliminated latent virus pool, and in consequence, we predicted that the MCMV-specific CD8s would come to display the familiar phenotype seen in cleared virus infections such as lymphocytic choriomeningitis virus (LCMV). We were surprised to find that removing VIPRs had little detectable effect on the course of virus infection or on the antiviral CD8 T cell response.

Materials and Methods

Mice

Female C57BL/6 (B6) mice were purchased from Simonson (Gilroy, CA) or The Jackson Laboratory (Bar Harbor, ME); mice from a single vendor were used for each experiment. B cell-deficient (μ mt) mice were purchased from The Jackson Laboratory. OT-1 transgenic mice were kindly provided by D. Hinrichs (Veterans Affairs Hospital, Portland, OR). All mice were housed in an isolation suite and used at age 6 wk or greater. Sentinel mice were routinely tested for mouse pathogens, and none were found in the course of this study.

Cells

Mouse embryo fibroblasts (MEFs) were grown from trypsin-digested day 12–14 mouse embryos, and used between passages 2 and 6. NIH 3T3 cells (CRL-1658), BALB 3T3 cells (CCL-163), and JAWSII cells (CRL-11904)

were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM/10% FBS. JAWSII cells were maintained in α -MEM supplemented with 20% FBS, sodium pyruvate, nonessential amino acids, and 5 ng/ml GM-CSF.

Viruses

MCMV Smith was purchased from American Type Culture Collection (no. 1399-VR). Δ MS94.5 (lacking ORFs *m151–165*) (43), wild-type BAC MCMV MW97.01 (44), and Δ 04+m06+m152 (6) have been described. Viruses were grown on B6 MEFs. Stocks were prepared by sonication of infected cells. Virus was titered without centrifugal enhancement on BALB-3T3 cells to determine PFU for all stocks. Aliquots of the same virus stock were used for each experiment. Mice were infected i.p. with either 5×10^4 or 5×10^6 PFU of MCMV, as described in the figures. The lower dose was used in earlier experiments in which we assessed CTL responses only (see Figs. 1, 5, and 6). The higher dose was necessary to detect robust virus replication in B6 mice, and hence was used in the later experiments when virus load was assessed in addition to monitoring CD8 T cell responses (see Figs. 2–4). A recombinant vaccinia virus expressing chicken OVA (vv-ova) (a gift from J. Yewdell, National Institutes of Health, Bethesda, MD) was grown on L929 cells and titered on Vero cells.

Detection of virus in tissues

Organs were dissected and immediately flash frozen in liquid nitrogen (see Fig. 2b), or placed on ice and homogenized by passing through a cell strainer and frozen. DNA was extracted using DNA Blood mini-kit (no. 51106; Qiagen, Valencia, CA) or High Pure Viral Nucleic Acid kit (no. 1858874; Roche, Basel, Switzerland). Real-time PCR was performed to detect the MCMV IE1 gene exon 1 using the following primers: primer 1, TCG CCC ATC GTT TCG AGA; primer 2, TCT CGT AGG TCC ACT GAC GGA. PCR product was detected with the probe ACT CGA GTC GGA CGC TGC ATC AGA AT labeled with 6-FAM and black hole quencher-1, manufactured by Biosearch Technologies (Novato, CA). Copy numbers were determined by reference to a standard curve obtained using plasmid DNA expressing IE1, a gift from J. Nelson (Oregon Health and Science University).

Immunosuppression to reactivate latent MCMV

For the experiment in Fig. 5, mice were infected with 5×10^6 PFU of MCMV. Thirty-seven (for μ mt mice) or 42 (for B6 mice) days later, mice were injected with cyclophosphamide (150 mg/kg) and 0.3 ml of anti-lymphocyte serum (M4529; Sigma-Aldrich, St. Louis, MO). They were then injected with 0.3 ml of anti-lymphocyte serum and hydrocortisone succinate (125 mg/kg) every 2 days until sacrifice 14 days later. To detect viable virus, salivary gland homogenates were plated onto MEFs until most of the monolayer showed cytopathic effect. Cells were then scraped and pelleted, and DNA was extracted using a Qiagen DNA Blood mini-kit. Viral genes were detected by PCR using the following primers: IE1, primer 1, CAC CAT GGA GCC CGC CGC A, and primer 2, CTT CTT GCT CTT CTT CTT GGG C; m4, primer 1, CAC CAT GTC TCT CGT ATG TCG GCT GGT GTT GGT G, and primer 2, GTT ACT CTT AAG CGG TTT GAA GTT C; m6, primer 1, CAC CAT GCC CAG TTG GAG CGA T, and primer 2, TTT GGT AAG CAA GGG GGA AGT; and m152, primer 1, CAC CAT GCT GGG CGC TAT CA, and primer 2, CCA CAC GCG GCA GTT GAT GTA

CTL clones and assay

MCMV-specific CTL clones have been previously described (20). The ^{51}Cr release assay was conducted as previously described (14), using IFN- γ -pretreated MEF targets infected for 16 h with virus at a multiplicity of infection of 50 in the presence of phosphonoacetic acid to prevent late gene expression.

OT-1 T cell transfer

A total of 2×10^7 whole splenocytes from naive OT-1 TCR transgenic mice was transferred i.v. into unmanipulated naive B6 mice that were either left uninfected or immediately injected i.p. with 5×10^4 PFU of Δ MS94.5 or 2×10^5 PFU of vv-ova. Splenocytes were analyzed by FACS 7 days later.

Intracellular cytokine staining (ICS) and tetramer staining

For ICS, JAWSII cells were infected with MCMV (Δ MS94.5 at a multiplicity of infection of 30–50) for 16 h in the presence of phosphonoacetic acid. Effector splenocytes were isolated from MCMV-infected mice and incubated at a ratio of 1:1 with infected or uninfected APCs for 6 h in the presence of brefeldin A (GolgiPlug; BD Pharmingen, San Diego, CA).

Cells were washed, incubated with FcBlock (BD Biosciences, Mountain View, CA), and surface stained with Abs to the CD8 α chain conjugated to PE-Cy5 (no. 15-0081-83; eBioscience, San Diego, CA) and to NKG2A/C/E using the mAb 20d5 (no. 550520; BD Biosciences), or to CD43 (no. 558761; BD Biosciences), CD11c (no. 557400; BD Biosciences) or CD62L (no. 553150; BD Biosciences), all FITC conjugated. Cells were then fixed and permeabilized using BD Pharmingen's Cytofix/Cytoperm kit before staining with an Ab to IFN- γ (no. 12-7311-82; BD Biosciences). For BrdU staining, the BD Pharmingen BrdU Flow kit (2345KK) was used according to the manufacturer's protocol. CD8 T cells were analyzed by flow cytometry using CellQuest software (BD Biosciences). All further analyses were performed using FlowJo software (Treestar, San Carlos, CA). K b -SIINFEKL tetramers were purchased from the Trudeau Institute Molecular Biology Core Facility (Saranac Lake, NY). D b -HGIRNASFI tetramers were used to stain T cells specific for the immunodominant M45 epitope (31). Tetramers were generated using HGIRNASFI peptide, following the previously described protocols (45, 46), and coupled to streptavidin-PE.

BrdU labeling

Mice infected for 1 day or 30 wk were injected i.p. with 1.8 mg of 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) on day 1 and subsequently fed 0.8 mg/ml BrdU in their drinking water for 7 days before sacrifice.

Results

Assays used to monitor the CD8 T cell response to MCMV infection

In this study, we examined the course of infection and immune responses of B6 mice following infection with wild-type MCMV and compared this to the response mounted against viruses lacking effective VIPR function. The virus Δ MS94.5 lacks ORFs 151–165 (43) and is readily detected by MCMV-specific CTL (20, 43). Recently, BAC technology was used to construct a virus lacking all three VIPRs, Δ m4+m6+m152 (6). Δ m4+m6+m152 has identical growth kinetics in vitro to its parental BAC-derived wild-type virus, and is unimpaired in growth in vivo in immune-depleted animals (6). Whereas wild-type MCMV-infected cells were not lysed by MCMV-specific CTL clones, cells infected with Δ m4+m6+m152 were readily lysed, indicating a substantial loss of VIPR function (Fig. 1a).

We adapted the ICS assay to quantitatively assess the total CD8 T cell response to MCMV using virus-infected cells (Fig. 1b). For APCs, JAWSII dendritic cells were infected with the Ag presentation-competent mutant MCMV Δ MS94.5. The region deleted in Δ MS94.5 does not contain immunodominant epitopes for MCMV in B6 mice (M. W. Munks, manuscript in preparation). In some experiments, we also assessed the response to the immunodominant D b -restricted M45_{985–93} epitope (31). A D b -M45_{985–93} tetramer was constructed, and Fig. 1c demonstrates the specificity of this reagent: only CD8 T cells from MCMV-infected mice are stained by this tetramer.

Because ICS using infected APCs may underestimate the true number of MCMV-specific CD8 T cells, we also assessed expression of the inhibitory receptor NKG2A as an indication of Ag experience. NKG2A is expressed after activation on a high percentage of CD8 T cells and remains expressed long after clearance of Ag on a high percentage of Ag-specific CD8 T cells (47–50), although the level of expression decreases with time after Ag exposure (50). To determine the usefulness of NKG2A expression as a surrogate marker for MCMV specificity, we assessed expression of NKG2A on bystander CD8 T cells during MCMV infection. OVA-specific CD8 T cells from OT-1 TCR transgenic mice were adoptively transferred into naive B6 recipients. In the experiment shown in Fig. 1d, MCMV induced expression of NKG2A on only 4% of OT1 T cells, and in a second experiment (not shown), there was no increase in the percentage of OT1 T cells expressing NKG2A after MCMV infection. We conclude that the increase in

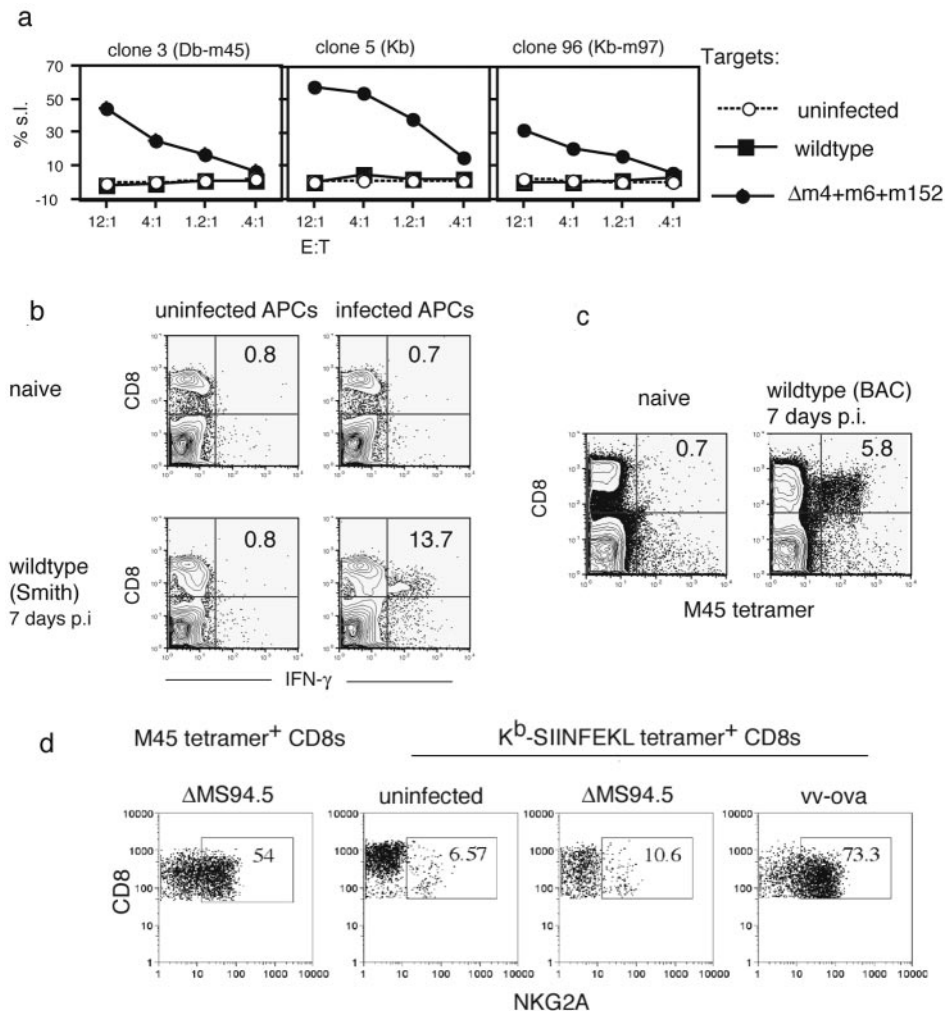


FIGURE 1. Methods used to analyze MCMV-specific CD8 T cell response in B6 mice infected with wild-type or $\Delta m4+m6+m152$ virus. *a*, Cells infected with $\Delta m4+m6+m152$ virus are recognized by MCMV-specific CTL, whereas wild-type MCMV-infected cells are not. MEFs were infected with wild-type MCMV (BAC-derived MW97.01) or $\Delta m4+m6+m152$, or left uninfected. MCMV-specific CD8 T cell clones of three different specificities were used in a standard Cr release assay. *b*, ICS assay using infected APCs detects MCMV-specific CD8 T cells. Splenocytes from naive mice or mice infected for 1 wk with MCMV were incubated with $\Delta MS-94.5$ -infected or uninfected APCs, and stained for CD8 and IFN- γ . *c*, M45-HGIRNASFI-Db tetramer staining. Splenocytes from naive mice or mice infected for 1 wk with MCMV were stained with the M45-D^b tetramer and anti-CD8. *d*, MCMV induces little up-regulation of NKG2A on bystander CD8 T cells. OVA-specific OT-1 transgenic T cells were transferred into B6 mice, which were then infected with MCMV, vv-ova, or left uninfected ($n = 2$ per group). One week postinfection, splenocytes were stained with SIINFEKL-K^b tetramer (to identify OVA-specific cells), M45-D^b tetramer, anti-CD8, and anti-NKG2A. OVA-specific CD8 T cells averaged $3.2 \pm 0.1\%$ of CD8 T cells in uninfected mice, $1.3 \pm 0.3\%$ of CD8s in MCMV-infected mice, and $3.7 \pm 1.0\%$ of CD8s in vv-ova-infected mice. NKG2A staining is shown for one representative mouse per group, gating on tetramer⁺ cells. The percentage of OVA-specific CD8 T cells expressing NKG2A averaged $7.4 \pm 1.2\%$ in uninfected animals (the same as the total CD8 population) and $11.4 \pm 1.1\%$ in MCMV-infected mice. In a duplicate experiment (not shown), the average frequency of K^b-SIINFEKL⁺ CD8s that expressed NKG2A was $7.8 \pm 0.6\%$ without treatment and $7.9 \pm 2.1\%$ after infection with MCMV.

percentage of CD8 cells expressing NKG2A after MCMV infection is a useful surrogate marker for MCMV-specific CD8 T cells.

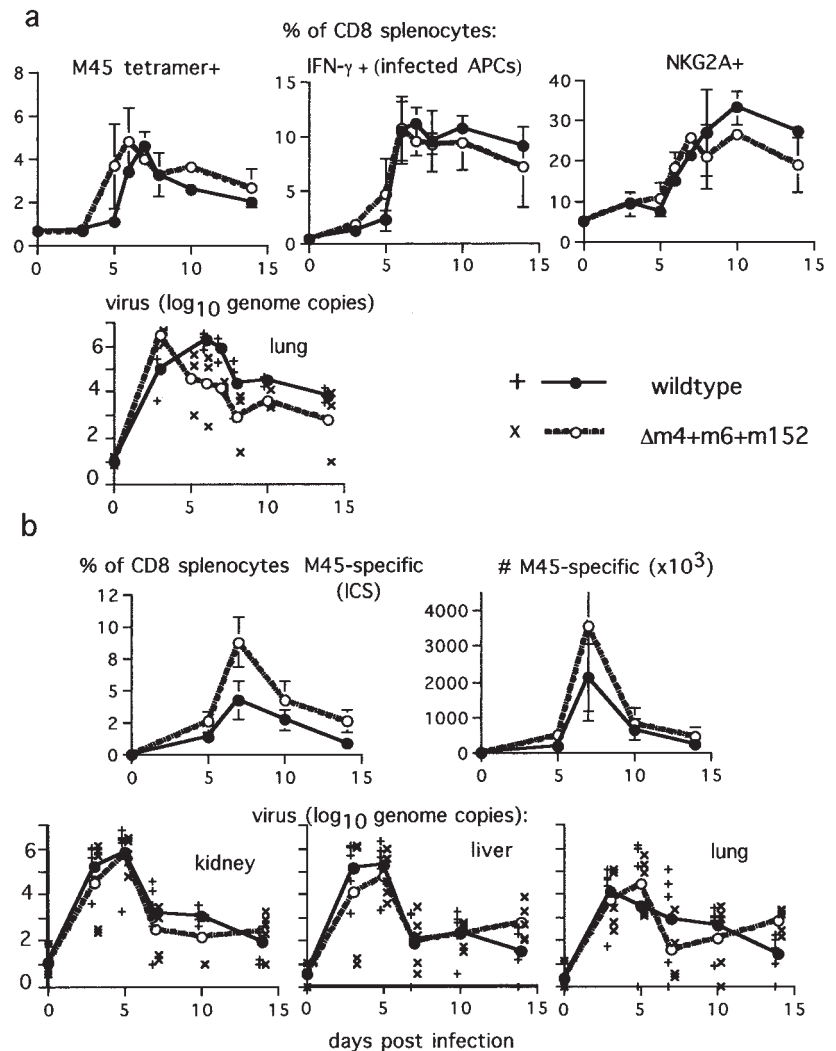
Comparison of VIPR-deficient and wild-type virus during acute infection

We have previously reported that the peak size and immunodominance of the CD8 T cell response to M45 does not differ between wild-type infection and infection with a virus lacking the single VIPR *m152*. In this study, we compared wild-type infection with the $\Delta m4+m6+m152$ virus, which lacks all three VIPRs, and performed a kinetic analysis of virus levels and the CD8 T cell response during the first 2 wk of infection. Fig. 2*a* shows an experiment in which mice were infected with 5×10^6 PFU of wild-type or $\Delta m4+m6+m152$ MCMV. Over a 14-day period, the CD8 T cell response to M45 was measured by tetramer staining, and total

MCMV-specific CD8s assessed by ICS for IFN- γ after incubation with infected APCs. The percentage of CD8 T cells expressing NKG2A is also shown. Virus levels were assessed in the lungs, an organ in which virus is particularly sensitive to CD8 T cell control. Fig. 2*b* shows a second experiment, in which the CD8 response to M45 was assessed by ICS after incubation with the M45 peptide. Virus levels in kidneys, liver, and lungs are shown.

As has been previously described, virus levels peaked at day 3 and declined thereafter. The CD8 T cell response peaked later, at day 7, and then declined. The peak of NKG2A positivity was a little slower, because CD8 T cells only express NKG2A after several days of activation. In multiple assays, we observed that size of the peak CD8 T cell response to $\Delta m4+m6+m152$ was frequently (e.g., Fig. 2*b*) but not always (e.g., *a*) greater than the response to wild-type viruses. Also, small differences in viral titers were sometimes seen between

FIGURE 2. Comparison of acute infection with wild-type and $\Delta m4+m6+m152$. *a*, Experiment 1. B6 mice were infected i.p. with 5×10^6 PFU of wild-type or $\Delta m4+m6+m152$ MCMV from 0 to 14 days before sacrifice. The *top three panels* show percentage of CD8 T cells staining with M45 tetramer (mean \pm SD ($n = 3$)), percentage of CD8 T cells expressing IFN- γ after incubation with $\Delta MS-94,5$ -infected APCs, and percentage of CD8 T cells staining for NKG2A. Less than 0.9% of cells stained positive for IFN- γ after exposure to uninfected cells, and background tetramer staining on splenocytes from naive mice was $<0.8\%$. The next panel shows virus genomes in the lung; geometric mean is shown as a line graph, crosses represent individual animals. *b*, Experiment 2. Five mice per group were infected as for experiment 1. M45-specific CD8 T cells were identified by ICS after incubation with peptide. Percentage of M45-specific CD8 T cells, and number of M45-specific CD8 T cells per spleen are shown (mean \pm SD). The *bottom three panels* show as line graphs the mean genome copies per $10 \mu\text{l}$ of DNA extracted from kidney, liver, and lung; crosses indicate individual animals.



wild-type and $\Delta m4+m6+m152$ at some time points. However, overall, the remarkable feature of these experiments was the similarity between wild-type and $\Delta m4+m6+m152$ infections.

Comparison of VIPR-deficient and wild-type viruses during the chronic/latent phase of infection

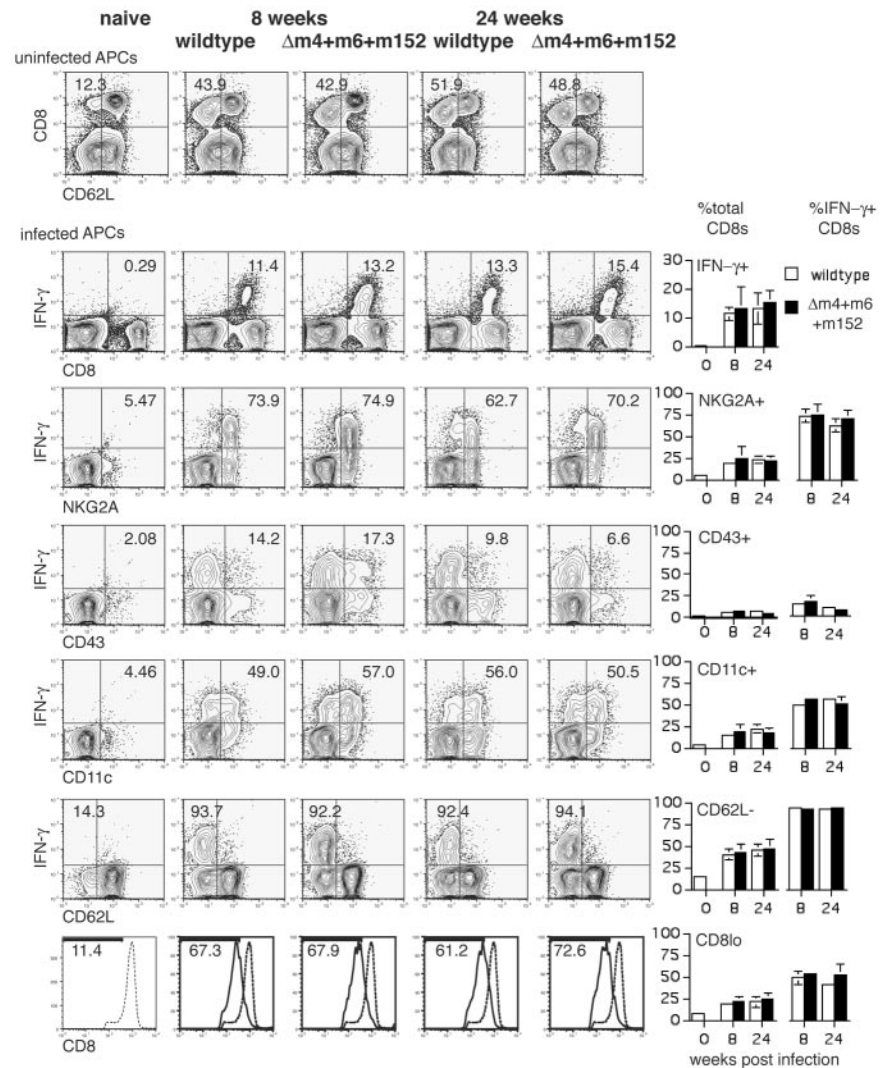
We were surprised that VIPR-mediated impairment of infected cells had little effect on the course of acute MCMV infection and on the antiviral CD8 T cell response. However, as with many other virus infections, the initial control of MCMV infection may be primarily a function of NK cells: an effective CD8 response does not develop until day 5 postinfection, a time at which virus titers are already falling (Fig. 2). Furthermore, we have previously suggested that, in MCMV infection, naive CD8 T cells may be initially primed *in vivo* primarily through cross-presented Ag (31), which would explain why VIPR function had little effect on the size of the initial CD8 T cell response. However, because cells infected with $\Delta m4+m6+m152$ are readily detected and lysed by CTL (Fig. 1*a*), we expected that the virus would be eventually cleared and that the numbers of MCMV-specific CD8 T cells would then decline and show the characteristic features of a CD8 T cell response to a cleared virus infection. Specifically, we expected that MCMV-specific CD8 T cell numbers in the memory phase would be 5–10% of those seen at the peak, and the phenotype would revert in time to a resting, central memory phenotype

(CD62L⁺). In contrast, we expected that mice infected with wild-type virus in which the VIPRs are functional would maintain a higher level of virus activity, resulting in higher numbers of memory CD8 T cells, and that these cells would display the characteristic effector memory phenotype (CD62L⁻) that has been described in chronic CMV infection in mice and humans.

Fig. 3 shows an experiment in which the number and activation status of MCMV-specific CD8 T cells was assessed at 8 and 24 wk postinfection. The M45_{985–93} response becomes very subdominant during chronic infection (M. W. Munks and M. C. Gold, unpublished data), and so Ag-specific responses could only be measured by the ICS assay with infected APCs. During chronic infection, we have not observed CD69 expression on MCMV-specific CD8s (data not shown). Staining for CD43, CD11c, NKG2A, and CD62L is shown. We also quantified the level of CD8 expression. The CD8 coreceptor is down-regulated after T cell activation, but returns to near naive levels within 30–60 days after the acute response to cleared pathogens such as LCMV and *Listeria monocytogenes* (45, 51). We assigned a CD8^{low} gate to determine the percentage of cells whose intensity of CD8 staining was clearly below that seen on naive cells.

The *top panel* of Fig. 3 shows the CD8 and CD62L staining of cells incubated with uninfected APCs, where alteration in staining levels cannot be attributed to *in vitro* TCR triggering. These plots demonstrate the dramatic, permanent alteration in the CD8 T cell

FIGURE 3. Comparison of virus-specific CD8 T cell response to chronic infection with $\Delta m4+m6+m152$ or wild type. Mice were infected with wild-type or $\Delta m4+m6+m152$ virus for 8 or 24 wk before sacrifice. Splenocytes were incubated with uninfected or ΔMS 94.5-infected APCs, and stained for the cell surface markers shown and intracellular IFN- γ . FACS plots are shown for one representative mouse per group. The bar graphs show the mean \pm SD of each group of three mice, first as a percentage of the total CD8⁺ gate, and then as a percentage of the IFN- γ ⁺CD8⁺ gate. The *top panel* shows staining of splenocytes exposed to uninfected APCs, to demonstrate the direct ex vivo phenotype. The numbers indicate the percentage of total CD8⁺ cells that were CD62L⁻. The second panel shows IFN- γ staining after exposure to infected APCs. Background IFN- γ positivity after exposure to uninfected APCs was <0.5%, except for one mouse in which it was 1.08%. The next four panels are gated on CD8⁺ cells and show staining for NKG2A, CD43, CD11c, and CD62L. The numbers indicate the percentage of IFN- γ ⁺ cells displaying the indicated marker. The *bottom panel* shows the CD8 histogram of IFN- γ ⁺ CD8⁺ cells (solid line), overlaying the CD8 histogram from a representative naive mouse (dashed line). The number indicates the percentage of IFN- γ ⁺ CD8⁺ cells scored as CD8^{low} using the gate shown. Three similar experiments with two or more mice per group showed similar results.



compartment engendered by MCMV infection: mice infected with MCMV had a much expanded CD62L⁻CD8^{low} effector memory CD8 T cell population at both 8 and 22 wk. Strikingly, the pattern was very similar for wild-type and $\Delta m4+m6+m152$ viruses. The rest of the panels show staining of cells exposed to infected APCs. Around 13% of CD8 T cells made IFN- γ in response to infected APCs at both 8 and 22 wk. Most of these cells were NKG2A⁺, indicating Ag experience. About one-half expressed CD11c, which is up-regulated on effector CD8 T cells (52–54). About one-half were CD8^{low}, suggesting that they had encountered Ag within ~60 days. Only a small percentage (6–17%) displayed the acute effector marker CD43. Interestingly, consistent with previous reports, the IFN- γ ⁺ CD8 T cells were almost exclusively CD62L negative, which is considered the hallmark of the effector memory phenotype. Although some CD62L loss may have occurred during the 6-h incubation with brefeldin A, most of this loss is likely to have occurred in vivo, as a high proportion of CD8 T cells were CD62L⁻ without exposure to Ag (Fig. 3, *top panel*). Thus, a large proportion of the MCMV-specific CD8 T cells in chronic infection were CD62L⁻, NKG2A^{high}, and CD8^{low}, characteristic of an effector memory phenotype and suggestive of repeated exposure to viral Ag. Remarkably, the number and phenotype of the CD8 T cells in $\Delta m4+m6+m152$ -infected mice were strikingly similar to wild-type infection.

Reactivation of wild-type and $\Delta m4+m6+m152$ viruses from mice infected for 6 wk

During chronic infection, virus cannot be cultured from mice (55). We have occasionally detected viral DNA from homogenized organs by real-time PCR in both wild-type and VIPR-deficient infections, but frequency of positive samples for both infections is too low in our hands for statistical comparison.

To assess whether the virus persisted but was undetectable due to the ongoing immune response, we immunosuppressed chronically infected mice to enable reactivating virus to replicate. In addition to B6 mice, we used B cell-deficient (μmt) mice, in which reactivating virus is easier to detect because it cannot be eliminated by neutralizing Ab (28). Mice were infected with either wild-type or $\Delta m4+m6+m152$ MCMV for 6 wk. They were then immunosuppressed for 2 wk with a combination of cyclophosphamide, hydrocortisone, and anti-lymphocyte globulin. After sacrifice, viral load in liver, lungs, kidneys, and salivary glands was measured by real-time PCR. For μmt mice, virus was detected in four of four wild-type and four of four $\Delta m4+m6+m152$ -infected mice. For B6 mice, virus was detected in three of three wild-type and three of four $\Delta m4+m6+m152$ -infected mice. Salivary gland extracts from B6 mice were plated onto monolayers until viral CPE was detected; DNA was extracted and analyzed for presence of viral

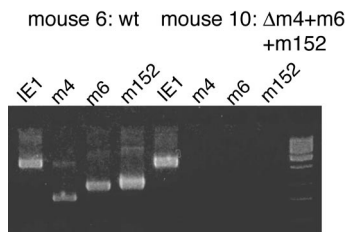


FIGURE 4. Confirmation of genotype of viable virus recovered from mice chronically infected with wild-type or $\Delta m4+m6+m152$ virus. After immunosuppression, virus was cultured from the salivary glands of B6 mice infected for 6 wk with wild-type or $\Delta m4+m6+m152$ virus as described in *Results*. DNA was extracted from cultured virus and analyzed by PCR for presence of m4, m6, m152, and IE1, to confirm that the recovered virus was of the expected genotype. In each case, the genotype was that of the infecting virus. PCR from two representative animals are shown.

genes. In each case, the reactivated virus was of the expected genotype: Fig. 4 shows a representative PCR for two animals. These experiments do not allow us to quantify the level of latent or persistent virus in the animals before immunosuppression, but they provide formal proof that $\Delta m4+m6+m152$ virus was not eradicated by 6 wk postinfection in six of seven animals.

Comparison of wild-type infection with a virus lacking 15 ORFs (*m151–m165*)

We also analyzed infections with the virus $\Delta MS94.5$, which lacks the VIPR *m152* and also lacks *m157* and 13 other ORFs, many of which are likely to be immunomodulatory (four have MHC class I homology (16)). Unlike wild-type virus, $\Delta MS94.5$ is readily detected in vitro by MCMV-specific CTL (20, 43). Fig. 5 shows two experiments. At the peak of the response to each virus, ~ 5 million $CD8^+$ splenocytes were virus specific, based on $IFN-\gamma$ production following in vitro stimulation with MCMV-infected cells. This resolved to a chronic response that was relatively stable over the

time assessed (over 1 year). Many of these cells displayed a $CD8^{low}$ phenotype. The small number of animals in these experiments does not allow statistically significant comparison between the two infections. However, we can conclude both from the size of the chronic response relative to the acute response, and from the phenotype of the cells, that the response to both viruses suggested ongoing virus infection. Furthermore, over the multiple time points analyzed, the kinetics of the response appears similar for both viruses, suggesting that a similar dynamic equilibrium between virus and host immune response was reached in both infections. These experiments are of interest because 1) they provide the first report of the CD8 T cell response in B6 mice to MCMV lacking *m157*, 2) they will also be of interest as the function of the other genes missing from $\Delta MS94.5$ is determined, and 3) we performed more detailed and protracted kinetic analyses of the CD8 T cell response to this virus than to the more recent $\Delta m4+m6+m152$ virus.

BrdU staining of CD8 T cells in mice infected with wild-type and $\Delta MS94.5$ virus

To assess whether MCMV-specific CD8 T cells were actively dividing, mice that had been infected for 1 or 30 wk were fed BrdU for 1 wk before being sacrificed. Splenocytes from these mice were restimulated as above, and costained for CD8, $IFN-\gamma$, and BrdU. Fig. 6 shows that at wk 1 after infection with either wild-type or $\Delta MS94.5$, 90% of Ag-specific cells had incorporated BrdU, indicating they had recently replicated their DNA. However, at 30 wk, BrdU incorporation in $IFN-\gamma^+$ CD8 T cells was not distinguishable from naive animals. Thus, these cells were not cycling rapidly enough to be detected by this assay, suggesting that the CD8 T cell pool was not turning over rapidly.

Discussion

The experiments described in this paper show that, for an experimental infection of B6 mice with MCMV, the absence of VIPRs resulted in very little difference in the course of viral titers during

FIGURE 5. Comparison of the CD8 T cell response to wild type and $\Delta MS94.5$. Mice were infected i.p. with 5×10^4 PFU of wild-type (Smith) or $\Delta MS94.5$ (which lacks ORFs 151–165) at the intervals shown before sacrifice. Two separate experiments are shown. The percentage of $CD8^+$ splenocytes that were $IFN-\gamma^+$ after incubation with $\Delta MS94.5$ -infected APCs is shown in the *top panel*, and the total number of $IFN-\gamma^+ CD8^+$ splenocytes is shown in the *second panel*. Background frequencies of $IFN-\gamma^+ CD8^+$ cells obtained with uninfected targets ranged from 0.1 to 1.1% in the first experiment to 0.1 to 1.3% in the second. The *third panel* shows the percentage of $IFN-\gamma^+$ splenocytes that were $NGK2A^+$. The *fourth panel* shows the percentage of total $CD8^+$ splenocytes that were $NGK2A^+$. The *fifth panel* shows the percentage of $IFN-\gamma^+ CD8$ splenocytes that had down-regulated CD8, assessed using a similar gate to that shown in Fig. 3. The asterisk in the *fifth row* denotes the number of total naive $CD8$ cells that were gated as $CD8^{low}$ in each experiment.

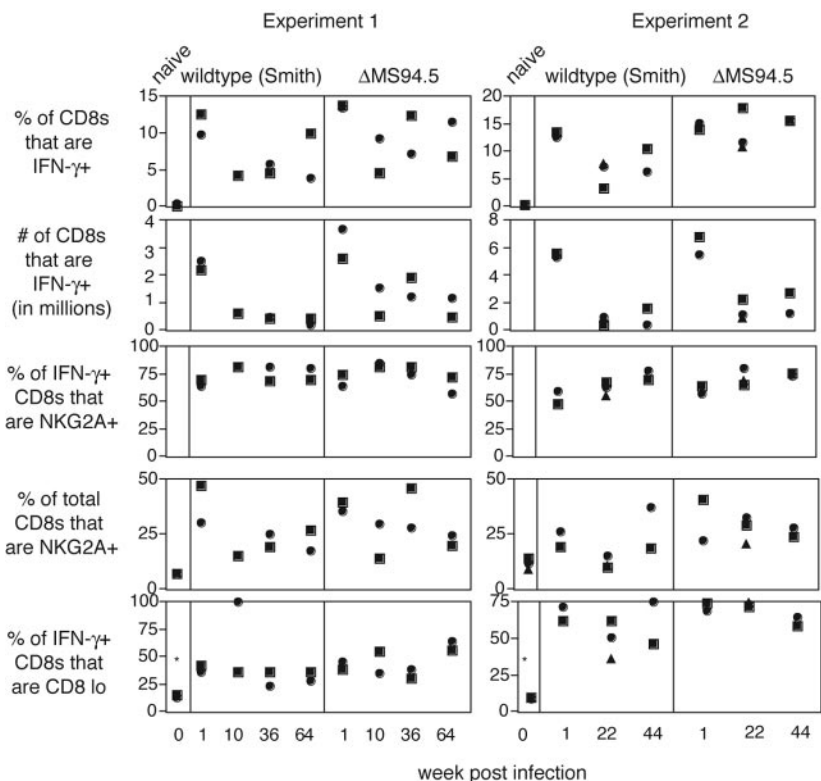
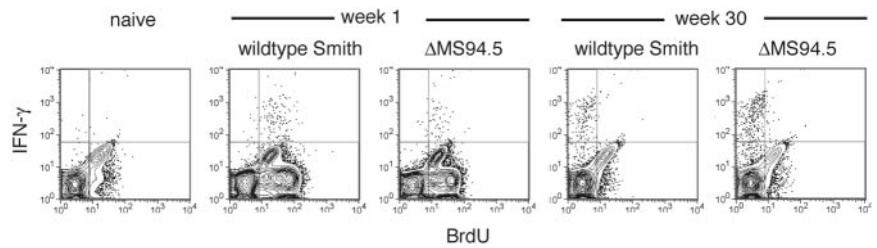


FIGURE 6. MCMV-specific CD8s are not actively cycling in mice chronically infected with wild-type Smith or Δ MS94.5. Mice were infected for 1 or 30 wk, and fed BrdU for 1 wk before sacrifice. After incubation with infected APCs, splenocytes were surface stained for CD8, permeabilized, and costained for BrdU and IFN- γ . Plots are gated on CD8⁺ cells. This experiment was repeated twice with two animals per group with similar results; representative animals are shown.



the acute stages of infection. VIPRs also had little effect on either the numbers or the kinetics of the CD8 T cell response. More surprisingly, the kinetics, numbers, and phenotype of the chronic CD8 T cell response to MCMV were also largely unaffected by the absence of VIPRs: for both wild-type and Δ m4+m6+m152 virus, the size and phenotype of the chronic CD8 response was characteristic of chronic virus infection, or at least repeated Ag presentation. At 6 wk postinfection, we could readily detect both viruses after immunosuppression, formally demonstrating that the Δ m4+m6+m152 virus was not eradicated by an effective CD8 T cell response. Some sort of VIPR function has been described for all herpesviruses looked at closely, implying that this function is crucial for the herpesvirus lifestyle (2, 3, 5). It has generally been assumed that VIPRs would be necessary for herpesviruses to persist in an immunocompetent host. However, our results show that, even though MCMV VIPRs effectively inhibit lysis of infected cells by MCMV-specific CTL *in vitro*, a virus without these genes was still able to establish latent or persistent infection. Furthermore, there was no discernible effect of VIPR removal on the CD8 T cell response to infection *in vivo*.

These results are obviously very different from those we had expected, which was that the Δ m4+m6+m152 virus would be cleared and that the CD8 T cell response would in turn become typical in size and phenotype of a cleared virus infection. This has caused us to look more closely at the assumptions that formed the basis of this expectation. In the following, we list some of the issues that we believe need to be clarified or explored further.

*Demonstration of the ability of VIPRs to impact CD8 T cell recognition relevant cell types *in vivo**

The MCMV genes *m4*, *m6*, and *m152* have a profound effect on the ability of CTL to lyse infected cells in Cr release assays in several cell types (Fig. 1; Refs. 20–22 and 43), and an *m152*-deletant virus was better controlled by CD8 T cells *in vivo* (22, 56), indicating that VIPRs do indeed function as predicted in important tissues *in vivo*. However, the strength of VIPR-mediated inhibition of Ag presentation *in vivo* to CD8 T cells of different specificities, and in APCs as well as somatic cells, should be determined.

Correlation of the effector memory phenotype of CD8 T cells in MCMV infection with virus activity

The size of the CD8 T cell response to CMV and the characteristic effector memory phenotype of the cells in both humans and mice has been accepted to mean that the T cells have been repeatedly restimulated, either by replicating virus or at the very least by proteins produced by abortively reactivating virus. Because CMV is known to persist, even though it is often undetectable without immunosuppression, this explanation of the CD8 T cell phenotype seems logical. In fact, it has been suggested that the CD8 T cell response may be the best indicator of virus activity in chronic CMV infection (39). We have formally demonstrated persistent viable Δ m4+m6+m152 at 6 wk postinfection. When the same immunosuppression protocol was used on B6 mice that had been

infected for 6 mo, we did not detect virus. Detection of reactivating virus from long-term infected B6 mice is difficult, perhaps due to the presence of neutralizing Ab, and we are attempting to improve our protocols. However, in none of these experiments did we find any significant difference in our ability to detect virus in animals infected in parallel with wild-type or Δ m4+m6+m152 virus. Thus, beyond 6 wk postinfection, the phenotype of the CD8 T cell response is our main evidence suggesting that virus activity in chronic infection is not affected by VIPR removal. The relationship between the CD8 T cell phenotype and virus activity in MCMV infection needs to be formally demonstrated. The phenotype of CD8 T cells in peripheral organs that support CMV reactivation, such as salivary gland and lungs, and the extent to which T cells recirculate between peripheral tissues and central lymphoid organs will also be of interest. It has been well documented in other models, particularly acute LCMV infection, that in the absence of continued antigenic stimulation, memory CD8 T cells come in time to re-express CD62L and to up-regulate CD8 (51, 54, 57). Thus, it seems likely that the CD62L⁺CD8^{low} phenotype in MCMV infection—both Δ m4+m6+m152 and wild-type—does indeed result from continued Ag exposure. If this proves not to be the case, a different problem will need to be addressed, i.e., how does CD8 T cell priming in MCMV infection differ from other models to cause CD8 T cells to retain this unique phenotype?

The source of viral Ag and the nature of Ag presentation that drives the CD8 T cell response in chronic infection

The fact that we are forced to rely on the CD8 T cell response to compare Δ m4+m6+m152 and wild-type virus activity in chronic infection raises some important issues. We are using a single measurement to assess the effect of VIPRs on two separate processes: T cell priming, and the impact of effector CD8 T cells on virus load. CD8 T cells can be primed either by directly infected APCs or by APCs that take up proteins synthesized in infected cells and cross-present them. VIPR function should affect priming by directly infected cells, but not by cross-presentation. It follows that the size and phenotype of the CD8 T cell response would not be affected by VIPR function 1) if cross-presentation were the only means by which CD8 T cells could be primed and 2) if the amount of viral protein being made were the same in wild-type and Δ m4+m6+m152 infections. It has been shown that MCMV encodes immune evasion genes other than VIPRs that impair the ability of infected dendritic cells to prime T cells (58, 59), so cross-priming may indeed be the only mechanism by which MCMV-specific CD8 T cells can be primed. Even so, we would think that the Δ m4+m6+m152 virus should be more readily controlled by CD8 T cells, limiting the amount of viral protein available for cross-presentation. In contrast, the amount of viral protein available for cross-presentation during chronic infection with both viruses could be the same if the major source of viral Ag is abortively reactivating virus, and the size of the latent virus pool, established early during acute infection, is similar for both viruses. Another explanation could be that, if directly infected cells do play

a role in priming, a smaller amount of $\Delta m4+m6+m152$ virus in chronic infection would be able to activate more CD8 T cells. In other words, opposite effects on T cell priming and virus control could lead to a similar final picture. These permutations highlight the need to define experimentally 1) the source and amount of viral Ag in chronic infection, 2) the relative importance of direct and cross-priming, and 3) the ability of CD8 T cells to affect the amount of wild-type and $\Delta m4+m6+m152$ virus load in chronic infection.

The evolutionary advantage of VIPRs

VIPR functions are too widely expressed among herpesviruses (2, 3, 5) for us to readily dismiss the notion of interference with Ag presentation as laboratory artifact. At present, our favored explanation is that VIPRs function in concert with other types of immunoevasins, and that removing one layer of a complex strategy has not been enough to have a major impact on viral fitness. This may especially be the case because of the artificial context of inbred laboratory mice and experimental infection conditions (high virus dose and i.p. infection).

Nevertheless, the lack of impact of VIPR removal causes us to at least wonder whether we have looked in the wrong place to understand their evolutionary advantage for this virus. It is possible that the greatest impact of VIPRs may be on virus transmission. In other words, VIPR function may be necessary for latent virus to occasionally replicate sufficiently in the salivary gland to generate an infectious inoculum and spread to a new host. The observation that, in the salivary gland, wild-type (VIPR-containing) MCMV is particularly resistant to CD8 T cell control (60) might support this hypothesis. Another possibility is that VIPRs may lessen the risk of immunopathology engendered by the massive size of the CMV-specific memory CD8 T cell population. Persistent viruses depend on their hosts' health for their own persistence and transmission. CD8 T cell immune responses can be hazardous. Although we saw no gross pathology in our chronically infected animals, it is possible that, in some circumstances, CD8 T cell effector functions, such as recurrent IFN- γ and TNF- α release (which would presumably occur more often in VIPR-deficient infections), could damage host tissues such as great vessels and lungs over a lifetime. Yet another possibility is that VIPRs are necessary for superinfection. A strange feature of CMV epidemiology, demonstrated in mice (61, 62), humans (63), and monkeys (64), is the ability to reinfect an already-infected animal. Although the evolutionary advantage of superinfection remains to be determined, VIPR function may be necessary for it to occur.

Conclusions

CMVs have been coevolving with their mammalian hosts at least since the mammalian radiation ~60 million years ago (65, 66). It is apparent that, in this time, they have fine-tuned a complex relationship that we are far from understanding. Given the ubiquity of CMV infection and the size of the immune response to it, understanding its immunobiology must be part of understanding the normal immune system. Although there are differences in genetic mechanisms, at a functional level, the mouse model seems to closely parallel human infection. This is fortunate because sorting out most of the issues raised above requires *in vivo* experiments. Understanding the normal immunobiology of CMV will be important as we attempt to manipulate or exploit it through such interventions as vaccination.

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