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The Context of Gene Expression Defines the Immunodominance Hierarchy of Cytomegalovirus Antigens

Iryna Dekhtiarenko,* Michael A. Jarvis,† Zsolt Ruzsics,‡ and Luka Čičin-Šain*.§

Natural immunity to CMV dominates the CD4 and CD8 memory compartments of the CMV-seropositive host. This property has been recently exploited for experimental CMV-based vaccine vector strategies, and it has shown promise in animal models of AIDS and Ebola disease. Although it is generally agreed that CMV-based vaccine vectors may induce highly protective and persistent memory T cells, the influence of the gene expression context on Ag-specific T cell memory responses and immune protection induced by CMV vectors is not known. Using murine CMV (MCMV) recombinants expressing a single CD8 T cell epitope from HSV-1 fused to different MCMV genes, we show that magnitude and kinetics of T cell responses induced by CMV are dependent on the gene expression of CMV Ags. Interestingly, the kinetics of the immune response to the HSV-1 epitope was paralleled by a reciprocal depression of immune responses to endogenous MCMV Ags. Infection with a recombinant MCMV inducing a vigorous initial immune response to the recombinant peptide resulted in a depressed early response to endogenous MCMV Ag. Another recombinant virus, which induced a slowly developing “inflationary” T cell response to the HSV-1 peptide, induced weaker long-term responses to endogenous CMV Ags. Importantly, both mutants were able to protect mice from a challenge with HSV-1, mediating strong sterilizing immunity. Our data suggest that the context of gene expression markedly influences the T cell immunodominance hierarchy of CMV Ags, but the immune protection against HSV-1 does not require inflationary CD8 responses against the recombinant CMV-expressed epitope. The Journal of Immunology, 2013, 190: 000–000.

Cytomegaloviruses are ubiquitous herpesviruses, characterized by broad cell tropism, strict species specificity, and an ability to establish lifelong latency (1). Human CMV (HCMV) induces a potent cellular immune response, which can dominate the memory compartment of the infected patients (2). Experiments in the mouse model of infection with the murine CMV (MCMV) have shown that CMV-specific CD8 T cells accumulate in tissues (3) and in the blood (4) at times when the virus is latent, resulting in an ongoing expansion of CMV-specific T cells called “memory inflation” (4). Similarly, higher frequency of CMV-specific CD8 T cells could be observed with progressing age in blood samples of healthy humans (5), suggesting that HCMV-specific cellular immunity may similarly increase, or “inflate,” with age.

The cloning of CMV as bacterial artificial chromosomes (BAC) in Escherichia coli (6) allowed quick and efficient targeted mutagenesis of the CMV genome, as well as the generation of CMVs expressing defined heterologous target Ags (7). Initial results showed that these recombinants were able to induce T cell responses to peptides derived from other viruses, such as influenza A or lymphocytic choriomeningitis virus (7), and that provided immune protection against recombinant poxviruses expressing the target Ag. This concept has more recently been successfully applied to develop recombinant CMVs that target the simian immunodeficiency (8, 9) and Zaire Ebola virus (10) in rhesus and murine models of infection, respectively. Taken together, these studies open the possibility that recombinant HCMVs may be used as a vaccination strategy to target clinically important human and veterinary pathogens that require cellular immunity for efficient immune protection.

Parameters that define the immunogenicity of the heterologous Ag targets are largely unexplored. Munks et al. (11) have shown that CMV-encoded peptides induce CD8 T cell responses with diverse kinetics, with some peptides, such as the m139-encoded TVYGFCLL or the ie3-encoded RALEYKNL, inducing inflationary responses, whereas others, such as the M45-encoded HGIRNASFI, induced acute responses that dominated the response repertoire early upon infection but contracted rapidly and were thereafter maintained at low levels. Therefore, it stood to reason that peptide immunodominance, and consequently immune protection, may depend on the context of gene expression. To the best of our knowledge, this has not been experimentally validated.

The rational design of CMV-based vectors faces another critical question in relationship to immunodominance. It remains unclear whether recombinant CMVs may offer immune protection against multiple targets, or whether the insertion of multiple Ags within the same CMV vector would result in suboptimal immune responses to the antigenic targets due to a competition of T cells for immune responses. We have recently observed that memory inflation of an immunodominant peptide does not reflect an overall expansion of the memory pool, but rather occurs while the size of the memory pool is stable (12). This result indicated that memory inflation is not an expansion of the CMV-specific memory compartment, but rather reflects the ongoing focusing toward a selected few antigenic epitopes. In that case, multiple target Ags expressed by the same
recombinant CMVs would compete for immune responses and potentially limit its protective capacity.

In this study we use a series of recombinant CMVs expressing an identical immunodominant HSV-1 target peptide Ag fused to distinct MCMV genes to examine the role of gene expression kinetics as well as Ag competition in CMV T cell immunity. We show that the insertion of the same immunodominant peptide in the context of the viral genes MCMV immediate early 2 (ie2) or M45 induced immune responses that drastically diverged in magnitude and kinetics. HSV-1 peptide fusion to the M45 gene induced a strong initial response, followed by immune contraction. In contrast, fusion to the ie2 gene resulted in memory inflation. We also show that HSV-1 peptide expression reduced CD8 responses toward endogenous MCMV peptides, and that the inflammatory response to the transgenic peptide reduced inflammatory responses to MCMV peptides, whereas the same peptide introduced into the M45 gene reduced the initial CD8 response to multiple genes but had no effect on memory inflation. Finally, we show that both insertion loci could still mediate strong protective immunity.

**Materials and Methods**

**Mouse strains**

129S2/SvPas Cr1 (129/Sv) mice were purchased from Charles River (Sulzfeld, Germany). C57BL/6 mice were purchased from Janvier (Le Genet Saint Isle, France). Mice were housed and handled in accordance with good animal practice as defined by Federation of Laboratory Animal Science Associations and the national animal welfare body Die Gesellschaft für Versuchstierkunde/Society of Laboratory Animals. All animal experiments were approved by the responsible state office (Lower Saxony State Office of Consumer Protection and Food Safety) under permit no. 33.9-42502-04-11/0426.

**Cells**

M2-10B4 (CRL-1972), Vero (CCL-81), and NIH 3T3 fibroblasts (CRL-1658) (all from American Type Culture Collection) were maintained in DMEM supplemented with 10% FCS, 1% glutamine, and 1% penicillin/streptomycin. C57BL/6 murine embryonic fibroblasts (MEFs) were prepared and maintained as described previously (15).

**Viruses and viral mutagenesis**

BAC-derived wild-type MCMV (MCMV WT) was propagated as described previously (14). HSV-1 strain 17 obtained from Dr. J. Nikolich-Zugich (University of Arizona, Tucson, AZ) was grown and titrated on Vero cells (15). MCMVrecSL and MCMVM45SL were generated using two-step E/T BAC-based mutagenesis using galK selection as described in Warning et al. (16) with some modifications. For MCMVrecSL, a linear PCR-derived recombination fragment encoding galK and kanamycin resistance, was inserted into the MCMV genome at the 3′ end of the MCMV ie2 gene (nucleotide positions 187296–187297 of the MCMV WT genome). This recombination fragment was generated by amplifying a linear PCR product from the pGPS/galKn plasmid (17) using primers ie2GalK forward and ie2GalK reverse (for primer sequences, see Supplemental Table I). Targeting of the recombination fragment was ensured by incorporation of homologous sequences flanking the desired site of insertion on the primers’ 5′ ends. Recombinant BAC clones were then selected by growth on kanamycin plates. The recombination fragment for the second step was synthesized by Euforins MWG Operon and consisted of the SSIEFARL sequence (5′-TCATCGATTGAATTCGCCCGCCTC-3′) flanked by areas of homology to the insertion site. This recombination resulted in the “in-frame” fusion of SSIEFARL to the C terminus of the ie2 gene product. Recombinant BAC clones were selected by their growth on minimal plates in the presence of 2-deoxy-D-galactose as described (16). For MCMVM45SL, we inserted the same SSIEFARL encoding sequence at the 3′ end of M45 gene (between nucleotides 59513 and 59514) as described above. The linear fragment for the first step of this mutagenesis was generated by PCR using primers H5-M45gB (forward) and H3-M45gB (reverse). The recombination fragment for the second mutagenesis step was cloned by “assembly” PCR, with the two necessary PCR products being generated by amplification of MCMV sequences flanking the M45 stop codon. In addition to the necessary SSIEFARL-encoding nucleotides at their 5′ end, PCR primers contained unique restriction sites to facilitate subsequent cloning (see Supplemental Table I). The product of primers 5M45 forward and 5M45 reverse was cleaved with SpeI and Clal, whereas the product of primers 3M45 forward and 3M45 reverse was cleaved by Clal and HindIII. PCR products were ligated together and inserted into the SpeI/HindIII acceptor sites of a Litmus28 plasmid (NEB). The recombination fragment was cleaved from this construct by Sall and applied in the second round of mutagenesis, as described above.

**Peptides**

The peptides M45 (H-2Dd–restricted, 498HVIRNASFI935, m139 (H-2Kb–restricted, 319TYVGFCFLL326), IE3 (H-2Kb–restricted, 416RALEYKMK147), M38 (H-2Kb–restricted, 318SSPMFRV323) (11) and the HSV-1 glycoprotein–derived epitope gB (H-2Kb–restricted, 498SSIEFARL505) (18) were synthesized and HPLC purified (65–95% purity) at the HZI peptide-synthesis platform.

**Restoration of MCMV from recombinant BACs and preparation of virus stocks**

Recombinant viruses were reconstituted from BACs by transfection of BAC DNA into MEFs using FuGENE transfection reagent (Promega) according to the manufacturer’s protocol. Viruses were propagated as described (19) and virus stocks were prepared from M2-10B4 lysates purified on a sucrose cushion as described previously (20). Virus titers were determined on MEFs by plaque assay.

**In vitro infection**

Monolayers of NIH 3T3 cells were infected at a multiplicity of infection of 0.1 with MCMV recombinants or MCMV WT. After 1 h, the inoculum was removed, cells were washed with 1× PBS, supplied with fresh medium, and incubated for 6 d. At selected time points postinfection (p.i.), supernatants were harvested and stored at −70°C until titration.

**In vivo infection**

Male 6- to 12-wk-old mice were infected with purified, tissue culture–derived virus and housed in specific pathogen-free conditions throughout the experiment. Infected mice showing very weak immune priming (2 SDs below average) at 7 d p.i. were regarded as outliers owing to suboptimal infection and were excluded from the study. For experiments involving the establishment of infectious virus titers, organs were dissected under sterile conditions and stored at −70°C until titration.

**Infectious virus quantification**

MCMV from organ homogenate or tissue culture supernatants was titrated on MEFs as described previously (21). For identification of HSV-1 titer in organs, titration was performed on Vero cells as described before (22).

**Peptide stimulation**

Cells were stimulated with peptides (1 μg/ml) in 100 μl RPMI 1640 (10% FCS supplemented) for 6 h at 37°C with brefeldin A (Cell Signaling Technology) added at a concentration 10 μg/ml for the last 5 h stimulation. Negative control samples were generated for all tested groups by incubating cells in the same conditions, but in the absence of any peptide. Cells were tested for cytokine responses by intracellular cytokine staining using flow cytometry. Cytokine responses observed in unstimulated samples were considered background noise due to unspecific Ab binding and were subtracted from the values observed in test samples.

**Cell surface, intracellular cytokine staining, and flow cytometry**

Blood cells were stained for 30 min at 4°C with SSIEFARL tetramer-allophycocyanin (National Institutes of Health Tetramer Facility) and the following surface Abs: anti–CD4-Pacific Blue (clone GK1.5; BioLegend), anti–CD8a-PerCP/Cy5.5 (clone 53-6.7; BioLegend), anti–CD44–Alexa Fluor 700 (clone IM7; BioLegend), anti–CD11a-PE-Cy7 (clone 2D7; BD Biosciences), and anti–CD3–allophycocyanin-eFluor 780 (clone 17A2; eBioscience). For staining with Streptamer, the MHC class I–Strep molecules were first oligomerized (incubation for 45 min at 4°C with Streptactin allophycocyanin; IBA Life Sciences). Afterward, cells were stained with the oligomerized MHC class I–Strep molecules for 15 min at 4°C with subsequent staining with surface Abs for additional 30 min. For intracellular cytokine staining, cells were first surface stained for 30 min at 4°C with anti–CD4-eFluor 605NC (clone GK1.5; eBioscience), anti–CD8a-PerCP/Cy5.5 (clone 53-6.7; BioLegend), anti–CD44–Alexa Fluor 700 (clone IM7; BioLegend), anti–CD11a-PE-Cy7 (clone 2D7; BD Bio-
sciences), and anti–CD3-allophycocyanin-eFluor 780 (clone 17A2; eBioscience) Abs. Cells were subsequently fixed for 5 min with 100 μl IC fixation buffer (eBioscience), followed by 3 min permeabilization with 100 μl permeabilization buffer (eBioscience) and overnight incubation with anti–IFN-γ-allophycocyanin (clone XMG1.2; BioLegend). Cells were washed and acquired in an LSR-II cytometer (BD Biosciences). Cytometric results were analyzed with FlowJo software (version 9.5.3).

**Counting the cell populations in absolute terms**

Aliquots (100 μl) of blood leukocytes were stained for CD8 and CD4 expression with Abs and with streptamers to detect the M38-specific CD8 cells. The count of CD8+CD4− leukocytes or of M38-specific CD8+ cells per microliter of blood was assessed in an Accuri C6 flow cytometer (BD Biosciences).

**Statistics**

Two-way ANOVA was used to analyze weight loss data. Kruskal–Wallis analysis followed by Dunn’s post hoc analysis was used to compare multiple samples at single time points. Comparisons between two groups were performed using the Mann–Whitney U test (two-tailed). Statistical analysis was performed using a GraphPad Prism program.

**Results**

**Generation of recombinant viruses: rationale, construct design, and stability in vivo**

The CD8-restricted response to CMV Ags shows a distinctive hierarchy of immunodominance and a highly reproducible kinetic of individual peptide responses (11). To ascertain the role of gene expression context, rather than intrinsic peptide properties, in the kinetic and immunodominance hierarchy, we generated recombinant MCMVs expressing a single defined HSV-1 H-2b–restricted CD8 T cell epitope fused to the C terminus of different MCMV open reading frames. To further remove the effect of the intrinsic nature of the peptide, we selected a highly immunodominant Kβ-restricted peptide derived from the glycoprotein B of HSV-1 (SSIEFARL) (18).

**FIGURE 1.** Generation of recombinant MCMVs expressing the SSIEFARL peptide. (A) The MCMV genome area at ~kb 186–187 corresponds to the MCMV gene ie2 (enlarged above), whereas the gene M45 is found at ~kb 58–59 (enlarged below). A construct encoding the SSIEFARL peptide (DNA sequence was inserted by means of traceless BAC mutagenesis at the very end of either gene) and the inserted nucleotide sequence (black letters) as well as the corresponding amino acid sequence (gray letters) are shown. The inserted DNA sequence was designed to contain a ClaI restriction site (underlined part of the DNA sequence) to allow a diagnostic assay of the mutagenesis efficiency and of the stability of the construct. (B) DNA was isolated from salivary glands of mice 21 d upon infection with 10⁶ PFU indicated viruses and used as a PCR template with primers flanking the peptide sequence insertion site. PCR products were purified, digested with ClaI restriction enzyme (where indicated), and run on a horizontal agarose gel.
We inserted the HSV-1 peptide in-frame at the C terminus of either the ie2 or the M45 gene (Fig. 1A) by BAC-based mutagenesis, which generated MCMVie2SL and MCMVM45SL, respectively. The ie2 gene is expressed with immediate early kinetics, and fusion of heterologous target epitopes to the ie2 open reading frame has been used previously by ourselves and by others to induce inflationary responses against these epitopes (7, 10). The M45 gene is expressed with early kinetics and encodes a peptide that induces noninflationary CD8 T cell responses (11). To avoid major differences in the proteasomal processing of the SSIEFARL peptide encoded by the two genes, the epitope was inserted at the protein C terminus of each gene. To allow monitoring of the stability of the modified gene in the recombinant viruses, the coding sequence was designed to include a ClaI restriction site (Fig. 1A). The in vivo stability of the inserted peptide sequence was then assessed by diagnostic PCR of the respective MCMV-encoded gene (either ie2 or M45), followed by a ClaI digestion. These studies were performed on DNA isolated from the salivary glands of mice at 21 d p.i. with MCMV WT, MCMVie2SL, or MCMVM45SL. The PCR products obtained from mice infected with either of the recombinant viruses were larger than MCMV WT samples and sensitive to ClaI cleavage (Fig. 1B), consistent with stable in vivo maintenance of both mutant MCMVs.

**MCMVie2SL and MCMVM45SL in vitro and in vivo replication is comparable to WT MCMV**

To ensure that recombinant MCMVs maintained a comparable level of fitness, virus growth was initially assessed by a multistep growth kinetic assay in NIH 3T3 cells. As shown in Fig. 2A, both recombinants replicated comparably to MCMV WT. We then analyzed the viral replicative capacity in vivo by virus titration in lung and lungs of mice (at 5 d p.i.) and in salivary glands (at 21 d p.i.). Fig. 2B shows that in vivo recombinant MCMVs replicated at levels comparable to MCMV WT in all organs tested. In summary, the insertion of the SSIEFARL peptide does not interfere with viral replication in vivo, and any differences in T cell responses can therefore not be explained by a difference in the availability or organ distribution of infected cells.

**Recombinant viruses show expected immunological phenotype**

Next, we characterized CD8 T cell responses to the SSIEFARL peptide expressed in the context of the different viral genes. To exclude the possibility that CD8 T cell response may be influenced by Ly49H-m157 signaling (23), we used the 129/Sv mouse strain, which is H-2b restricted but lacks the Ly49H receptor. SSIEFARL-specific CD8 T cells in blood samples were identified by peptide/MHC complex tetramer staining and multiparametric flow cytometry (the complete gating strategy is shown in Supplemental Fig. 1). The response to the peptide in the CD8 T cell pool (tetramer CD1La+) was compared between mice infected with either MCMVie2SL, MCMVM45SL, HSV-1 (positive control), or MCMV WT (negative control) at 17 and 180 d p.i. (Fig. 3A). MCMV WT resulted in background tetramer staining comparable to levels observed prior to infection, and HSV-1 infection induced a CD8 response on day 7 p.i., with some responding cells still present at 180 d p.i. The magnitude of the HSV-1–specific CD8 responses in mice infected with recombinant MCMVs was increased in comparison with HSV-1–infected mice. However, the two recombinant viruses were distinct in both the magnitude and kinetics of their HSV-1 immune response. At day 7 p.i., the HSV-1 response induced by MCMVM45SL was stronger than the response induced by MCMVie2SL (Fig. 3A). However, by day 180 p.i. this situation had been reversed, with the HSV-1–specific T cell response induced by MCMVie2SL, being increased ∼4-fold relative to MCMVM45SL. Taken together, these results suggest that expression of the HSV-1 peptide with expression characteristics of ie2 results in memory inflation, whereas peptide expression characteristics comparable to M45 results in a noninflationary response that had contracted by day 14 p.i. and thereafter remained at a constant low level. This observation was confirmed by monitoring the kinetics of the HSV-1 tetramer–positive CD8 T cells over an entire time course of infection (Fig. 3B), which showed memory contraction by day 14 p.i. in groups...
FIGURE 3. The kinetic of immunodominance depends on the context of MCMV gene expression. Mice (129/Sv) were infected with $2 \times 10^5$ PFU MCMV WT, MCMVie2SL, MCMV^M45SL, or $10^5$ PFU HSV-1. Blood leukocytes were stained for CD3, CD4, CD8, CD11a, CD44, and SSIEFARL-Kb tetramer and analyzed by flow cytometry. (A) Representative dot plots of CD3^+CD4^+CD8^+ lymphocytes (the complete gating strategy is shown in Supplemental Fig. 1). Cell staining with anti-CD11a and SSIEFARL peptide/MHC complex tetramers at day 7 (left column) or 180 p.i. (right column) is shown. (B) Group means of CD11a^+tetramer^+ cells at 7, 14, 28, 60, 90, 120, and 180 d p.i. Pooled averages from five mice per group are shown. Error bars indicate SEM. (C) Cells from mice shown in (A) were additionally gated on CD44 and CD11a to identify primed CD8 cells. Representative dot plots of tetramer-positive cells at day 7 (left column) or day 180 (right column) for the indicated infection conditions are shown. (D) Group means at indicated days after infection are connected; error bars indicate SEM.
infected with HSV-1 or MCMVM45SL, but inflation of HSV-1–specific responses in the group infected with MCMVie2SL (Fig. 3B).

More detailed analysis of peptide-specific T cell responses, which examined responses in the compartment of primed CD8 T cells (CD44+CD11a+), showed that the percentage of the SSIEFARL-specific cells in the memory pools was essentially identical in MCMVM45SL– and HSV-1–infected mice at both day 7 and 180 p.i. (Fig. 3C). In contrast, MCMVie2SL infection resulted in lower percentages of peptide-specific memory T cells at day 7, but higher levels at day 180 p.i. (Fig. 3C). The kinetics of development of the peptide-specific cells in the memory compartment (Fig. 3D) were similar to those described for the total CD8 T cell pool (see Fig. 3B). HSV-1 infection resulted in identical percentages of the SSIEFARL-specific cells as in the MCMVM45SL infection. MCMVie2SL infection resulted in a very quick enrichment of peptide-specific cells in the pool of the primed CD8 T cells, which reached ~50% of this pool by day 60 p.i. and stayed constant thereafter. Taken together, these results indicate that gene context is critical in defining the immunodominance of Ags encoded by MCMV.

**FIGURE 4.** CD8 T cells specific for the SSIEFARL peptide encoded by either of the recombinant MCMVs stay functional. Mice were injected with $2 \times 10^5$ indicated viruses or 200 μl PBS. Blood leukocytes were stimulated with SSIEFARL peptide at 7, 14, 28, 60, 90, 120, and 180 d p.i. Cells were surface stained for CD3, CD4, CD8, CD11a, and CD44 and intracellularly for IFN-γ expression and analyzed by flow cytometry. (A) Representative dot plots showing IFN-γ+ cells in the pool of primed CD8+ T cells at day 7 (left column) and day 180 (right column) upon infection with indicated viruses. To identify the percentage of peptide-specific cells in the pool of primed cells, the CD8+ T cells were pregated on a CD11a+CD44+ and afterward gated on IFN-γ+CD11a+ gate. (B) The experiment was performed independently three times (five mice per group in each experiment), and grouped means ± SEM are shown. (C) Geometric mean fluorescence intensity of the IFN-γ signal in the IFN-γ+–gated CD8 T cells on day 7 and 180 p.i. in mice infected with MCMVie2SL or MCMVM45SL. Each symbol represents one mouse; group median values are shown as horizontal lines. Significance was assessed by a Mann–Whitney $U$ test. ***$p < 0.001$. 

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**PEPTIDE COMPETITION IN CMV INFECTION**

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FIGURE 5. Expression of the SSIEFARL peptide from the MCMV genome influences the CD8 T cell responses to endogenous MCMV M45 and m139 peptides. Blood of mice infected with the indicated viruses was analyzed for the fraction of CD8 T cells responding to in vitro restimulation with MCMV peptides M45 and m139. (A) Representative dot plots showing the intracellular expression of IFN-γ at days 7 and 180 p.i. upon M45 peptide stimulation. (B) Grouped means ± SEM of cells responding to the M45 peptide at days 7, 14, 28, 60, 90, 120, and 180 p.i. (C) Representative dot plots of IFN-γ cell response to m139 peptide stimulation at 7 or 180 d p.i. with indicated virus. (D) Grouped means ± SEM of cells responding to the m139 peptide at days 7, 14, 28, 60, 90, 120, and 180 p.i. The experiment was performed three times independently with five mice per group in each experiment, and grouped averages from all three experiments are shown.
**CD8 T cells specific for the SSIEFARL peptide encoded by either of the recombinant MCMVs are functional**

MCMV has been shown to suppress immunity by modulation of PD-L1 expression on dendritic cells, which is associated with diminished CD8 and CD4 T cell responses (24), and by induction of suboptimal CD8 IFN-γ responses (25). It was therefore necessary to determine whether expression context affected SSIEFARL-specific CD8 T cell effector functionality. Blood leukocytes were stimulated in vitro with peptide for 6 h and then stained for IFN-γ. The fraction of IFN-γ responding cells in the pool of primed CD8 T cells was comparable to the size of the tetramer-positive population at day 7 p.i., but was somewhat lower in mice infected with either recombinant virus at day 180 (Fig. 4A). Kinetic analysis (Fig. 4B) showed that the CD8 T cells responding to the SSIEFARL peptide by production of IFN-γ paralleled the tetramer responses, although the percentage of responding cells was lower than those identified by tetramers beginning at day 28 p.i. (Fig. 3D). Interestingly, the geometric mean fluorescence intensity of the IFN-γ producing CD8 T cells in mice infected with MCMV	extsuperscript{M45SL} was significantly higher than in MCMV	extsuperscript{ie2SL} infected groups at day 180 (p < 0.001), but not at day 7 p.i. (Fig. 4C), indicating that noninflationary CD8 T cells produced more IFN-γ upon restimulation.

**Insertion of immunodominant peptide in the MCMV genome results in the inhibition of CD8 T cell responses to endogenous viral peptides**

SSIEFARL peptide expression from the MCMV genome resulted in a strong CD8 T cell response on day 7 p.i., when the peptide was expressed from the early M45 gene, or in an accumulation of peptide-specific primed T cells during latency, when the peptide was expressed from the ie2 gene (see Figs. 3, 4). To determine whether this expression of an additional heterologous immunodominant peptide would affect CD8 responses to endogenous MCMV Ags, we compared the responses to endogenous MCMV peptides (either noninflationary M45-derived peptides or inflationary m139-derived peptides) in mice infected with MCMV	extsuperscript{M45SL}, MCMV	extsuperscript{ie2SL}, or MCMV WT. For these studies, blood samples were divided into equal parts and stimulated with the respective HSV-1 or endogenous MCMV-derived peptides. We also assessed the percentage of IFN-γ+ cells in CD8 T cells at 7 and 180 d p.i. (representative gating in Fig. 5A and 5C for M45 and m139 peptides, respectively). Comparison of the kinetics of the T cell response to each peptide in different groups showed a reciprocal relationship between HSV-1 and endogenous MCMV peptide responses. CD8 responses to the endogenous M45 peptide were impaired only on day 7 p.i. and only in mice infected with the MCMV	extsuperscript{M45SL} (Fig. 5B). Responses to the endogenous inflationary m139 peptide (Fig. 5D) were impaired in both groups infected with the recombinant viruses early upon infection, but they then recovered to levels observed in MCMV WT infected controls by 150 d p.i. in MCMV	extsuperscript{M45SL}-infected mice. Consistent with the maintained inflationary nature of responses against the HSV-1 epitope in MCMV	extsuperscript{ie2SL}-infected mice, responses against the m139 peptide were depressed for the length of the experiment. Statistical analysis of responses at day 7 p.i. showed a significantly impaired M45 response in MCMV	extsuperscript{M45SL}-infected but not in MCMV	extsuperscript{ie2SL}-infected mice (Fig. 6A), as well as lower m139-specific responses in either mutant infection, but not to a level that would reach statistical significance (Fig. 6B). This response pattern was essentially recapitulated when peptide-specific responses were expressed as a fraction of the primed CD8 compartment (Supplemental Fig. 2). The situation at day 180 p.i. was different, with responses to the endogenous peptide M45 being low for all viruses (data not shown), but responses to the m139 peptide being low only in the total CD8 pool of MCMV	extsuperscript{ie2SL}-infected mice (Fig. 7A). To understand whether this phenomenon was exclusive to the m139 peptide, we tested additional MCMV-derived inflationary peptides derived from the genes ie3 and M38 (11). On day 150 p.i., the blood CD8 responses were lower for both IE3 (Fig. 7B) and M38 (Fig. 7C) peptides in mice infected with MCMV	extsuperscript{ie2SL} mutant, and the situation was identical when the peptide response was calculated as a fraction of the primed CD8 compartment (Supplemental Fig. 2). MCMV	extsuperscript{M45SL} infection resulted in no significant reduction of the responses to the IE3 and m139 peptides. The response to the M38 peptide was reduced, but not as significantly as in the infection with MCMV	extsuperscript{ie2SL} (Fig. 7, Supplemental Fig. 2).

We considered the possibility that infection with recombinant viruses resulted in higher absolute counts of CD8 T cells. In this case, the percentages of CD8 T cells specific for immunodominant MCMV peptides might have been decreased whereas their absolute numbers remained unchanged, which would imply that the SSIEFARL peptide increased the pool of MCMV-specific cells without competing with endogenous peptides. We addressed this question experimentally by comparing the absolute counts of the total CD8 and M38-specific T cells in the blood of mice infected for >1 y with MCMV WT, MCMV	extsuperscript{ie2SL}, or MCMV	extsuperscript{M45SL} (Supplemental Fig. 3). We observed that there was no increase in the count of total CD8 T cells in mice infected with either of the SSIEFARL mutants (Supplemental Fig. 3A), whereas the counts of M38-specific CD8 T cells were significantly lower in the group infected with MCMV	extsuperscript{ie2SL} (Supplemental Fig. 3B), reflecting the data obtained by analyzing the relative amounts (Fig. 7C). Taken together, our data strongly suggest that the induction of strong inflationary responses to heterologous peptides may weaken the existing inflationary responses.

**Expression of the SSIEFARL peptide from either ie2 or M45 genes induces protective immunity in the HSV-1 infection model**

Previous studies have shown that the insertion of a single peptide in the ie2 gene may result in strong immune protection against pox-
viruses (7) or even Ebola virus (10). To assess whether mice infected with MCMV\(^{M45SL}\) mutant are protected on the same level as the one with MCMV\(^{ie2SL}\), we challenged mice that had been infected with the SSIEFARL encoding mutants or with MCMV WT with HSV-1 at 1 y p.i. 129/Sv male mice are highly susceptible to HSV-1 (26), and $5 \times 10^6$ PFU HSV-1 corresponded to $\sim 100$-fold the LD\(_{50}\) (data not shown). Disease was monitored on the basis of clinical signs and weight loss over the 7-d postchallenge period. Significant ($p < 0.001$) weight loss was observed by day 7 p.i. in MCMV WT-infected mice, but not in mice infected with the recombinant MCMV viruses (Fig. 8A). MCMV WT-infected mice also showed pronounced symptoms of disease, including lethargy, ruffled fur, abdominal bloating, and keratitis. To confirm that the disease signs were induced by HSV-1, we assayed its replication in organs. We observed that only the MCMV WT control group showed high viral titers in the brain and lungs (Fig. 8B) whereas mice infected with either of the mutant MCMVs expressing the SSIEFARL peptide were completely protected against HSV-1 infection and did not show any detectable virus in the tested organs.

**Discussion**

It has long been speculated that low-level transcription of immediate early CMV genes during latency (27) may be a key driver of memory inflation (4). The recent observation that long-term suppression of DNA replication by antiviral therapy has the potential to suppress memory inflation (28) suggests that genes involved in viral lytic replication are necessary for the induction of memory inflation (28). The \(M45\) gene is expressed with early kinetics commencing at $\sim 12$ h p.i (29), whereas \(ie2\) gene expression is most abundant immediately after infection (30) and can be observed during virus latency (27). By removing variables associated with intrinsic differences in the target epitope, to our knowledge, our study provides the first direct evidence that memory inflation is critically dependent on the context of viral gene expression.

**FIGURE 7.** Expression of the SSIEFARL peptide from the \(ie2\) MCMV gene inhibits CD8 T cell responses to endogenous inflationary MCMV peptides. Blood leukocytes of mice infected with indicated virus were stimulated with MCMV peptides m139 (A) on day 180 p.i. Blood from the same mice was also collected on day 150 and stimulated with IE3 (B) or M38 (C) peptides. Cells were stained with surface and IFN-\(\gamma\)-specific Ab and analyzed by flow cytometry. The percentage of IFN-\(\gamma^+\) cells in the pool of total CD\(_8^+\) T cells was defined by flow cytometry. Three independent experiments were performed and the results were grouped for statistical analysis. Each symbol represents a mouse; horizontal lines indicate medians. Significance was assessed by a Kruskal–Wallis test followed by Dunn’s post hoc analysis for indicated columns. $^*p < 0.05$, $^{**}p < 0.001$.

**FIGURE 8.** Infection of mice with recombinant MCMVs expressing SSIEFARL peptide protects mice against a systemic HSV-1. Mice (129/Sv, infected for 1 y with MCMV WT, MCMV\(^{ie2SL}\), or MCMV\(^{M45SL}\)) were challenged with $5 \times 10^6$ PFU HSV-1 per mouse. (A) Weight loss kinetic was monitored daily for 7 d, and weight, expressed as percentage of weight at HSV-1 challenge, is shown as average $\pm$ SEM. (B) Seven days after challenge, mice were sacrificed and brain and lung homogenates were titrated. Each symbol represents one mouse; group median values are shown as a horizontal line. Significance was assessed by a Kruskal–Wallis test followed by Dunn’s post hoc analysis for indicated columns. $^{**}p < 0.01$. DL, Detection limit.
results cannot completely exclude the possibility that subtle differences in proteasomal processing influence the efficiency of HSV-1 peptide presentation and thereby T cell induction. However, insertion of the peptide at the same C-terminal position in both proteins reduces this possibility, and the observation that both constructs induced substantial responses against the HSV-1 epitope, albeit with different kinetics, further supports that the epitopes are being processed in an equivalent fashion. In summary, our data are consistent with a model wherein expression of the SSIEFARL peptide with ie2, but not in M45, gene kinetics results in repeated exposure of T cells to the HSV-1 target Ag during viral latency (as a consequence of stochastic reactivation) (27), which results in the observed differences in T cell immunity. These observations are also consistent with the hierarchical temporal expression of peptides in latently infected cells resulting in CD8-mediated block of downstream viral gene transcription, which fits the observation that deletion of the anchoring residue of the immunodominant, Ld-restricted, icl-encoded MCMV peptide YPHFMPNTL simultaneously abrogated memory inflation against this peptide and increased ie3 transcription during virus latency (31).

High levels of CD8 T cell immunity induced by CMV-based vaccine vectors are thought to be instrumental in the induction of protective immunity against target pathogens (10). However, the impact of targeting of immune responses to novel immunodominant Ags on responses against existing CMV Ags remains unclear. During the preparation of this manuscript, we became aware of the results by Hill and colleagues (32). Both this study and that of Hill and colleagues show that the induction of inflationary responses against immunodominant heterologous target Ags may compromise responses against other (in this case, endogenous) Ags expressed by CMV. In our study, we observed a decrease in memory inflation toward endogenous peptides in mice infected with the recombinant MCMV expressing the HSV-1 peptide in an inflationary context (i.e., fused to ie2), but not where the HSV-1 peptide was noninflationary (i.e., fused to M45). Results from Hill and colleagues, using a recombinant MCMV expressing an OVA-derived immunodominant peptide (SHINKFEKL) fused to the C terminus of the ie2 gene, showed a similar result from expression of this immunodominant epitope. Moreover, they also observed that suppression of inflationary responses only occurs against endogenous Ags expressed by the recombinant virus, presumably due to coexpression of heterologous and endogenous Ags within the same cell. Suppression of inflationary endogenous responses was not observed when mice are coinfected with the WT and the recombinant MCMV. This observation implies that memory inflation is induced by the direct presentation of peptides on cells harboring latent viral genomes, and not by cross-presentation, in line with previous studies by Reddhease and colleagues (33) and by Oxenius and colleagues (34) showing that memory inflation was dependent on Ag presentation by nonhematopoietic cells. Consistent with this model, CD8 recognition of a peptide that is expressed at immediate early times (i.e., with ie2 context) would block the expression of peptides expressed later in the virus replication cycle, which fits the difference between the inhibitory effects of MCMVie2SL- and MCMVM45SL-encoding artificial chromosome. Cytomegaloviruses: Molecular Biology and Immunology. Caister Academic Press, Norfolk, United Kingdom.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary table I: List of primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
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<tr>
<td>ie2GalK</td>
<td>Forward 5’-GTGTACTAGTGCTCTTCCGGAACCACCGGTTTTCTTTCTTTGACCAGAGACCTGTTGCCAGCAGAAGATTCACTCGGTGTTGACAAATTAATCATCGGCA-3’&lt;br&gt;Reverse 5’-GTGTAAGCTTGCTCTTTCTTCTCCTGATGGAATAAAACCTCTTTATTATTGATTTAAAACCATGACATACCTCGTCGCGCAAGTGTGCAACACATCGGCA-3’</td>
<td>Generation of the construct for the first step of mutagenesis of MCMV^{ie2SL} virus</td>
</tr>
<tr>
<td>H3-M45gB</td>
<td>Forward 5’-TGTCCGTCGGAGTATATAATGTAGTATGCCCCCCCTTCGCTGAAATTATCGCGCAGCTGTTGACAAATATCTCGGCATACATCGGCA-3’&lt;br&gt;Reverse 5’-AAGCCCACTCGAGCGAGCAATAAGACTCGTTTTTTGAGCGACGAGTCTTCGGACAAATGATCATCGGCA-3’</td>
<td>Generation of the construct for the first step of mutagenesis of MCMV^{M45SL} virus</td>
</tr>
<tr>
<td>H5-M45gB</td>
<td>Reverse 5’-AAGCCCACTCGAGCGAGCAATAAGACTCGTTTTTTGAAGCGACGAGGTCGGACAAATATGCATCGGCA-3’</td>
<td></td>
</tr>
<tr>
<td>5M45</td>
<td>Forward 5’-GTGTACTAGTGCTCTTCCGGAACCACCGGTTTTCTTTCTTTGACCAGAGACCTGTTGCCAGAAGATTCACTCGGTGTTGACAAATTAATCATCGGCA-3’&lt;br&gt;Reverse 5’-CCTCATCGATTGAGTTCGCCCGCCTCTGACAACTCGTCGCCAAAAACGAG-3’</td>
<td>Generation of the construct for the second step of mutagenesis of MCMV^{M45SL} virus</td>
</tr>
<tr>
<td>3M45</td>
<td>Forward 5’-ACTCAATCGATGAGGTGCTTTGATGCGATAATTCACGGAAGGG-3’&lt;br&gt;Reverse 5’-GTGTAAGCTTGCTCTTTCTCCTCGAAAGAGTCCCGAC-3’</td>
<td>Generation of the construct for the second step of mutagenesis of MCMV^{M45SL} virus</td>
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LEGENDS TO SUPPLEMENTARY MATERIALS

**Figure S1. Representative gating strategy.** To define tetramer specific cells in the whole pool or in the pool of primed CD8 T-cells, cells were first gated on a forward/side scatter gate, followed by gating for singlets. Next cells were gated on a CD3+ gate, where somewhat lower expression of CD3 was tolerated to accommodate for the decrease of CD8 expression on CMV-specific cells. CD3+ cells were gated by CD4 and CD8 expression into a CD4CD8+ gate, from which we either identified tetramer positive cells as CD11ahi tet+, or proceeded to progressively gate the CD11a’CD44+ cells into a subset which was analysed for the presence of tet+ cells. The same gating strategy was applied for the detection of cytokine responses, substituting the tet+ gate with the IFNγ+ one.

**Figure S2. Expression of the SSIEFARL peptide from the ie2 MCMV gene lowers the percentage of memory CD8 T-cell responses to endogenous inflationary MCMV peptides.** Blood leukocytes of mice infected with indicated virus were stimulated with MCMV peptides M45 and m139 on day 7 p.i. (A), or with MCMV peptides IE3, M38 or m139 at late time points (day 150 p.i. for M38 and IE3, day 180 p.i. for m139). Cells were stained with surface and IFNγ specific antibodies and analysed by flow cytometry. The percentage of IFNγ+ cells in the pool of the primed CD8+ T-cells was defined by flow cytometry. Three independent experiments were performed and the results were grouped for statistical analysis, except for stimulation with the IE3 peptide where results were pooled from two independent experiments. Each symbol represents a mouse, horizontal lines indicate medians. Significance was assessed by Kruskal-Wallis test followed by Dunns post-analysis for indicated columns (ns – p>0.05, * - p<0.05, ** - p<0.01, *** - p<0.01).

**Figure S3. Absolute counts of total CD8 and M38+ cells within total CD8 T cells in blood of MCMV WT, MCMVie2SL or MCMVM45SL -infected mice.** In mice (5 per group) infected for more than 1 year with 2x10⁵ PFU of MCMV WT, MCMVie2SL or MCMVM45SL, we counted the absolute number of total CD8 T cells (A) or CD8 T cells specific for the M38 peptide (B) in an aliquot corresponding to (100 µl) of whole blood. Each dot represents data from a single mouse, horizontal lines show medians. Significance was assessed by Kruskal-Wallis test followed by Dunns post-analysis for indicated columns (ns – p>0.05, * - p<0.05).