Competition for Antigen at the Level of the APC Is a Major Determinant of Immunodominance during Memory Inflation in Murine Cytomegalovirus Infection

Lila A. Farrington, Tameka A. Smith, Finn Grey, Ann B. Hill and Christopher M. Snyder

*J Immunol* published online 1 March 2013
http://www.jimmunol.org/content/early/2013/02/28/jimmunol.1203151
Competition for Antigen at the Level of the APC Is a Major Determinant of Immunodominance during Memory Inflation in Murine Cytomegalovirus Infection

Lila A. Farrington,* Tameka A. Smith,* Finn Grey,† Ann B. Hill,* and Christopher M. Snyder‡

The unique ability of CMV to drive the expansion of virus-specific T cell populations during the course of a lifelong, persistent infection has generated interest in the virus as a potential vaccine strategy. When designing CMV-based vaccine vectors to direct immune responses against HIV or tumor Ags, it becomes important to understand how and why certain CMV-specific populations are chosen to inflate over time. To investigate this, we designed recombinant murine CMVs (MCMVs) encoding a SIINFEKL-enhanced GFP fusion protein under the control of endogenous immediate early promoters. When mice were infected with these viruses, T cells specific for the SIINFEKL epitope inflated and profoundly dominated T cells specific for nonrecombinant (i.e., MCMV-derived) Ags. Moreover, when the virus encoded SIINFEKL, T cells specific for nonrecombinant Ags displayed a phenotype indicative of less frequent exposure to Ag. The immunodominance of SIINFEKL-specific T cells could not be altered by decreasing the number of SIINFEKL-specific cells available to respond, or by increasing the number of cells specific for endogenous MCMV Ags. In contrast, coinfection with viruses expressing and lacking SIINFEKL enabled coinflation of T cells specific for both SIINFEKL and nonrecombinant Ags. Because coinfection allows presentation of SIINFEKL and MCMV-derived Ags by different cells within the same animal, these data reveal that competition for, or availability of, Ag at the level of the APC determines the composition of the inflationary response to MCMV. SIINFEKL’s strong affinity for H-2Kb, as well as its early and abundant expression, may provide this epitope’s competitive advantage.

The Journal of Immunology, 2013, 190: 000–000.

Cytomegalovirus establishes an asymptomatic latent or persistent infection, which is characterized by the lifelong accumulation of a large number of virus-specific T cells. This process is termed memory inflation and has led to the exploration of CMV as a vaccine vector for HIV and for tumor Ags, with significant initial success in the SIV model (1, 2). The fact that memory inflation occurs postinfection with a single-cycle CMV (3) indicates that CMV-based vaccines may be safely used even in immunosuppressed cancer patients, further increasing the appeal of this approach. The vaccine potential of this virus has elevated the importance of understanding how inflammatory CMV-specific responses are selected and maintained during infection.

C57BL/6 mice mount a response to at least 20 viral Ags during acute infection with murine CMV (MCMV) (4). Most of these responses, including those to the immunodominant M45 Ag, then decline precipitously and leave small central memory populations. In contrast, memory inflation is dominated by only three responses: those to M38, m139 and IE3, all of which are subdominant to M45 during acute infection (5). These same three epitopes display memory inflation postinfection with the single cycle ΔgL-MCMV (3), which implies that nonproductively infected cells harboring the viral genome can drive memory inflation.

We presume that ongoing presentation of viral epitopes must be involved in memory inflation. We have shown that memory inflation is sustained by repeated production of short-lived effectors derived from a pool of memory cells established early in infection (6). However, the reason that inflationary responses focus on just a few Ags is not well understood.

MCMV has a highly ordered sequence of lytic cycle gene expression, which starts with the transcription of immediate early (IE) genes and is followed by the synthesis of early (E) and then late (L) gene products. However, latent MCMV infection in the lungs and liver is characterized by sporadic expression of IE genes without evidence of E or L gene expression (7, 8). This is thought to be abortive reactivation, in which the virus initiates the standard lytic gene cascade, but gene expression is aborted at the IE stage (9). This scenario predicts that IE gene products would be the most abundant during latent infection and thus immunodominant, which is at least partly the case: IE3 becomes progressively more immunodominant over time in C57BL/6 mice, and pp89 (IE1)-specific responses inflate somewhat more than do those specific for the E Ag m164 in BALB/c mice. Furthermore, recombinant epitopes expressed behind IE promoters provoke inflationary responses (10). However, M38 and m139, both E Ags, also provoke immunodominant inflationary responses in C57BL/6 mice, as does m164 in BALB/c mice (5). Likewise in humans, T cells target epitopes expressed with IE, E, and L kinetics (11), and cells specific for the L gene product pp65 are frequently immunodo-
minant (12–14). The viral gene expression program that drives these diverse responses is not yet clear.

Our data suggest that viral gene expression, and not productive replication, is sufficient to promote inflation of T cells specific for E gene products. This is evidenced by the ability of a single cycle \( \Delta G \)-MCMV to stimulate inflation of T cells specific for the E genes M38, m139, and m164 (3). Abortive reactivation may sometimes proceed to expression of E genes, as suggested by Simon et al. (9). An alternate possibility is that a completely different gene expression program occurs in some infected cells. Indeed, in the rat CMV heart transplant model, expression of a subset of E genes without production of infectious virus has been described (15). It is interesting that this “persistent” pattern of gene expression involved very little IE gene expression. Similarly, expression of some viral genes in the absence of IE gene expression is reported in monocytocytes latently infected with human CMV (16). Hence, inflammatory responses to E epitopes may be driven by different cells harboring a different program of gene expression than those that drive the IE responses.

There is also some evidence that T cells can influence the pattern of immunodominance during memory inflation. Indeed, Holtappels et al. (17) described a “conditional” immunodominant response specific for the viral m145 gene product in BALB/c mice, which appeared when the immunodominant m164- and IE1-derived epitopes were deleted. In line with this, Simon et al. (9) have suggested that T cells directly limit the cascade of viral gene expression. Thus, immunodominant T cell responses may restrict other epitopes from being produced. Inflammatory T cell responses of particularly high avidity, either due to expression of high-affinity TRCs or to abundant Ag expression, might enforce a selective advantage by suppressing expression of additional epitopes.

In this study, we describe memory inflation in response to recombinant MCMVs that encode a SIINFEKL-GFP fusion protein under immediate early control. Not only did SIINFEKL promote memory inflation, it became the sole inflationary epitope during chronic infection. We used this model to explore the determinants of immunodominance in the inflammatory T cell response to MCMV.

Materials and Methods

**Mice**

C57BL/6 mice were purchased from The Jackson Laboratory. B6.SJL-CD45.1 congenic (B6.SJL-Ptgpc-2 Pepc/BoyJ) mice were also purchased from The Jackson Laboratory and bred to C57BL/6 mice in-house to generate CD45.1/CD45.2 F1 mice. Mice were between the ages of 6 and 16 wk old. All studies were approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at Oregon Health and Sciences University.

**Virus strains and infections**

Mice were infected i.p. with \( 2 \times 10^5 \) PFU virus, except in coinfection experiments, where mice were infected with \( 1 \times 10^5 \) PFU of each virus used. Virus labeled MCMV–wild-type (WT) bacterial artificial chromosome (BAC) was of the strain MWM97.01, which is derived from a BAC of the Smith strain (19). MCMV–GFP-SL8 and MCMV–GFP-MSL8 were used. MCMV–GFP-SL8 and MCMV–GFP-MSL8 were generated from the MCMV-WT BAC (strain MW97.01) (22) using homologous recombination. Stocks of this virus were produced on GL-3T3 cells, which provide gL in trans (3), and titred by plaque assay on GL-3T3s without centrifugal enhancement. The individual virus stocks used in Fig. 3 were checked for reversion by infecting murine embryonic fibroblasts, a noncomplementing cell line, and then passaging and monitoring these infected cells for 30 d. The growth of cells not infected by the initial inoculum confirmed the inability of the gL-deficient virus to spread from cell to cell.

**Intracellular cytokine staining and FACS analysis**

For measurement of intracellular IFN-\( \gamma \), peripheral blood was collected at the indicated time points. RBCs were lysed with 3 ml lysis buffer (150 mM NH\(_4\)Cl, 10 mM NaHCO\(_3\)) and the remaining cells were incubated for 5–6 h at 37°C in the presence of 10 \( \mu \)M of the indicated peptide and brefeldin A (GolgiPlug; BD Pharmingen). Surface staining was done overnight at 4°C, and cells were fixed and permeabilized for intracellular cytokine staining with Cytofix/Cytoperm (BD Pharmingen). The following fluorescently conjugated Abs were used: CD8a (clone 53-6.7), CD27 (clone LG.7F9), CD23 (clone 145-2C11), CD127 (clone A7R34), KLH41 (clone 2F1), IFN-\( \gamma \) (clone XMG1.2) (all Abs were purchased from BD Biosciences, eBioscience, or BioLegend). Samples were acquired on an LSR II or a FACScalibur (both BD Biosciences) and analyzed with FlowJo software (Tree Star).

**Adoptive transfers**

Splenocytes from congenic mice infected for 7 d with MCMV-WT BAC were harvested, passed through a 70-\( \mu \)m cell strainer, washed twice with T cell media (RPMI 1640 with l-glutamine, 10% FBS, 1% penicillin/streptomycin, and 5 \times 10^{-5} \) M-2-ME), and resuspended in PBS at 5 \times 10^{7} cells/ml. One hundred microliters of this unfractionated splenocyte suspension was injected intravenously into each congenic recipient via the retro-orbital route. These mice were infected with either MCMV-GFP-SL8 or MCMV-WT BAC the following day.

**RMA-S peptide binding and stabilization assays**

For binding assays, TAP-deficient RMA-S cells were plated at 1 \times 10^{5} cells/well in 96-well plates and cultured for 16 h at 25°C in T cell media buffered with 25 mM HEPES. The cells were then washed with T cell media, incubated with different concentrations of the indicated peptides at 25°C for 2 h, and then incubated for an additional 2 h at 37°C. After this incubation, cells were washed once and stained on ice with 1 h with PE-conjugated Y3 mAb, which binds to the class I MHC 1-2K\(_{a}\). The cells were then washed twice with PBS, fixed with BD Fix/Perm solution (BD Biosciences), and analyzed on a FACScalibur (BD Biosciences).

**Quantitative real-time PCR**

Murine embryonic fibroblasts (1 \times 10^{5}) were infected with WT MCMV or MCMV-GFP-SL8 at a multiplicity of infection of 10. Cells were harvested at 0, 1, 2, 3, 4, 8, 18, and 24 h after infection, and RNA was extracted using the Qiagen RNeasy Mini kit. On-column DNAse treatment was performed as described in the Qiagen protocol. cDNA was generated using the Invitrogen SuperScript III First-Strand Synthesis Supermix protocol. For measurement of intracellular IFN-\( \gamma \), RNA was subjected to reverse transcriptase to ensure that there was no DNA contamination. cDNA was then stored at -20°C. Quantitative PCR was performed using Platinum SYBR Green qPCR SuperMix-UGD with ROX, using the primers at a concentration of 250 nM. The samples were run on an ABI Prism 7700 sequence detection system (Applied Biosystems). Relative gene expression was determined by normalizing each gene to \( \beta \)-actin and comparing the gene expression relative to cells at 0 h. The calculations were made following the method described in user bulletin no. 2 on the ABI Prism 7700 sequence detection system (Applied Biosystems; subject, relative quantitation of gene expression). Primer sequences were as follows: SL9, forward, 5'-ACAAAGGCGCAACAGTTC-3', reverse, 5'-TGAATTCAGGCAGCCAACC-3'; SL9, forward, 5'-GTTCAACCCGGCTTGTAG-3', reverse, 5'-GATAATTCAGGCAGCCAACC-3'; M38, forward, 5'-TGATATTAGGCTGCTTTGA-3', reverse, 5'-GGGCTGCTTGTGACAGATT-3', reverse, 5'-ACGACGCAACAATGGGA-3'.

was encoded with the major IE promoter of human CMV and targeted to replace exon 3 of the m128 gene in MCMV. Stocks of these viruses were produced from murine embryonic fibroblasts and titred by plaque assay on Balb3T3s without centrifugal enhancement.

To produce the \( \Delta G \) viruses, an amplicon gene fragment was inserted into the M115 (gl) gene of the MCMV-WT BAC (strain MW97.01) (22) using homologous recombination. Stocks of this virus were produced on GL-3T3 cells, which provide gL in trans (3), and titred by plaque assay on GL-3T3s without centrifugal enhancement. The individual virus stocks used in Fig. 3 was checked for reversion by infecting murine embryonic fibroblasts, a noncomplementing cell line, and then passaging and monitoring these infected cells for 30 d. The growth of cells not infected by the initial inoculum confirmed the inability of the th gL-deficient virus to spread from cell to cell.
Results

SIINFEKL-specific CD8+ T cells dominate memory inflation postinfection with MCMV-GFP-SL8

We generated a recombinant strain of MCMV expressing a GFP-SIINFEKL fusion construct under the control of the endogenous MCMV IE2 promoter (H.M. Turula, F. Grey, K. Zurbach, and C.M. Snyder, submitted for publication). After infection with this virus (MCMV-GFP-SL8), the SIINFEKL-specific CD8 T cell responses in C57BL/6 mice steadily inflated over time, becoming the dominant inflationary T cell population in these animals at chronic time points (Fig. 1A, 1B). We also generated a virus in which the GFP-SIINFEKL fusion construct is under control of the human CMV major IE promoter (MCMV-GFP-MSL8), resulting in ~10- to 20-fold greater GFP fluorescence after in vitro infection (not shown). SIINFEKL-specific T cells dominated the inflationary response in mice infected with this virus as well (data not shown). Responses to IE3- and M38-derived peptides were barely detectable in these animals, whereas T cells specific for these epitopes each comprised ~5% of the CD8 T cell compartment in mice infected with WT MCMV (Fig. 1A and Ref. 5). Notably, the size of M38- and IE3-specific T cell populations were similarly reduced when measured as a frequency of all cells in the blood (not shown). Moreover, the proportion of CD8+ T cells specific for M45, which are resting memory cells that do not inflate during MCMV infection, were comparable in mice infected with either virus (Fig. 1 and not shown). These data indicate that proportional changes in T cell numbers cannot explain the disappearance of IE3 and M38 inflation. Thus, the presence of the SIINFEKL epitope and the resulting T cell response suppressed inflation of IE3- and M38-specific T cells, despite evidence that M38-specific T cells were successfully primed during acute infection.

SIINFEKL responses dominate memory inflation when SIINFEKL is expressed in a single-cycle MCMV

To determine whether the profound immunodominance of SIINFEKL would also occur in a single-cycle MCMV, we produced a version of MCMV-SL8-GFP lacking gL, a glycoprotein necessary for cell entry and spread. The gL-SL8 virus was confirmed to be spread-deficient as described in Materials and Methods, but it still induced SIINFEKL-specific T cells to inflate and become dominant (Fig. 1C), indicating that productive infection is not needed for the immunodominance of this response.

Phenotype of cells specific for inflationary epitopes after infection with MCMV-GFP-SL8

Inflationary CD8+ T cells express KLRG1 and have low levels of the IL-7 receptor (CD127) and the costimulatory molecule CD27 (6). This terminally differentiated effector phenotype is consistent with recent or repeated Ag exposure. Conversely, T cells comprising the memory response to noninflationary epitopes M45 and M57 exhibit a memory phenotype (KLRG1+, CD27+, and CD127+), which suggests that they are rarely exposed to Ag after the acute phase of infection. Because responses to M38 and IE3 contract sharply after acute MCMV-GFP-SL8 infection, we wondered whether they also would develop a memory phenotype.

Fig. 2 shows that SIINFEKL-specific CD8s at week 18 postinfection exhibit the classic phenotype of inflationary MCMV T cells, with upregulated expression of KLRG1 and downregulation of CD27 and CD127. In contrast, T cells specific for M45 mostly lacked KLRG1 and retained expression of CD127 and CD27, although some cells were KLRG1+. This is similar to their phenotype in WT infection. Strikingly, the small M38-specific population found in MCMV-GFP-SL8–infected mice had a similar phenotype to the M45-specific cells: most cells lacked KLRG1 and retained CD27 and high levels of CD127. IE3-specific cells were so infrequent that an accurate assessment of their phenotype was impossible. These results suggest that SIINFEKL-specific cells have seen Ag recently or repeatedly and that M45- and M38-specific cells encounter Ag rarely.

Altering the ratios of functional, epitope-specific cells available to respond to infection does not influence the immunodominance of SIINFEKL-specific T cells

The precursor frequency of Ag-specific T cells (either naive or memory) is a major determinant of immunodominance during acute infections and also affects proliferation and memory CD8 T cell lineage decisions (23). We wondered whether we could modify the immunodominance of the SIINFEKL response during chronic infection by altering the ratios of functional, epitope-specific CD8 T cells prior to infection. We explored this possibility in three ways.

First, we used mice that express OVA as a self-Ag behind the mouse mammary tumor virus promoter. When these mice were infected with MCMV-GFP-MSL8, the acute response to SIINFEKL was approximately a third of that in WT mice (Fig. 3A), consistent with a lower number of SIINFEKL-specific precursors. Nevertheless, during chronic infection with either MCMV-GFP-MSL8 (not shown) or MCMV-GFP-SL8, the SIINFEKL response inflated at the expense of the M38 and m139 responses (Fig. 3A).

Next, we reduced the number of naive CD8+ T cells capable of responding to SIINFEKL during acute infection by i.v. injection of SIINFEKL peptide prior to infection. Intravenous peptide provides a large amount of Ag (signal 1) in the absence of costimulation (signal 2), resulting in anergy or deletion of cognate T cells (24–26). Mice were injected i.v. with 10 µg SIINFEKL peptide on each of the 3 d prior to infection. SIINFEKL-specific
T cells were not detected by intracellular cytokine staining or tetramer staining 7 d postinfection, indicating profound suppression and probable deletion of SIINFEKL-specific cells, whereas T cells specific for MCMV epitopes were primed normally (Fig. 3B). However, by week 12, SIINFEKL responses had risen to the same percentage of total CD8s as those of mice left untreated, and responses to IE3 and M38 were barely detectable (Fig. 3B).

In a third experiment, we asked whether increasing the number of T cells available to respond to IE3 and M38 would enable those responses to inflate postinfection with the SIINFEKL-expressing virus. Splenocytes from CD45.2+ donor mice that had been infected with WT MCMV 7 d previously were adoptively transferred into CD45.1+CD45.2+ F1 naive recipients. These mice were then infected with MCMV-GFP-SL8. A control group received splenocytes from the same donors but was infected with WT MCMV instead. Fig. 3C shows that the SIINFEKL-specific response still dominated memory inflation at the expense of the IE3 and M38 responses. This was not because the transferred cells were unable to proliferate, as the donor cells expanded and contributed to inflation in WT-infected mice (Fig. 3C). Thus, pre-expanding T cells specific for MCMV epitopes were not able to override the profound immunodominance of SIINFEKL-specific CD8 T cells in chronic infection.

Taken together, these results suggest that the frequency of epitope-specific cells available prior to infection is not the most significant factor in determining the size of the SIINFEKL response relative to other MCMV responses during chronic infection with MCMV-GFP-SL8.

**Competition for Ag shapes immunodominance during chronic MCMV infection**

Because precursor frequency did not explain the dominance of SIINFEKL during chronic infection, we asked whether the phenomenon was the result of competition between T cells at the level of the APC. This phenomenon has been termed immunodomination (27). To test this, we coinfeected mice with both WT MCMV and MCMV-GFP-SL8. Previous work has shown that coinfection with two viruses yields distinct foci of infection with each individual virus (28). Thus, in our experiments, WT MCMV and MCMV-GFP-SL8 should largely infect different cells within the same host and their epitopes should be presented to T cells by different APCs. This eliminates competition between T cells of different specificities at the level of the APC.

In mice receiving both viruses, responses to SIINFEKL and to the MCMV epitopes IE3 and M38 were codominant during chronic infection (Fig. 4A). We interpreted this to mean that T cells specific for endogenous MCMV gene products were able to inflate when these epitopes were not presented by APCs also presenting SIINFEKL. However, a trivial explanation for this would be that a much faster replicating WT virus would result in a greater abundance of MCMV epitopes in coinfected mice. Indeed, MCMV-SL8-GFP does grow with slightly delayed kinetics in vitro (Turula et al., submitted for publication).

To ensure that this was not the case, we repeated these coinfection experiments with a single-cycle virus, ΔgL-MCMV, in
place of WT MCMV. Despite lacking gL, this virus can still promote memory inflation during chronic infection (Fig. 4B and Ref. 3). Nevertheless, in mice coinfected with MCMV-GFP-SL8 and ΔgL-MCMV, Ags from MCMV-GFP-SL8 would clearly be more abundant. Fig. 4B shows that at 18 wk postinfection, responses to IE3, M38, and m139 were similar in coinfected mice and mice infected with ΔgL-MCMV alone. These data indicate that the results in Fig. 4A are not due to differing rates of viral replication. We therefore conclude that competition at the level of the APC influences inflation and immunodominance during MCMV infection.

**FIGURE 3.** Precursor frequency does not contribute significantly to the immunodominance of SIINFEKL-specific CD8 T cell responses in chronic infection. (A) OVA transgenic mice were infected i.p. with the indicated viruses. Virus-specific CD8 T cells were measured in the blood on day 7 and at week 18 postinfection using intracellular cytokine staining. (B) C57BL/6 mice were injected with 10 μg SIINFEKL peptide i.v. on days −3, −2, and −1 prior to infection with MCMV-GFP-SL8. Responses were measured in the blood on day 7 and at week 18 postinfection. (C) CD45.2+, CD45.1+ naïve recipients received 2–5 × 10⁶ unfractionated splenocytes from mice infected for 7 d with WT MCMV. Recipients were infected with WT MCMV or with MCMV-GFP-SL8 and virus-specific responses were measured in the blood at week 18 postinfection. Total CD8 T cell responses are shown on the left and percentages of CD45.2+ donor cells contributing to either IE3 or M38 responses are shown on the right. Bars represent four to five mice per group. The experiments were done twice.

**FIGURE 4.** Competition for Ag shapes immunodominance during chronic MCMV infection. (A) C57BL/6 mice were infected i.p. with WT MCMV and MCMV-GFP-SIINFEKL at the same time. Virus-specific T cells were measured in the blood at the indicated times after infection using intracellular cytokine staining. (B) Mice were infected i.p. with ΔgL-MCMV or both ΔgL-MCMV and MCMV-GFP-SIINFEKL. Virus-specific CD8 T cell responses were measured in the blood at the indicated times after infection. The graph on the left shows the T cell responses at the indicated weeks after coinfection. The graph on the right shows the data from all groups at week 18. Individual bars represent four to five mice per group. The experiments were done twice.

**SIINFEKL is expressed earlier and has a higher MHC binding affinity than do endogenous MCMV epitopes**

The above data established that SIINFEKL is able to outcompete endogenous MCMV epitopes to promote T cell inflation when presented on the same APC. The mechanisms that cause the immune system to narrowly focus T cell responses on a few immunodominant epitopes are not completely understood. That being said, some factors are obviously important: peptides that are more abundantly presented, either due to expression, processing, or binding affinity, are more likely to be the focus of these responses (29, 30).

To compare the MHC binding affinity of SIINFEKL and the MCMV-derived inflammatory epitopes, all of which are presented by H-2Kb, we evaluated the ability of these peptides to stabilize Kb on the surface of the TAP-deficient cell line RMA-S. Fig. 5A shows that SIINFEKL bound Kb most strongly, followed by M38 and m139, with IE3 binding with the weakest affinity. Thus, a better ability to bind Kb would favor SIINFEKL presentation.

Epitope presentation is also affected by the amount of parent protein available for degradation and presentation. Because SIINFEKL dominated memory inflation postinfection with the single cycle ΔgL-SL8, we presume that cells harboring the latent viral genome, or their progeny, are responsible for the Ag presentation that drives memory inflation. Because the identity of these cells is...
unknown, it is not possible to definitively describe Ag synthesis and presentation at this site. However, as described above, sporadic expression of IE genes in the absence of detectable E or L genes has been described in latently infected lungs (7, 9). Preferential expression of IE genes is likely the explanation for the immunodominance of IE-encoded Ags during memory infection. In MCMV-GFP-SL8, SIINFEKL is encoded behind the IE2 promoter. Thus, we predicted that SIINFEKL expression would be dominant during memory infection. However, in the context of MCMV gene expression influences whether an epitope generates an inflationary response. Dekhtiarin et al. (31) infected mice with one of two recombinant viruses expressing the gB epitope from HSV-1, linked to the C terminus of either IE2 or M45. Inflating gB responses were seen only when expression was controlled by the IE2 promoter. When gB was linked to M45, an E gene, gB T cell responses dominated only during acute infection. This study lends support to the idea that ordered, temporal viral gene transcription results in immune recognition of the first viral gene products and immune silencing of downstream transcription, resulting in a bias of the T cell response toward IE Ags.

A similar scenario is likely at play in our system, where the IE2 promoter controls SIINFEKL expression. Additionally, SIINFEKL may be more abundant than other MCMV epitopes as a result of higher MHC affinity and greater transcription levels. However, in both the BALB/c model and the C57BL/6 model, inflationary responses developed to both SIINFEKL and endogenous MCMV epitopes. This happened either because these cells had more Ag available to them or because they were better able to access Ag. Thus, competition for, or availability of, Ag at the level of the APC plays a significant role in the selection of inflationary responses during chronic MCMV infection.

This competition may be won by the SIINFEKL response, at least in part because patrolling SIINFEKL-specific CD8+ T cells see Ag first and go on to terminate further gene transcription. The silencing/desilencing and immune sensing hypothesis proposed by Simon et al. (9) suggests that T cells specific for the IE1-derived epitope in BALB/c mice prevent further MCMV gene transcription. Consistent with this, only IE1 and IE2 transcripts have been found in latently infected lung tissue from BALB/c mice (8). IE3 and gB were found at low levels only when the IE1 epitope was mutated such that it could no longer be presented to T cells (9).

Indeed, work from Čičin-Sain and colleagues (31) shows that the context of MCMV gene expression influences whether an epitope generates an inflationary response. Dekhtiarin et al. (31) infected mice with one of two recombinant viruses expressing the gB epitope from HSV-1, linked to the C terminus of either IE2 or M45. Inflating gB responses were seen only when expression was controlled by the IE2 promoter. When gB was linked to M45, an E gene, gB T cell responses dominated only during acute infection. This study lends support to the idea that ordered, temporal viral gene transcription results in immune recognition of the first viral gene products and immune silencing of downstream transcription, resulting in a bias of the T cell response toward IE Ags.

**Discussion**

We have shown that a GFP-SIINFEKL fusion construct, when inserted into MCMV under IE control, completely dominates the inflationary memory response during chronic infection with this virus. The number of SIINFEKL-specific T cells available prior to infection was not the main determinant of immunodominance because the SIINFEKL response was still dominant in mice expressing SIINFEKL as a self-Ag or after specific peptide tolerization. Conversely, adoptive transfer to increase the number of T cells specific for endogenous MCMV-derived peptides did not enable them to inflate in response to the SIINFEKL-expressing virus. However, when mice were coinfected with WT MCMV and our recombinant MCMV expressing SIINFEKL, inflationary responses developed to both SIINFEKL and endogenous MCMV epitopes. This indicated that when different cells in the same animal were infected with each of the individual viruses, and thus WT-infected APCs were able to present MCMV epitopes without the competing influence of SIINFEKL, T cells recognizing these epitopes were able to inflate alongside the SIINFEKL response. However, when both sets of epitopes were encoded by the same virus and presumably expressed on the same APC, T cells responding to SIINFEKL outcompeted the MCMV-specific responses. This happened either because these cells had more Ag available to them or because they were better able to access Ag. Thus, competition for, or availability of, Ag at the level of the APC plays a significant role in the selection of inflationary responses during chronic MCMV infection.

**FIGURE 5.** MHC binding affinity of MCMV epitopes and kinetics of expression. (A) BALB/c fibroblasts with WT MCMV or MCMV-GFP-SL8, harvested RNA was used for qRT-PCR analyses. The experiment was done twice. Shown is the normalized mean fluorescence intensity of class I MHC on the surface of cells. (B) Murine embryonic fibroblasts were infected with the indicated viruses and RNA was harvested at the time points listed on the y-axis. cDNA was made in parallel with no reverse transcriptase controls for each sample, and quantitative RT-PCR was done for the indicated gene products. No signal was obtained from the no reverse transcriptase controls. The experiment was done twice.
at the level of the infected APC dictates the selection of epitopes that drive memory inflation. This hypothesis implies that after WT MCMV infection, IE1-specific T cells (in BALB/c mice) and IE3-specific T cells (in C57BL/6 mice) fail to completely silence MCMV E gene expression.

When considering the use of MCMV and eventually human CMV as a vaccine vector, these results emphasize the importance of gene expression kinetics and epitope availability in determining the size of inflationary memory responses to individual Ags.

Acknowledgments

The authors have no financial conflicts of interest.

Disclosures

The authors have no financial conflicts of interest.

References