The Journal of Immunology

Four Distinct Patterns of Memory CD8 T Cell Responses to Chronic Murine Cytomegalovirus Infection

Michael W. Munks, Kathy S. Cho, Amelia K. Pinto, Sophie Sierro, Paul Klenerman and Ann B. Hill

J Immunol 2006; 177:450-458; ;
doi: 10.4049/jimmunol.177.1.450
http://www.jimmunol.org/content/177/1/450

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Four Distinct Patterns of Memory CD8 T Cell Responses to Chronic Murine Cytomegalovirus Infection[1]

Michael W. Munks,* Kathy S. Cho,* Amelia K. Pinto,* Sophie Sierro,† Paul Klenerman,† and Ann B. Hill[2]*

CMVs are β herpesviruses that establish lifelong latent infection of their hosts. Acute infection of C57BL/6 mice with murine CMV elicits a very broad CD8 T cell response, comprising at least 24 epitopes from 18 viral proteins. In contrast, we show here that the CD8 T cell response in chronically infected mice was dominated by only five epitopes. Altogether, four distinct CD8 T cell kinetic patterns were evident. Responses to some epitopes, including M45, which dominates the acute response, contracted sharply after day 7 and developed into stable long-term memory. The response to m139 underwent rapid expansion and contraction, followed by a phase of memory inflation, whereas the response to an M38 epitope did not display any contraction phase. Finally, responses against two epitopes encoded by the immediate early gene *IE3* were readily detectable in chronically infected mice but near the limit of detection during acute infection. CD8 T cells specific for the noninflationary M45 epitope displayed a classic central memory phenotype, re-expressing the lymph node homing receptor CD62L and homeostatic cytokine receptors for IL-7 and IL-15, and produced low levels of IL-2. Responses to two inflationary epitopes, m139 and IE3, retained an effector memory surface phenotype (CD62L^{low}, IL-7Rα$^-$, IL-15Rβ$^-$) and were unable to produce IL-2. We suggest that immunological choices are superimposed on altered viral gene expression profiles to determine immunodominance during chronic murine CMV infection. *The Journal of Immunology,* 2006, 177: 450–458.

Cytomegaloviruses are ubiquitous, large DNA viruses of the β subfamily of herpesviruses. They establish species-specific lifelong infection in the majority of individuals in most mammalian species. These infections are usually asymptomatic, with pathology only occurring when the immune system is compromised. However, it has recently become apparent that these benign lifelong companions of the mammalian immune system trigger an extraordinarily large response from their hosts' T cell compartments.

Using overlapping peptides covering the entire human CMV (HCMV)[3] genome, it has been demonstrated recently that a median of 4.0% of CD4 T cells and 4.6% of CD8 T cells from seropositive adults are HCMV specific (1). It has also been demonstrated that the size of the HCMV-specific T cell response increases with age, a phenomenon dubbed "memory inflation" (2, 3). Consistent with this, T cells specific for HCMV have a characteristic phenotype indicative of repeated Ag exposure (3–8). They are mostly CCR7$^-$, CD27$^-$, and CD28$^-$, placing them in the effector memory (T_{EM}) compartment (8, 9), although many re-express the so-called "naïve" isoform of CD45, CD45RA. Finally, many express CD57 and CD85j, markers associated with replicative senescence (3, 10). In the elderly, CMV-specific responses can become so large that they dominate the T cell repertoire (11). The precise consequences of these enormous responses to HCMV are not yet clear, but HCMV infection is associated with impaired influenza vaccination (12–14), diminished T cell responses to EBV (2), and may contribute to immunosenescence in the elderly (15).

Because CMVs are highly species specific, the natural mouse pathogen murine CMV (MCMV) is used as a model of human infection. MCMV infection can be divided into three phases: 1) an acute phase, in which virus replicates in many organs, lasting ~1 wk; 2) a persistent phase, lasting several weeks in susceptible mouse strains such as BALB/c, in which virus continues to replicate almost exclusively in the salivary gland; and 3) a chronic phase in which infectious virus cannot be recovered. Since no virus replication can be detected in this third phase, infection is referred to as latent. Because CMV infection in humans is usually asymptomatic, studies of T cell immunity are usually restricted to individuals whose primary infection occurred many years earlier. For most of these subjects, CMV infection is clinically latent, i.e., virus cannot be cultured from any site. One major advantage of studying the murine model is that it enables us to follow the course of the CD8 T cell response during the transition from acute to clinically latent infection.

The acute CD8 T cell response to MCMV in BALB/c mice is dominated by responses to two epitopes, from the immediate early protein IE1/pp89 and the early protein m164 (16). These responses "inflate" over time and also dominate the chronic response (16, 17). IE1/pp89- and m164-specific CD8 T cells in chronic infection display a T_{EM} phenotype (18, 19). They are CD62L^{low} and express low levels of the costimulatory molecules CD27 and CD28. They also lack expression of IL-7Rα (CD127) and IL-15Rβ (CD122), which are important for homeostatic maintenance of memory CD8 T cells (19).

*Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR 97239; and †The Peter Medawar Building for Pathogen Research, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom

Received for publication January 5, 2006. Accepted for publication April 12, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health (NIH) Grants AI47206 and AI50099 (to A.B.H.) and NIH Training Grant AI007472 (to M.W.M.).

[2] Address correspondence and reprint requests to Dr. Ann B. Hill, Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Mail Code L220, 3181 Southwest Sam Jackson Park Road, Portland OR 97239. E-mail address: hillan@ohsu.edu

[3] Abbreviations used in this paper: HCMV, human CMV; T_{EM}, effector memory; MCMV, murine CMV; T_{CM}, central memory; ORF, open reading frame; ICS, intracellular cytokine staining; VIPR, viral gene that interfere with Ag presentation.

0022-1767/06/$02.00

CD8 T cell epitopes have also been identified from m18 (20), M45 (21), m04 (22), M83 (23), and M84 (24) in BALB/c mice. M45 elicits a moderate although subdominant response during acute infection (19, 21), but the responses to the other epitopes are very small. Responses to these epitopes do not undergo memory inflation, instead decreasing to low or undetectable levels during chronic infection. Sierro et al. (19) studied the CD8 T cell response to one of these epitopes, M45, in chronic infection and found that its phenotype contrasted with that of IE1/pp89 and m164-specific responses. Whereas the inflationary responses displayed a T_{EM} phenotype, CD8 T cells specific for M45 regained a central memory (T_{CM}) phenotype, gradually re-expressing CD62L. M45-specific CD8 T cells were also $CD27^+$, $CD28^+$, IL-7Rα^+ (CD127), and IL-15Rβ^+ (CD122). Thus, during chronic infection of BALB/c mice, two patterns of CD8 T cell responses could be discerned. Some responses that had dominated the acute response subsequently inflated and displayed a T_{EM} phenotype, whereas other responses contracted severely, and at least in the case of M45, displayed a classic T_{CM} phenotype. However, the data to date are limited and the question remains as to which type of response is more typical, and what the underlying differences are between responses to inflationary and noninflationary epitopes.

We recently described a definitive map of the acute CD8 T cell response to MCMV in C57BL/6 mice by screening an expression library of MCMV open reading frames (ORFs) (25). Of the 18 minimal epitopes defined in that study, we demonstrate here that CD8 T cell responses to only two of these epitopes underwent memory inflation. Furthermore, a screen of the ORF library with CD8 T cells from chronic MCMV infection revealed a new inflationary Ag, IE3. Altogether, the C57BL/6 response to MCMV consists of four distinct memory CD8 T cell patterns, three of which are inflationary and one which is not. This work will facilitate dissection of how these responses arise in an experimental system and help determine the impact on subsequent immune responses.

Materials and Methods

Mouse and virus strains

C57BL/6 mice were purchased from The Jackson Laboratory. $K^{b-/-}D^{b+/+}$ and $K^{b+/+}D^{b-/-}$ mice on a C57BL/6 were a gift from D. Raulet (University of California, Berkeley, CA). Mice aged 6–12 wk were infected i.p. with 1–2 $\times$ 10^6 pfu MCMV strain MW97.01 (26). MW97.01 is derived from a bacterial artificial chromosome of Smith strain MCMV. Virus stocks were grown on mouse embryo fibroblasts, sonicated, and frozen. Titers were determined on BALB 3T3 cells (American Type Culture Collection) without centrifugal enhancements. All mice were housed at Oregon Health & Science University and all studies were approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee.

MCMV ORF library

The MCMV ORF library used in Fig. 1 has been described previously (25).

Peptide synthesis

The peptides used for this article are listed in Table I. All 8-mer, 9-mer and 10-mer peptides were synthesized as crude peptides (65–95% pure by HPLC) by GeneMed or JPT and confirmed by mass spectrometry. Overlapping 15-mer peptides were synthesized by JPT at 50 nmol scale.

Stimulation of splenocytes with transfected cells or peptides

K41 cells, an *SV-40*-transformed *H-2^b* fibroblast cell line (gift of M. Michalek, University of Alberta, Edmonton, Alberta, Canada) were plated at 5000 cells per well in 96-well flat-bottom plates. One day later, each well was transfected with 500 ng of plasmid DNA and 1.25 μl of FuGene 6 (Roche). Two days later, 8 $\times$ 10^5 splenocytes from infected mice were added per well in the presence of brefeldin A (GolgiPlug; BD Pharmingen) and incubated for 6 h at 37°C. Duplicate wells were combined into a single well in 96-well round-bottom plates for intracellular cytokine staining (ICS). For peptide stimulation, peptide was added to splenocytes for 6–7 h in the presence of brefeldin A, at a concentration of ~10 μM for overlapping 15 mers, 1 μg/ml for other peptides, or as indicated in the figure legend.

Surface and ICS

Splenocytes were surface stained with CD8α (53-6.7), CD62L (MEL-14), IL-15Rβ/CD122 (TM-b1), and/or IL-7Rα/CD127 (A7R34). M45-D^b tetramers and m139-K^b tetramers were generated as described previously (27). Kb:Ig dimers (BD Pharmingen) loaded with IE3 peptide were prepared according to the manufacturer's protocol. ICS for IFN-γ (XMG1.2), TNF-α (MP6-XT22), and/or IL-2 (PC61.5) was performed with the Cytofix/Cytoperm kit (BD Pharmingen). Samples were acquired on a FACSCalibur (BD Pharmingen) with CellQuest software and analyzed with FlowJo software (Tree Star).

Table I. *Peptides used in this study*

ORF	MHC	Residues	Sequence	Inflates?
M45	Db	985–993	HGIRNASFI	No
m139	Kb	419–426	TVYGFCLL	Yes
M57	Kb	816–824	SCLEFWQRV	No
m141	Kb	15–23	VIDAFSRL	No
M38	Kb	316–323	SSPPMFRV	Yes
M78	Kb	8–15	VDYSYPEV	No
M86	Db	1062–1070	SQNINTVEM	No
M33	Db	47–55	GGPMNFVVL	No
M112	Db	171–179	AAVQSATSM	No
M44	Db	130–138	ACVHNQDII	No
M100	Kb	72–79	RIIDFDNM	No
m164	Kb	283–290	GTTDFLWM	No
M97	Kb	210–217	IISPFPGL	No
m164	Db	267–275	WAVNNQAIV	No
M77	Db	474–482	GCVKNFEFM	No
m04	Db	1–9	MSLVCRLVL	No
M38	Kb	38–45	STYTFVRT	No
M36	Db	213–221	GTVINLTSV	No
IE3	Kb	416–423	RALEYKNL	Yes
IE3	Kb	461–475	VRKAVDETRARMGMR	Yes
M102	Db	431–445	SVAHIYVGVGGVCRI	No
M102	Kb	441–455	GVCRISIVDLRFAVL	No
M102	Kb	486–500	RLAHSSPRIFRRVRS	Yes

Results

A CD8 T cell epitope from IE3 is recognized in chronic but not acute MCMV infection

The phenomenon of CD8 T cell memory inflation, whereby the response to some epitopes increases with time, has been described previously in MCMV infection in BALB/c mice (16, 17, 19, 28). Examining the CD8 T cell response to MCMV in C57BL/6 mice, we consistently found that the response to m139 and M38 is as large or larger during chronic MCMV infection than in acute infection (Fig. 1*A*). However, the response to the 16 other recently described optimal epitopes decreased dramatically (Fig. 1*A* and data not shown).

In BALB/c mice, only the two codominant CD8 T cell epitopes in acute infection (IE1/pp89 and m164) undergo memory inflation in chronic infection. However, in C57BL/6 mice, the acutely immunodominant M45 is noninflationary, while two subdominant responses do undergo memory inflation (Fig. 1*A*). This raised the possibility that other CD8 T cell specificities may be dominant in chronic infection but low or undetectable in acute infection. We have described recently the cloning of each MCMV ORF into mammalian expression plasmids (25). This ORF library was transfected into *SV40*-transformed *H-2^b* fibroblasts (K41 cells) in a 96-well plate format and incubated with splenocytes from C57BL/6 mice that had been infected for 18 mo, which were then tested by

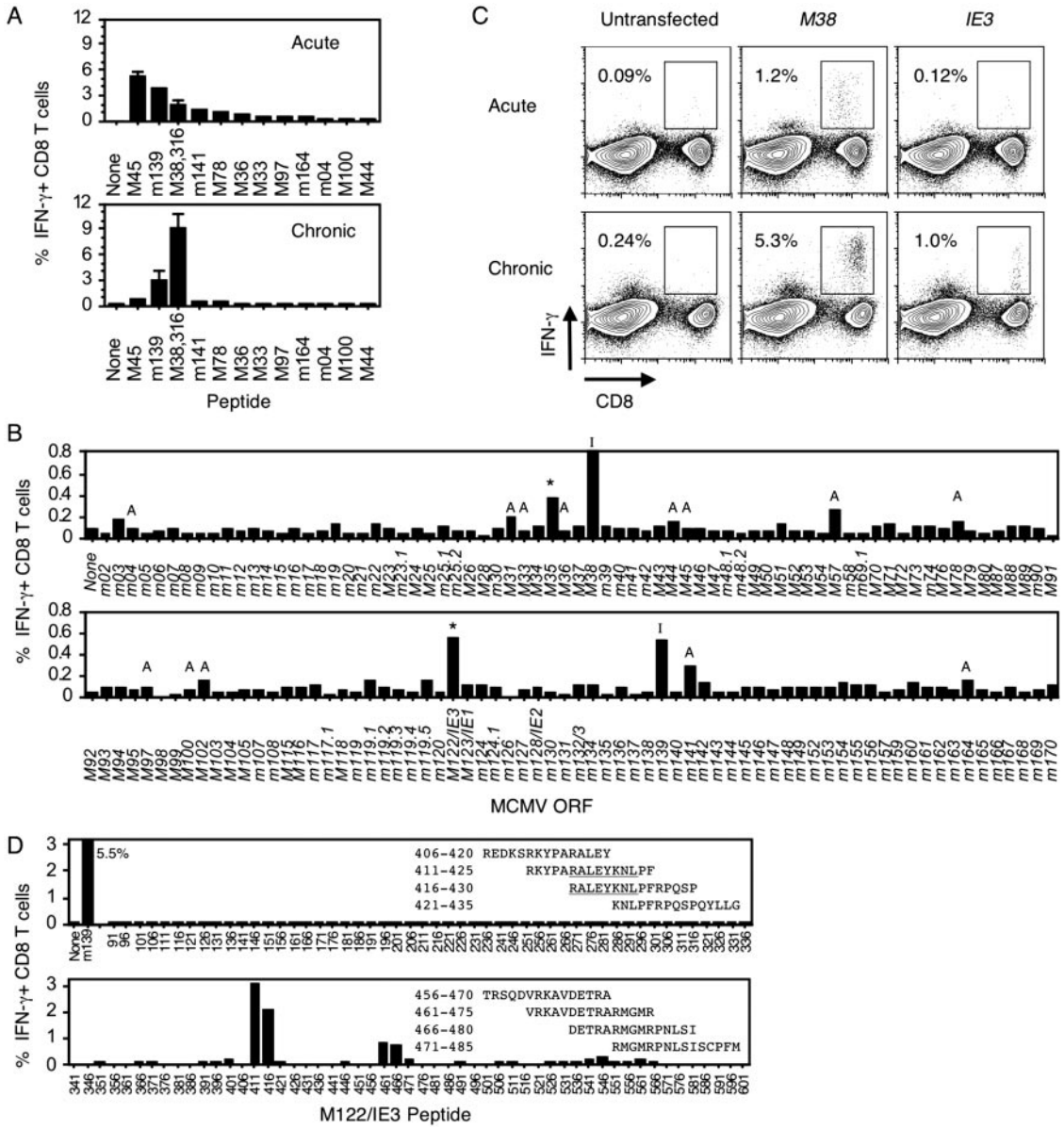

FIGURE 1. The CD8 T cell response to m139, M38, and IE3 is inflated during chronic MCMV infection. *A*, The CD8 T cell response to 12 defined *H-2^b* peptides was determined directly ex vivo from three acutely infected mice (day 7) and three chronically infected mice (1.5 years) by ICS for IFN-γ. *B*, Pooled splenocytes from three mice chronically infected with MCMV (1.5 years) were stimulated with K41 cells transfected with the indicated ORFs. After a 6-h incubation in the presence of brefeldin A, CD8 T cells were analyzed by ICS. "A" indicates ORFs known to contain noninflationary epitopes, "I" indicates epitopes identified as inflationary in *A*, and ∗ indicates ORFs not previously known to contain inflationary epitopes. *C*, Untransfected and *M38*- and *IE3*-transfected K41 cells were used to stimulate splenocytes from acute (day 7) or chronic (1.5 years) MCMV-infected mice. CD8 T cell IFN-γ production was analyzed by ICS. *D*, Pooled splenocytes from three mice chronically infected with MCMV were stimulated with overlapping 15-mer peptides spanning exon 5 of M122/IE3, then analyzed by ICS.

ICS for IFN-γ production (Fig. 1*B*). Although many of the recognized ORFs had also been detected in the previous screens of acutely infected mice (25), there were three important differences. First, the responses to some major acute Ags such as *M57* and *m141* were now minimal. Second, the responses to *M38* and *m139* were now dominant. And third, there were significant responses to two ORFs not previously recognized, *M122/IE3* and *M35* (Fig. 1*B*).

The response to *M35* was not consistent between experiments, but the chronic response to *IE3* was consistently seen between experiments. CD8 T cells from chronic but not acute MCMV infection responded to *IE3* (Fig. 1C), demonstrating that *IE3* is a bona fide chronic Ag and not simply undetected in our previous study. *IE3* contains four exons. One is noncoding and two are shared with *M123/IE1*, to which no response was detected (Fig. 1*B*). Thus, the scope of our search was narrowed to *IE3* exon 5. We tested 15-mer peptides overlapping by 5 aa that covered the entire exon and found that two regions were recognized by CD8 T cells from chronically infected mice (Fig. 1*D*). $K^{b+/+}D^{b-/-}$ mice chronically infected with MCMV responded to *IE3* whereas $K^{b-/-}D^{b+/+}$ mice did not, indicating that both peptides were K^b restricted (data not shown). Subsequent experiments determined the identity of the more dominant IE3 epitope as IE3 416–423, whereas the minimal determinant for the second epitope (IE3 466–475) remains to be identified.

CD8 T cells from chronically infected mice respond to five epitopes

We next examined the CD8 T cell immunodominance hierarchy in chronic MCMV infection using all 18 previously identified epitopes as well as the new IE3 epitope. Splenocytes from mice that had been infected with MCMV for 8 mo were examined for their response to each peptide (Fig. 2*A*), with the results listed from left to right according to their position in the immunodominance hierarchy at day 7 (25). The largest response was to m139, followed by M38 and IE3. This is in sharp contrast to the acute immunodominance hierarchy, which is dominated by M45, followed by m139, M57, and m141.

There are four additional epitopes from IE3 and M102 for which we have not yet identified the minimal peptide epitope. When 15-mer peptides were used to stimulate CD8 T cells from chronically infected mice, large responses were detected against two additional epitopes, IE3 461–475 and M102 486–500 (Fig. 2*B*). Thus, C57BL/6 mice chronically infected with MCMV make robust responses to five CD8 T cell epitopes: m139 419–426, M38 316–323, IE3 416–423, IE3 461–475, and M102 486–500.

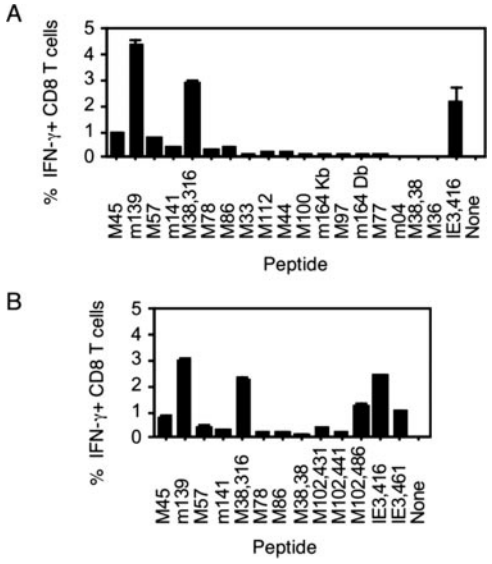

FIGURE 2. Five MCMV epitopes elicit memory CD8 T cell inflation in C57BL/6 mice. *A*, The CD8 T cell response to all 19 optimal *H-2*^{b} peptides was determined by ICS directly ex vivo from four chronically infected (8.5 mo) mice. Peptides are listed from left to right according to the acute immunodominance hierarchy (25). *B*, Nine optimal peptides (M45, m139, M57, m141, M38 316, M78, M86, and M38 38 and IE3 416) and four 15-mer peptides (M102 431–445, M102 441–455, M102 486–500, and IE3 461–475) were tested for their ability to stimulate IFN-γ production from chronic (11 mo) CD8 T cells.

CD8 T cells specific for different epitopes show distinct patterns of expansion and contraction

We wondered whether the responses to m139 and M38 actually underwent inflation during chronic MCMV infection or had simply failed to contract after the height of the acute response. We first examined the course of the response to a series of epitopes during the acute response. Epitopes such as M45 and m141 displayed kinetics similar to those that have been described for cleared infections such as LCMV or *Listeria monocytogenes*, reaching a peak at day 7 and then rapidly contracting (Fig. 3, *A* and *B*). The response to m139 also contracted rapidly after peaking at day 7, although less so than M45 or m141, indicating that the large response seen in chronic infection is due to true memory inflation. However, the response to M38 displayed a quite different pattern, being clearly detectable at day 7 but then continuing to increase thereafter, displaying little evidence of contraction even out to 30 days.

We next analyzed the CD8 T cell response to six epitopes over an extended period of time (Fig. 4). Four basic patterns of responses can be discerned. The first group is exemplified by M45 and M57. These displayed the classic kinetics of expansion, contraction, and stable memory that have been described for cleared infections. The second pattern is exemplified by m139, which peaked at day 7, rapidly contracted to a low point at day 35, then underwent memory inflation that has been described for IE1/pp89

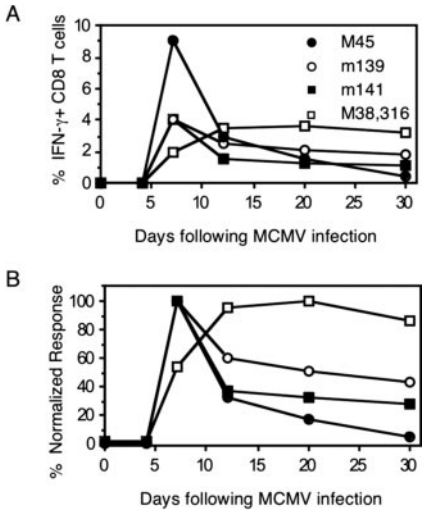

FIGURE 3. The CD8 T cell response to most MCMV epitopes contracts after day 7. *A*, The kinetics of the CD8 T cell response to two noninflationary epitopes (M45 and m141) and two inflationary epitopes (m139 and M38) was analyzed by ICS for IFN-γ. *B*, The response to each epitope was normalized to the maximum response for that epitope.

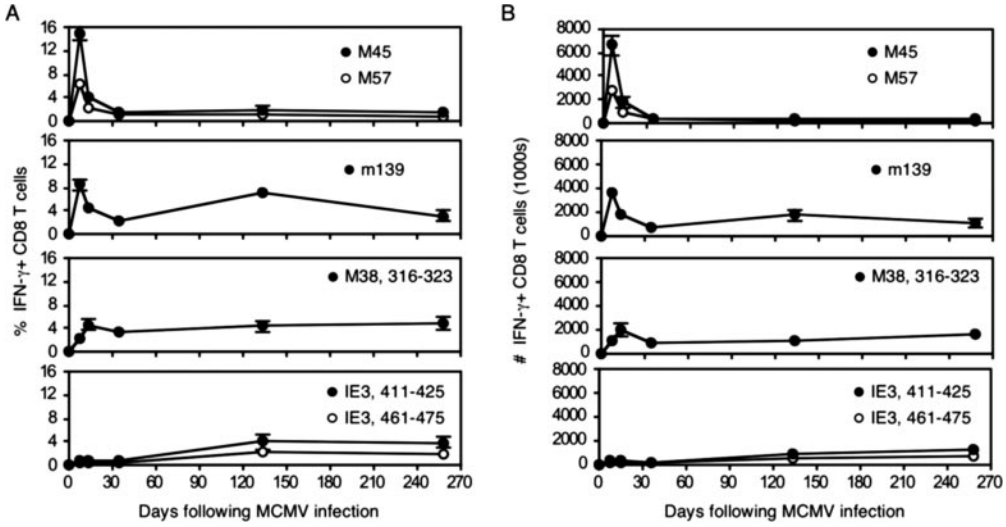

FIGURE 4. There are four distinct kinetics of CD8 T cell responses to MCMV. *A*, The frequency of CD8 T cells responding to two noninflationary MCMV epitopes (M45 and M57) and four inflationary epitopes (m139, M38 316, IE3 411–425, and IE3 461–475) was analyzed in mice infected for 7, 14, 35, 135, or 258 days ($n = 3$ mice per group per time point). *B*, From the experiment shown in *A*, the total number of CD8 T cells responding to each epitope at each time point was calculated per spleen.

and m164 in BALB/c mice. The apparent decline of the m139-specific response after day 120 in this experiment is not consistently observed; in general, m139 responses reach a plateau after ~4 mo. M38 represented the third pattern, not reaching its peak until day 14, undergoing only limited contraction by day 35, and subsequently reaching a long-term plateau. Finally, CD8 T cell responses to the two IE3 epitopes display a fourth pattern. Although responses to these epitopes were consistently detectable above background at days 7, 14, and 35 (range of 0.4–0.9% compared with <0.1% background), robust responses were only detected ≥4 mo after infection. It is currently unknown which of these patterns, if any, the response to M102 486–500 follows. We consider the first pattern, exemplified by M45 and M57, to be noninflationary and the other three patterns to be inflationary. In Table I, we have categorized each of the epitopes evaluated in this study as inflationary or noninflationary.

The fine details of the chronic CD8 T cell response varied between experiments. For example, in some experiments M38 was clearly dominant over m139 (Fig. 1), and the reverse was true in others (Fig. 2). We have not performed enough analyses of very long-term infections to conclusively compare the relative chronic immunodominance of m139 and M38. However, the basic pattern of kinetics shown in Figs. 3 and 4 is consistent. We typically found that in chronic infection (up to 18 mo), the largest responses were against m139 and M38 316, with lower but sizeable responses against the two IE3 epitopes and one M102 epitope.

Immunodominance during chronic infection is not a result of increased functional avidity

One mechanistic model for the phenomenon of CD8 T cell memory inflation is that high-avidity clones are selected during chronic infection. To test this possibility, we performed a dose-response titration, comparing the percentage of CD8 T cells that could produce IFN-γ at various peptide concentrations. As expected for a noninflationary epitope, the functional avidity of M45-specific CD8 T cells did not increase noticeably between acute and chronic infection (Fig. 5). For the m139 and M38 epitopes, the peptide titration curves for acute and chronic infection were also superimposable, indicating that there was no increase in the average functional avidity over the course of infection.

CD8 T cells specific for inflationary epitopes have a permanent effector memory phenotype

We next compared the cell surface phenotype of CD8 T cells specific for inflationary vs noninflationary epitopes. As described above, most CMV-specific CD8 T cells in humans and BALB/c mice lack expression of CD62L. Since CD62L is down-regulated by TCR stimulation, this marker is best assessed by costaining with peptide:MHC multimers, rather than during an ICS assay, which requires in vitro restimulation. We used MHC tetramers for M45 and m139, comparing acute and chronic responses. MHC-Ig dimers were used for IE3, but because this response is minimal during acute infection, only the chronic response was analyzed. Total CD8 T cells and Ag-specific CD8 T cells were assessed for CD62L and IL-15Rβ (CD122) (Fig. 6*A*) or IL-7Rα (CD127) (Fig. 6*B*). During acute infection, >95% of M45- and m139-specific CD8 T cells were CD62L^{low} (Fig. 6*A*). During chronic MCMV infection, ~50% of CD8 T cells specific for the noninflationary M45 epitope regained CD62L expression, whereas >90% of CD8 T cells specific for the inflationary m139 and IE3 epitopes remained CD62L^{low}. Consistent with this, chronic M45-specific CD8 T cells had increased IL-15Rβ (Fig. 6*A*) and IL-7Rα expression (Fig. 6*B*), whereas chronic m139- and IE3-specific CD8 T cells lost IL-15Rβ expression and remained IL-7Rα^{-}.

In addition to differences in their surface phenotype, CD8 T cells specific for inflationary epitopes also differed in function from those specific for noninflationary epitopes. CD8 T cells from acutely or chronically infected mice were stimulated with three noninflationary epitopes (M45, m141, and M86) and three inflationary epitopes (m139, M38, and IE3 416). Intracellular cytokine costaining was then used to determine the percentage of IFN-γ^{+} CD8 T cells that could also produce either TNF-α or IL-2. We found that CD8 T cells of all specificities could make some TNF-α, and this ability increased in chronic infection (Fig. 7, *A* and *B*). Notably, however, CD8 T cells specific for

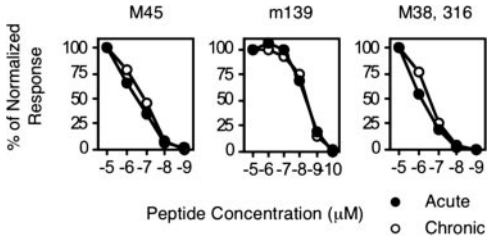

FIGURE 5. The functional avidity of CD8 T cells specific for inflationary epitopes does not increase in chronic infection. The concentration of peptide required to elicit a half-maximal frequency of IFN-γ^+ CD8 T cells was determined for acute (day 7) and chronic (8.5 mo) mice by performing a dose titration. Because the frequency of IFN-γ^+ CD8 T cells is different between acute and chronic infections, all responses were normalized to the response at the highest peptide concentration.

noninflationary epitopes were significantly more likely to produce TNF-α during chronic infection ($p = 0.03$). During acute infection, few CD8 T cells of any specificity produced IL-2 (Fig. 7, *C* and *D*). However, in chronically infected mice, there was a significant increase in the ability of CD8 T cells specific for noninflationary epitopes to produce IL-2 ($p = 0.05$), but there was no such increase in CD8 T cells specific for inflationary epitopes.

In summary, in mice with chronic MCMV infection, we observed two general categories of virus-specific CD8 T cells. Those directed against noninflationary epitopes acquire a T_{CM} phenotype associated with long-term, Ag-independent memory (CD62L^{high}, IL-7Rα^+, IL-15Rβ^+) and gain some IL-2 secretory capacity. In contrast, CD8 T cells specific for inflationary epitopes retain features associated with T_{EM} (CD62L^{low}, IL-7Rα^-, IL-15Rβ^-) and do not gain IL-2 secretory capacity.

Discussion

The data presented here indicate that the CD8 T cell response to MCMV undergoes a dramatic transition from the acute phase to the chronic, clinically latent phase of infection. Some major CD8 T cell specificities in the acute response such as M45 and M57 contract severely, displaying kinetics similar to those described for well-characterized acute, cleared infections. In contrast, the response to m139 contracted dramatically but then expanded again, whereas the response to M38 316 unexpectedly showed no obvious contraction phase. Finally, two epitopes from IE3 were identified to which substantial responses were only detected during the chronic phase of infection. For all of the specificities examined, the response remained relatively stable after the first 4 mo of infection. Importantly, this pattern of memory inflation was observed both in relative terms, as a percentage of the total CD8 T cell population, and also in terms of the total number of epitope-specific CD8 T cells per spleen.

The response to chronic MCMV also showed distinct surface and functional phenotypes. Most CD8 T cells specific for two inflationary epitopes, m139 and IE3 416–423, had a T_{EM} phenotype (CD62L^{low}, IL-7Rα^-, IL-15Rβ^-) and expressed IL-2 very infrequently. Both the phenotype and the inflating size of these specificities suggest that they are driven by repeated exposure to Ag. In contrast, ~50% of chronic CD8 T cells specific for M45 regained CD62L expression, typical of T_{CM}. Most M45-specific CD8 T cells were also IL-7Rα^+, IL-15Rβ^+, and some could produce IL-2 upon restimulation. This data suggest that M45-specific CD8 T cells traffic through secondary lymphoid organs and are capable of being maintained in the absence of antigenic stimulation by cytokine-driven homeostatic proliferation.

These results present an interesting comparison to the chronic CD8 T cell response to MCMV observed in BALB/c mice. In BALB/c mice, the two specificities that are codominant in acute infection (pp89 and m164) retain a T_{EM} phenotype and continue to dominate numerically in chronic infection (16, 18, 19). M45 elicits a significant but subdominant response during acute infection of BALB/c mice and contracts to a small, noninflating, T_{CM} population during chronic infection (16, 18, 19). Thus, in BALB/c mice, chronic infection leads to a focusing of the acute immunodominance hierarchy. In contrast, the pattern we observed for M45 in C57BL/6 mice, immunodominance in acute infection contracting to a small T_{CM} population in chronic infection, has no exact parallel in BALB/c mice.

These results also present an interesting comparison to the memory CD8 T cell phenotypic dichotomy that has been described for the gammaherpesviruses EBV and MHV-68 (29–31). Gammaherpesviruses have two distinct sets of genes, one responsible for lytic infection and a separate set of genes expressed during latent infection. Responses to lytic cycle Ags dominate acute infection; these responses contract and display both T_{EM} and T_{CM} phenotypes during chronic infection. In contrast, responses to latent cycle epitopes develop later, are present at higher frequencies during chronic infection, and uniformly display a T_{CM} phenotype. Thus, in gammaherpesvirus infection, the T_{CM}/T_{EM} dichotomy correlates with viral gene expression program, and in contrast to CMV, the responses that are most prevalent during chronic phase display a T_{CM} phenotype.

What processes are responsible for determining which CD8 T cell specificities will dominate in chronic MCMV infection? We find it useful to broadly distinguish between virological and immunological explanations.

MCMV encodes three MHC class I immune evasion genes, referred to as viral genes that interfere with Ag presentation (VIPRs) (32). One obvious virological explanation for memory inflation is that these epitopes are in some way resistant to the effects of MCMV's VIPRs. Consistent with this idea, both BALB/c inflationary epitopes, IE1/pp89 and m164, can be presented on some infected cells even when the VIPRs are present (33–35). However, this explanation does not seem likely to be correct since we have found that the chronic immunodominance hierarchy is not altered when mice are infected with either wild-type MCMV or a mutant lacking all three VIPRs (M. W. Munks, A. Pinto, A. Lang, C. Doom, J. Nikolich-Žugich, and A. Hill, submitted for publication).

A second virological explanation is that some viral gene products are much more abundantly expressed during latency than others. Although very little is known about CMV gene expression during latency, some available data do support this model. It is known that latently infected cells express can express *IE1*, *IE3*, and *IE2* transcripts without proceeding through the entire viral replication cycle, a phenomenon referred to as abortive reactivation (36, 37). It has been proposed previously that this explains the immunodominance of the IE1/pp89 epitope in chronic MCMV infection (16, 18). Interestingly, when C57BL/6 mice were infected with recombinant MCMV in which foreign epitopes were expressed behind an IE promoter (Refs. 19 and 28; G. Shellam, unpublished data), the CD8 T cell response to these recombinant epitopes resembled the response to IE3 observed in this study. Minimal CD8 T cell responses to the recombinant epitopes were detected during the acute response. However, the responses underwent memory inflation and became readily detectable during clinically latent infection, displaying a CD62L^{low} effector-memory phenotype. Thus, it appears that the process of memory inflation strongly prefers epitopes found within IE-encoded proteins expressed during abortive reactivation.

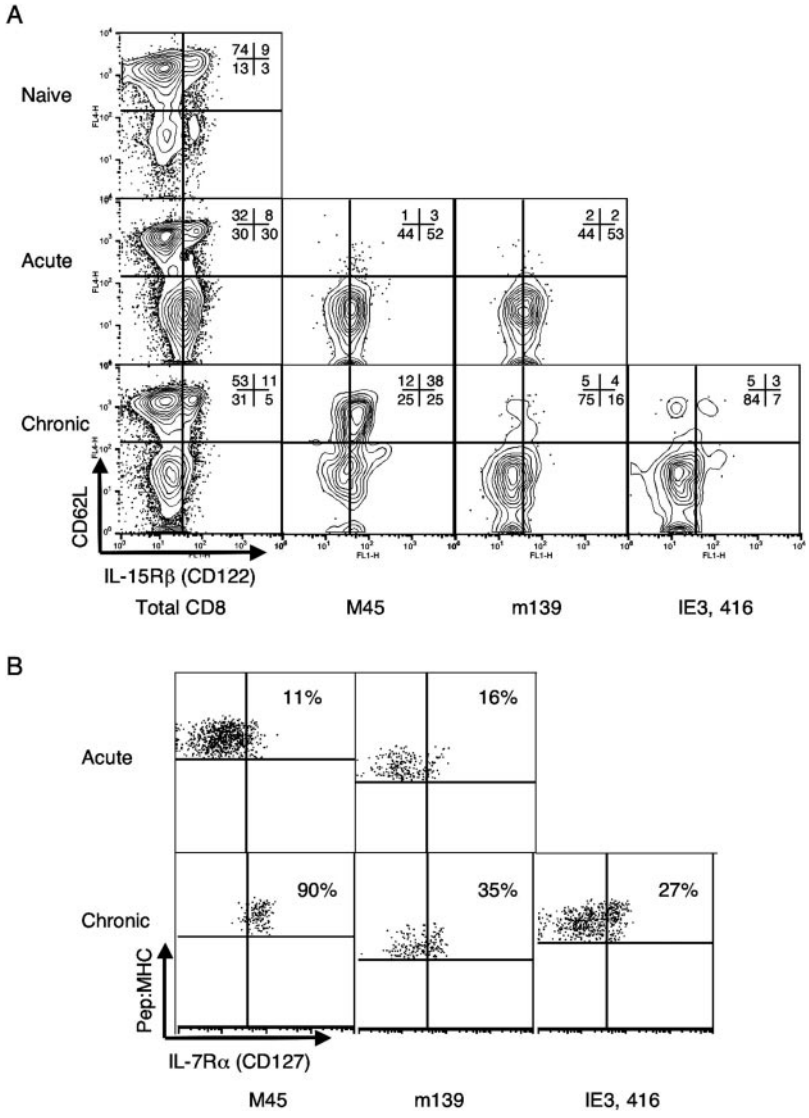

FIGURE 6. CD8 T cells specific for inflationary epitopes have a permanent effector-memory phenotype. *A*, CD8 T cells from naive mice, or mice infected acutely (day 7) or chronically (7.5 mo) with MCMV, were analyzed for CD62L and IL-15Rβ (CD122) expression. Total CD8 T cells (*left*) were gated only by CD8. M45- and m139-specific CD8 T cells were gated on $CD8^+$tetramer$^+$ cells, whereas IE3-specific CD8 T cells were gated on $CD8^+$Kb:peptide dimer$^+$ cells. *B*, Ag-specific CD8 T cells from acute (day 7) or chronic (7.5 mo) MCMV infection were analyzed for IL-7Rα (CD127) expression. Horizontal gates are not identical between different specificities due to differences in staining intensity between M45:D^b, m139:K^b, and IE3:K^b multimers.

Data from HCMV are largely consistent with this model. Sylwester et al. (1) examined the expression kinetics of ORFs that elicited CD4 and CD8 T cell responses in HCMV seropositive adults, presumably in the chronic phase of infection. They found that responses to IE Ags occur more frequently and are larger, on average, than responses to E and L Ags. This was particularly true for CD8 T cell Ags. A small study by Gibson et al. (38) lends additional support to this model. The frequency of CD8 T cells responding to the IE Ag IE1 and the L Ag pp65 was examined over time in HCMV-infected infants. In infants who mounted acute CD8 T cell responses to both IE1 and pp65, it was found that the IE1-specific response was always larger than the pp65-specific response by 1 year of age, regardless of which Ag was immunodominant upon initial infection.

But how does this model account for CD8 T cell inflation in response to m139, M38, and M102 epitopes in C57BL/6 mice and the m164 epitope in BALB/c mice? Nothing is known about expression of these genes during latency or reactivation. It is possible that these genes are expressed early in abortive reactivation or that they are expressed as part of a latency-specific gene expression program. Another possibility is that these genes are expressed in a cell-type specific manner, but the little that is known about their functions does not reveal an obvious explanation. M102 is homologous to HCMV UL102, which encodes a helicase-primase component (39, 40). m139 belongs to a family of genes implicated in MCMV replication in macrophages (41–43), and the functions of M38 and m164 are unknown. M45, the source of noninflationary epitopes in both BALB/c and C57BL/6 mice, is important for endothelial cell tropism (44).

There is currently no evidence to either support or refute these other virological explanations. CMV latency remains poorly understood. During the chronic/latent phase of infection, replicating virus cannot be detected in any tissue, and PCR detection indicates that viral genomes are rare. Hence, this phase of infection is characterized by an active, evolving T cell response apparently driven by virus activity that is below the threshold of detection with the most sensitive molecular techniques. Because of its exquisite sensitivity to low levels of Ag, it is an intriguing possibility that the T cell response may provide us with the best view of virus activity during this elusive phase of infection.

We believe that preferential viral gene expression during latency plays some role in determining chronic immunodominance, but other factors may also be involved. For example, M38 contains two epitopes to which CD8 T cell responses can be detected during acute infection (25), yet only one (M38 316–323) undergoes memory inflation. It is possible that different *M38* gene products are produced during acute and chronic infection. However, within

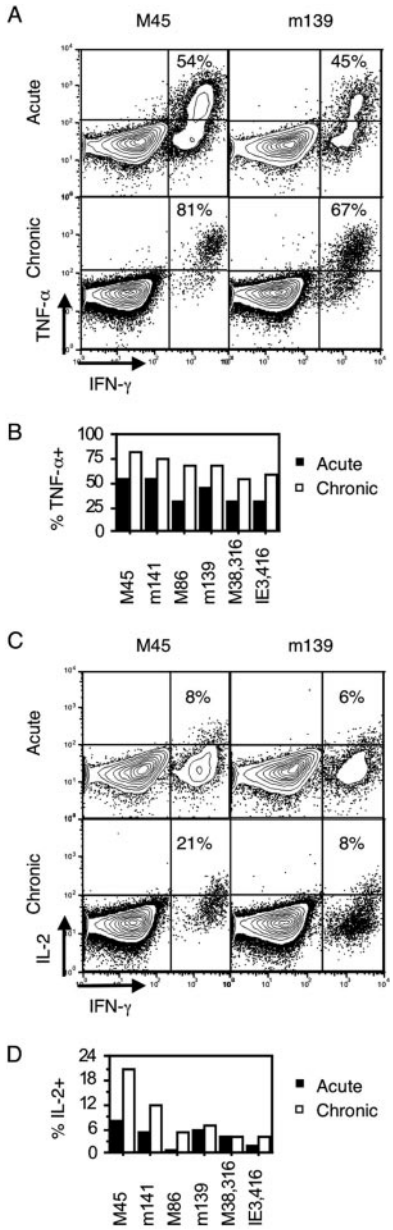

FIGURE 7. Only CD8 T cells specific for noninflationary epitopes gain the ability to produce IL-2. CD8 T cells from acute (day 7) or chronic (8.5 mo) MCMV infection were stimulated with the indicated peptides, then analyzed for IFN-γ and TNF-α production or IFN-γ and IL-2 production. *A* and *C*, Representative FACS plots of IFN-γ/TNF-α or IFN-γ/IL-2 costaining. *B* and *D*, IFN-γ/TNF-α or IFN-γ/IL-2 data for three noninflationary and three inflationary epitopes.

m164, the inflationary BALB/c epitope (257–265) is only separated from one noninflationary C57BL/6 epitope (267–275) by 2 aa. We find it very unlikely that these epitopes would not always be produced together and propose that other mechanisms are very likely to play a role in memory inflation, referred to here as immunological explanations. These assume that there is something the immune system prefers about certain MCMV epitopes, or else the CD8 T cells that respond to them, that causes these responses to dominate during chronic infection.

It does not appear that the functional avidity of T cells is an important factor. The CD8 T cell responses to M86 and M78 have the highest functional avidities in acute infection (25), yet these responses do not inflate. Also, the acute functional avidity of responses to m139 and M38 is not remarkable, compared with other major acute epitopes. More conclusively, Fig. 5 demonstrates that the functional avidity of the response to m139 and M38 does not change appreciably in chronic infection, compared with the acute response.

It has been reported recently hat the initial CD8 T cell precursor frequency dictates whether memory T cells will primarily have a T_{EM} or T_{CM} phenotype (45). T_{EM} generated from an initial low precursor frequency had a stable phenotype ($CD62L^{low}$), but T_{EM} generated from a high precursor frequency reverted to a T_{CM} phenotype ($CD62L^{high}$). We considered the possibility that M45-specific CD8 T cells, for example, might derive from a large number of precursors. In chronic infection, these would be capable of reverting to a T_{CM} phenotype. If m139- and M38-specific CD8 T cells were derived from a small precursor number, these CD8 T cells may be largely fixed with a T_{EM} phenotype. However, the current paradigm is that T_{CM}s are most capable of proliferation, both homeostatic and Ag driven, while T_{EM}s have little or no proliferative ability. In MCMV infection, it is those responses with a T_{EM} phenotype that undergo inflation whereas those with a T_{CM} phenotype do not. Thus, to reconcile the MCMV data with what is known about cleared infections will require further studies.

The basis of the immune system's choice of which particular MHC:peptide combinations to favor in chronic infection is poorly understood and likely to be complex. Multiple mechanisms may be at work, with the final immunodominance outcome determined by superimposing virological and immunological constraints.

In addition, other unanswered questions about the chronic CD8 T cell response to MCMV remain. Primary among these is formal demonstration that continued virus activity (i.e., Ag exposure) is required for MCMV-specific memory inflation and maintenance of the T_{EM} phenotype. If the response is driven by Ag, does presentation occur directly or via cross-presentation? And what program of viral gene expression is responsible? With the exception of the *IE* genes, the program(s) of viral gene expression that occur during latency, and the cell type(s) they occur in, are not known. In fact, the size, epitope specificity, and T_{EM} phenotype of the chronic CD8 T cell response stand in stark contrast to the absence of detectable viral activity. However, the power of the mouse model and advances in herpesvirus genetics means that these questions should be amenable to experimental dissection.

Disclosures

The authors have no financial conflict of interest.

References

1. Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P. R. Sleath, K. H. Grabstein, N. A. Hosken, F. Kern, et al. 2005. Broadly targeted human cytomegalovirus-specific $CD4^+$ and $CD8^+$ T cells dominate the memory compartments of exposed subjects. *J. Exp. Med.* 202: 673–685.
2. Khan, N., A. Hislop, N. Gudgeon, M. Cobbold, R. Khanna, L. Nayak, A. B. Rickinson, and P. A. Moss. 2004. Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. *J. Immunol.* 173: 7481–7489.
3. Northfield, J., M. Lucas, H. Jones, N. T. Young, and P. Klenerman. 2005. Does memory improve with age? CD85j (ILT-2/LIR-1) expression on CD8 T cells correlates with "memory inflation" in human cytomegalovirus infection. *Immunol. Cell Biol.* 83: 182–188.
4. Wang, E. C., P. A. Moss, P. Frodsham, P. J. Lehner, J. I. Bell, and L. K. Borysiewicz. 1995. $CD8^{high}CD57^+$ T lymphocytes in normal, healthy individuals are oligoclonal and respond to human cytomegalovirus. *J. Immunol.* 155: 5046–5056.
5. Wills, M. R., A. J. Carmichael, M. P. Weekes, K. Mynard, G. Okecha, R. Hicks, and J. G. Sissons. 1999. Human virus-specific $CD8^+$ CTL clones revert from $CD45RO^{high}$ to $CD45RA^{high}$ in vivo: $CD45RA^{high}CD8^+$ T cells comprise both naive and memory cells. *J. Immunol.* 162: 7080–7087.
6. Kern, F., E. Khatamzas, I. Surel, C. Frommel, P. Reinke, S. L. Waldrop, L. J. Picker, and H. D. Volk. 1999. Distribution of human CMV-specific memory T cells among the $CD8^+$ subsets defined by CD57, CD27, and CD45 isoforms. *Eur. J. Immunol.* 29: 2908–2915.

7. Gillespie, G. M., M. R. Wills, V. Appay, C. O'Callaghan, M. Murphy, N. Smith, P. Sissons, S. Rowland-Jones, J. I. Bell, and P. A. Moss. 2000. Functional heterogeneity and high frequencies of cytomegalovirus-specific $CD8^+$ T lymphocytes in healthy seropositive donors. *J. Virol.* 74: 8140–8150.
8. Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, et al. 2002. Memory $CD8^+$ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.* 8: 379–385.
9. Kuijpers, T. W., M. T. Vossen, M. R. Gent, J. C. Davin, M. T. Roos, P. M. Wertheim-van Dillen, J. F. Weel, P. A. Baars, and R. A. van Lier. 2003. Frequencies of circulating cytolytic, $CD45RA^+CD27^-$, $CD8^+$ T lymphocytes depend on infection with CMV. *J. Immunol.* 170: 4342–4348.
10. Brenchley, J. M., N. J. Karandikar, M. R. Betts, D. R. Ambrozak, B. J. Hill, L. E. Crotty, J. P. Casazza, J. Kuruppu, S. A. Migueles, M. Connors, et al. 2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of $CD8^+$ T cells. *Blood* 101: 2711–2720.
11. Ouyang, Q., W. M. Wagner, A. Wikby, S. Walter, G. Aubert, A. I. Dodi, P. Travers, and G. Pawelec. 2003. Large numbers of dysfunctional $CD8^+$ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old. *J. Clin. Immunol.* 23: 247–257.
12. Trzonkowski, P., J. Mysliwska, E. Szmit, J. Wieckiewicz, K. Lukaszuk, L. B. Brydak, M. Machala, and A. Mysliwski. 2003. Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination—an impact of immunosenescence. *Vaccine* 21: 3826–3836.
13. Goronzy, J. J., J. W. Fulbright, C. S. Crowson, G. A. Poland, W. M. O'Fallon, and C. M. Weyand. 2001. Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals. *J. Virol.* 75: 12182–12187.
14. Saurwein-Teissl, M., T. L. Lung, F. Marx, C. Gschosser, E. Asch, I. Blasko, W. Parson, G. Bock, D. Schonitzer, E. Trannoy, and B. Grubeck-Loebenstein. 2002. Lack of antibody production following immunization in old age: association with $CD8^+CD28^-$ T cell clonal expansions and an imbalance in the production of Th1 and Th2 cytokines. *J. Immunol.* 168: 5893–5899.
15. Pawelec, G., A. Akbar, C. Caruso, R. Effros, B. Grubeck-Loebenstein, and A. Wikby. 2004. Is immunosenescence infectious? *Trends Immunol.* 25: 406–410.
16. Holtappels, R., D. Thomas, J. Podlech, and M. J. Reddehase. 2002. Two antigenic peptides from genes m123 and m164 of murine cytomegalovirus quantitatively dominate CD8 T cell memory in the $H\text{-}2^d$ haplotype. *J. Virol.* 76: 151–164.
17. Karrer, U., S. Sierro, M. Wagner, A. Oxenius, H. Hengel, U. H. Koszinowski, R. E. Phillips, and P. Klenerman. 2003. Memory inflation: continuous accumulation of antiviral $CD8^+$ T cells over time. *J. Immunol.* 170: 2022–2029.
18. Holtappels, R., M. F. Pahl-Seibert, D. Thomas, and M. J. Reddehase. 2000. Enrichment of immediate-early 1 (m123/pp89) peptide-specific CD8 T cells in a pulmonary $CD62L^{lo}$ memory-effector cell pool during latent murine cytomegalovirus infection of the lungs. *J. Virol.* 74: 11495–11503.
19. Sierro, S., R. Rothkopf, and P. Klenerman. 2005. Evolution of diverse antiviral $CD8^+$ T cell populations after murine cytomegalovirus infection. *Eur. J. Immunol.* 35: 1113–1123.
20. Holtappels, R., N. K. Grzimek, D. Thomas, and M. J. Reddehase. 2002. Early gene m18, a novel player in the immune response to murine cytomegalovirus. *J. Gen. Virol.* 83: 311–316.
21. Reddehase, M. J. 2002. Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. *Nat. Rev. Immunol.* 2: 831–844.
22. Holtappels, R., D. Thomas, J. Podlech, G. Geginat, H. P. Steffens, and M. J. Reddehase. 2000. The putative natural killer decoy early gene m04 (gp34) of murine cytomegalovirus encodes an antigenic peptide recognized by protective antiviral CD8 T cells. *J. Virol.* 74: 1871–1884.
23. Holtappels, R., D. Thomas, and M. J. Reddehase. 2000. Identification of a K^d-restricted antigenic peptide encoded by murine cytomegalovirus early gene M84. *J. Gen. Virol.* 81: 3037–3042.
24. Holtappels, R., J. Podlech, N. K. Grzimek, D. Thomas, M. F. Pahl-Seibert, and M. J. Reddehase. 2001. Experimental preemptive immunotherapy of murine cytomegalovirus disease with CD8 T cell lines specific for ppM83 and pM84, the two homologs of human cytomegalovirus tegument protein ppUL83 (pp65). *J. Virol.* 75: 6584–6600.
25. Munks, M. W., M. C. Gold, A. L. Zajac, C. M. Doom, C. S. Morello, D. H. Spector, and A. B. Hill. 2006. Genome-wide analysis reveals a highly diverse CD8 T cell response to murine cytomegalovirus. *J. Immunol.* 176: 3760–3766.
26. Wagner, M., S. Jonjic, U. H. Koszinowski, and M. Messerle. 1999. Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. *J. Virol.* 73: 7056–7060.
27. Gold, M. C., M. W. Munks, M. Wagner, C. W. McMahon, A. Kelly, D. G. Kavanagh, M. K. Slifka, U. H. Koszinowski, D. H. Raulet, and A. B. Hill. 2004. Murine cytomegalovirus interference with antigen presentation has little effect on the size or the effector memory phenotype of the CD8 T cell response. *J. Immunol.* 172: 6944–6953.
28. Karrer, U., M. Wagner, S. Sierro, A. Oxenius, H. Hengel, T. Dumrese, S. Freigang, U. H. Koszinowski, R. E. Phillips, and P. Klenerman. 2004. Expansion of protective $CD8^+$ T cell responses driven by recombinant cytomegaloviruses. *J. Virol.* 78: 2255–2264.
29. Hislop, A. D., N. E. Annels, N. H. Gudgeon, A. M. Leese, and A. B. Rickinson. 2002. Epitope-specific evolution of human $CD8^+$ T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J. Exp. Med.* 195: 893–905.
30. Callan, M. F. 2003. The evolution of antigen-specific $CD8^+$ T cell responses after natural primary infection of humans with Epstein-Barr virus. *Viral Immunol.* 16: 3–16.
31. Obar, J. J., S. G. Crist, D. C. Gondek, and E. J. Usherwood. 2004. Different functional capacities of latent and lytic antigen-specific CD8 T cells in murine gammaherpesvirus infection. *J. Immunol.* 172: 1213–1219.
32. Yewdell, J. W., and A. B. Hill. 2002. Viral interference with antigen presentation. *Nat. Immunol.* 3: 1019–1025.
33. Hengel, H., P. Lucin, S. Jonjic, T. Ruppert, and U. H. Koszinowski. 1994. Restoration of cytomegalovirus antigen presentation by γ interferon combats viral escape. *J. Virol.* 68: 289–297.
34. Hengel, H., U. Reusch, G. Geginat, R. Holtappels, T. Ruppert, E. Hellebrand, and U. H. Koszinowski. 2000. Macrophages escape inhibition of major histocompatibility complex class I-dependent antigen presentation by cytomegalovirus. *J. Virol.* 74: 7861–7868.
35. Holtappels, R., N. K. Grzimek, C. O. Simon, D. Thomas, D. Dreis, and M. J. Reddehase. 2002. Processing and presentation of murine cytomegalovirus pORFm164-derived peptide in fibroblasts in the face of all viral immunosubversive early gene functions. *J. Virol.* 76: 6044–6053.
36. Kurz, S. K., M. Rapp, H. P. Steffens, N. K. Grzimek, S. Schmalz, and M. J. Reddehase. 1999. Focal transcriptional activity of murine cytomegalovirus during latency in the lungs. *J. Virol.* 73: 482–494.
37. Kurz, S. K., and M. J. Reddehase. 1999. Patchwork pattern of transcriptional reactivation in the lungs indicates sequential checkpoints in the transition from murine cytomegalovirus latency to recurrence. *J. Virol.* 73: 8612–8622.
38. Gibson, L., G. Piccinini, D. Lilleri, M. G. Revello, Z. Wang, S. Markel, D. J. Diamond, and K. Luzuriaga. 2004. Human cytomegalovirus proteins pp65 and immediate early protein 1 are common targets for $CD8^+$ T cell responses in children with congenital or postnatal human cytomegalovirus infection. *J. Immunol.* 172: 2256–2264.
39. Sherman, G., J. Gottlieb, and M. D. Challberg. 1992. The UL8 subunit of the herpes simplex virus helicase-primase complex is required for efficient primer utilization. *J. Virol.* 66: 4884–4892.
40. Lyons, P. A., P. B. Dallas, C. Carrello, G. R. Shellam, and A. A. Scalzo. 1994. Mapping and transcriptional analysis of the murine cytomegalovirus homologue of the human cytomegalovirus UL103 open reading frame. *Virology* 204: 835–839.
41. Cavanaugh, V. J., R. M. Stenberg, T. L. Staley, H. W. Virgin 4th, M. R. MacDonald, S. Paetzold, H. E. Farrell, W. D. Rawlinson, and A. E. Campbell. 1996. Murine cytomegalovirus with a deletion of genes spanning *Hin*dIII-J and -I displays altered cell and tissue tropism. *J. Virol.* 70: 1365–1374.
42. Hanson, L. K., J. S. Slater, Z. Karabekian, H. W. Virgin 4th, C. A. Biron, M. C. Ruzek, N. van Rooijen, R. P. Ciavarra, R. M. Stenberg, and A. E. Campbell. 1999. Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis. *J. Virol.* 73: 5970–5980.
43. Menard, C., M. Wagner, Z. Ruzsics, K. Holak, W. Brune, A. E. Campbell, and U. H. Koszinowski. 2003. Role of murine cytomegalovirus US22 gene family members in replication in macrophages. *J. Virol.* 77: 5557–5570.
44. Brune, W., C. Menard, J. Heesemann, and U. H. Koszinowski. 2001. A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. *Science* 291: 303–305.
45. Marzo, A. L., K. D. Klonowski, A. Le Bon, P. Borrow, D. F. Tough, and L. Lefrancois. 2005. Initial T cell frequency dictates memory $CD8^+$ T cell lineage commitment. *Nat. Immunol.* 6: 793–799.