Control of Gammaherpesvirus Latency by Latent Antigen-specific CD8$^+$ T Cells

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Abstract

The contribution of the latent antigen-specific CD8$^+$ T cell response to the control of gamma-herpesvirus latency is currently obscure. Some latent antigens induce potent T cell responses, but little is known about their induction or the role they play during the establishment of latency. Here we used the murine gammaherpesvirus system to examine the expression of the latency-associated M2 gene during latency and the induction of the CD8$^+$ T cell response to this protein. M2, in contrast to the M3 latency-associated antigen, was expressed at day 14 after infection but was undetectable during long-term latency. The induction of the M2$_{91-99}$/K^d CD8$^+$ T cell response was B cell dependent, transient, and apparently induced by the rapid increase in latently infected cells around day 14 after intranasal infection. These kinetics were consistent with a role in controlling the initial "burst" of latently infected cells. In support of this hypothesis, adoptive transfer of an M2-specific CD8$^+$ T cell line reduced the initial load of latently infected cells, although not the long-term load. These data represent the first description of a latent antigen-specific immune response in this model, and suggest that vaccination with latent antigens such as M2 may be capable of modulating latent gammaherpesvirus infection.

Key words: gammaherpesvirus • virus latency • CD8$^+$ T lymphocytes • viral antigens • viral load

Introduction

The CD8$^+$ T cell response is known to be of critical importance for the control of gammaherpesvirus infections. In both the human viruses, EBV and the Kaposi sarcoma–associated herpesvirus (HHV-8), lytic and latent cycle proteins are targets for the CD8$^+$ T cell response (1, 2), and recent data have shed light on the relative magnitude of the immune response against different viral proteins. In the dramatic T cell response that occurs during primary EBV infection (infectious mononucleosis), up to 44% of CD8$^+$ T cells are specific for an epitope in the lytic phase protein BZLF1 (3). In contrast, only 1–2% of CD8$^+$ T cells recognized an epitope from the latency-associated EBV nuclear antigen (EBNA)[1] 3A protein. In postconvalescent patients the response to the BZLF1 epitope declines to 10–20%; however, the response to the EBNA3A epitope remains at the same level. Given the strong immune response to lytic phase antigens, it seems likely that continual immune surveillance serves to recognize cells reactivating from latency, thus limiting the dissemination of the virus. In contrast, the role of latent antigen-specific CD8$^+$ T cells in the control of the infection remains enigmatic. Unlike lytic antigen-specific T cells, these cells have the potential to target latently infected cells directly and to modulate the latent virus burden.

The early events after gammaherpesvirus infection have been difficult to study in vivo, largely due to the lack of a tractable animal model. Humans display clinical symptoms of EBV infection several weeks after infection, a time when latency has likely already been established. It is therefore difficult to follow the onset of the immune reaction that is induced in response to latently infected cells. An understanding of this response is critical if our aim is to understand how the immune response can be harnessed to lower the load of latently infected cells in infected individuals.

The murine gammaherpesvirus 68 (MHV-68) system offers a very powerful tool to study fundamental aspects of

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[1]Abbreviations used in this paper: CFSE, carboxyfluorescein (diacetate) succinimidyl ester; EBNA, EBV nuclear antigen; ELISPOT, enzyme-linked immunospot; MHV-68, murine gammaherpesvirus 68; μMT, B cell–deficient; ORF, open reading frame; RT, reverse transcriptase.

gammaherpesvirus pathogenesis. After intranasal inoculation, the virus replicates productively in the lungs, then establishes a latent infection in lymphoid tissue, predominantly the spleen (4), although other cells can also maintain a latent infection (5–8). Latently infected cells increase in number rapidly up to ~14 d after infection, then they decline over the subsequent 2 wk to a low level. A low-level lytic replication can be detected in the spleen for ~1 mo after infection, which may be due to spontaneous reactivation from latency (9). Several candidate latency-associated genes have been identified (10–12), one of which, M2, has been shown to be associated exclusively with latent infection. Transcription of the M2 gene was detected in all cells of the latently infected cell line S11, whereas only a small proportion of cells expressed lytic cycle proteins. M2 is not expressed in lytically infected fibroblasts, but can be detected in spleen cells from latently infected mice in the absence of lytic cycle transcripts (10). We also detected transcription of a second putative latency-associated gene, M3, in latently infected spleen cells, although this gene was not uniformly expressed in latently infected S11 cells (10). Importantly, we showed that M2 contains an epitope ($M2_{91–99}$/ K^d) recognized by $CD8^+$ T cells (10). Therefore, using this model system we can measure the kinetics of induction of the latent antigen-specific $CD8^+$ T cell response, and test directly the impact of latent antigen-specific $CD8^+$ T cells during the infection.

In this study we report the kinetics of expression of the latency-associated M2 and M3 genes, and profile the initiation of the $CD8^+$ T cell response to M2. We present data showing that $M2_{91–99}$/K^d–specific $CD8^+$ T cells can contribute to the control of latently infected cells during the establishment of latency.

Materials and Methods

Mice and Virus. MHV-68 virus (clone G2.4) was originally obtained from Prof. A.A. Nash (University of Edinburgh). Virus was propagated and titered as described previously (4). BALB/c mice were purchased from The Jackson Laboratory or Bantin and Kingman. B cell–deficient (μMT) mice on a BALB/c background were bred at the Trudeau Institute Animal Breeding Facility from breeding pairs provided by Dr. W. Gerhard (The Wistar Institute, Philadelphia, PA). Mice were infected intranasally with 400 PFU or intraperitoneally with 10^6 PFU of MHV-68. All animal experiments were approved by The Trudeau Institute Animal Care and Use Committee.

Generation of the $M2_{91–99}$/K^d–specific $CD8^+$ T Cell Line. The S11 tumor cell line has been described previously (13). Spleen cells were prepared from BALB/c mice at 24 d after infection and depleted of $CD4^+$ and MHC class II^+ cells using anti–mouse CD4 (GK1.5, TIB207; American Type Culture Collection) and anti–mouse MHC class II (TIB120; American Type Culture Collection), followed by incubation with a mixture of anti–rat- and anti–mouse-conjugated Dynabeads (Dynal). Magnetically labeled cells were removed and the remaining cells were cultured with X-irradiated (3,800 rads) S11 cells with 10 U/ml recombinant human IL-2 (R&D Systems). Cells were restimulated with irradiated S11 cells supplemented with irradiated BALB/c spleen feeder cells and IL-2 every 5–8 d.

Chromium-release Assays. Assays were performed as described previously (14). In brief, target cells were loaded with 150 μCi $Na^{51}CrO_4$ with or without peptide for 18 h at 37°C. Target cells were washed and incubated with graded numbers of effector cells for 5 h at 37°C. Assays were performed in round-bottomed 96-well plates and each well received 10^3 target cells. Spontaneous and maximum release were measured by incubation with medium alone or 1% Triton X-100, respectively. The percentage of specific release was calculated using the formula: % specific release = [(experimental − spontaneous)/(maximum − spontaneous)] × 100.

Assays for Free and Latent Virus. Assays were performed essentially as described previously (4, 15). Plaque assay was used to measure free virus in the lungs or spleen. Serial dilutions of homogenized lung tissue or freeze–thawed spleen tissue were added to 3T3 monolayers in a minimal volume and left to adsorb for 1 h before overlaying with carboxymethylcellulose. Monolayers were fixed and stained after a 5-d incubation as described above. To confirm that the freeze–thaw procedure used in the estimation of spleen cell virus titer did not destroy the virus infectivity, we performed a control experiment in which virus was added to either medium alone or a spleen cell suspension. The titer obtained from frozen samples was then compared with that obtained from unfrozen samples. The freeze–thaw process reduced the titer by only 10% (data not shown). Latent virus was measured using an infective center assay. Graded numbers of spleen cells from infected mice were added to monolayers of NIH 3T3 cells and overlaid with carboxymethylcellulose. After 6 d, monolayers were fixed with methanol and stained with Giemsa stain. Samples of spleen cells were also subjected to freeze-thaw and used to measure the titer of free virus in the spleen.

Intracellular Staining for IFN-γ. Spleen cells from BALB/c mice infected with MHV-68 were stimulated in vitro with $M2_{91–99}$ peptide at a concentration of 1 μg/ml, then stained for the presence of intracellular IFN-γ according to previously published protocols (16). In brief, spleen cells were incubated with peptide plus 10 μg/ml brefeldin A and 10 U/ml IL-2 for 5 h. Fc binding was blocked using anti–mouse CD16/CD32 (BD PharMingen) and the cells were stained with Tricolor-conjugated anti-CD8 antibody (Caltag). After fixation with 2% formaldehyde, cells were permeabilized with 0.5% saponin (Sigma-Aldrich), then stained with anti–mouse IFN-γ or an isotype control IgG1 antibody (BD PharMingen) in the presence of 0.5% saponin. Samples were analyzed on a Becton Dickinson FACScan™ flow cytometer using CELLQuest™ software (Becton Dickinson). As a positive control, cells were treated with 50 ng/ml PMA plus 500 ng/ml ionomycin. Peptide synthesis was performed either by New England Peptide Inc. (Fitchburg, MA) or by the Center for Biotechnology at St. Jude Children's Research Hospital.

Carboxyfluorescein (Diacetate) Succinimidyl Ester Labeling and Culture Conditions. Spleen cells were labeled with carboxyfluorescein (diacetate) succinimidyl ester (CFSE) by incubation with 0.5–0.7 μM CSFE diluted in HBSS for 10 min in the dark. Cells were subsequently washed with HBSS or complete tumor medium (17) before use. These cells were restimulated in vitro at a 10:1 ratio with irradiated S11 cells in the presence of IL-2 at 10 U/ml (R&D Systems) at a cell density of 10^6/ml in 24-well plates.

MHC Tetrameric Reagents and Analysis. The construction of folded MHC class I–peptide complexes and their tetramerization have been described previously (18). Tetramers were generated by the Molecular Biology Core Facility at The Trudeau Institute. Two tetramers were used: K^d folded with $M2_{91–99}$ peptide

(GFNKLRSTL) and K^d folded with HA518–528 (IYSTVASSL) peptide derived from influenza A/PR/8/34. Tetramers were stored as aliquots at $-20°C$. Tetramers were titered using the S11-specific $CD8^+$ T cell line or spleen cells from mice transgenic for the K^d/HA$_{518-528}$ epitope (19); no cross-reactivity was detected between the two tetramers. Spleen cells were depleted of B cells by panning on goat anti–rat Ig (H and L chain transgenes)-coated flasks before staining. Cells were incubated with anti-CD16/CD32 Fc block (BD PharMingen) for 10 min on ice; staining with tetrameric reagents took place for 1 h at room temperature, followed by staining with anti-CD8–Tricolor on ice for 20 min. Stained samples were analyzed using a FACScan™ flow cytometer and CELLQuest™ software (Becton Dickinson).

Enzyme-linked Immunospot Assay.   The relative frequencies of IFN-γ–secreting cells derived from the spleen were determined after peptide stimulation in a standard enzyme-linked immunospot (ELISPOT) assay system (16). In brief, 96-well Multiscreen-HA nitrocellulose plates (Millipore) were coated overnight at 4°C with 100 μl per well of rat anti–mouse IFN-γ (clone R4-6A2; BD PharMingen) at a concentration of 10 μg/ml. Responder cells were prepared from spleens of BALB/c mice infected with MHV-68 for 95 d, and enriched for $CD8^+$ T cells by depleting $CD4^+$ and MHC class II^+ cells using antibodies TIB120 and GK1.5 followed by anti–rat Ig and anti–mouse Ig-coated Dynabeads (Dynal). The plates were then washed and blocked before the addition of a graded number of responding cells: irradiated (3,000 rads) syngeneic normal spleen cells, 10 μg/ml peptide M2$_{91-99}$, and 10 U/ml recombinant human IL-2. Plates were then incubated overnight at 37°C and developed overnight with a biotinylated detection antibody, rat anti–mouse IFN-γ (clone XMG1.2; BD PharMingen), followed by streptavidin–horseradish peroxidase (BD PharMingen) for 2 h at room temperature. Visible spots of IFN-γ–secreting cells were then enumerated using a SZH stereo zoom microscope system (Olympus). As a negative control in these assays, we used the HA$_{518-528}$/K^d epitope derived from influenza strain PR/8/34, which generally gave a background of <10 spots per well.

Reverse Transcriptase PCR Analysis from Infected Mouse Tissues. Lungs and spleens were removed and snap frozen at $-70°C$. A portion of tissue from each organ was removed and total RNA was prepared using RNAzol B (GIBCO BRL). RNA (2 μg) was treated with 10 U of RNase-free DNase 1 (Amersham Pharmacia Biotech) at 37°C for 30 min. cDNA was synthesized at 42°C with 200 U of Moloney murine leukemia virus reverse transcriptase (RT) (Superscript II; GIBCO BRL) in the presence of 100 pM random primers (Roche Molecular Biochemicals) and 100 nM dNTPs (Amersham Pharmacia Biotech). A portion of the cDNA (1.5 μl) was subjected to PCR with primer sets specific for the MHV-68 genes open reading frame (ORF)50, M2, and M3 (Table I). Separate PCR reactions for murine β-actin were done for each cDNA sample. PCR reactions were carried out with 1 U per reaction of Taq DNA polymerase (GIBCO BRL) in the presence of 100 nM dNTP, 2.0 mM MgCl$_2$, and 50 pmol of each primer in a total reaction volume of 50 μl. Cycling parameters were the same for each primer set used and were: 96°C, 45 s; 45°C, 60 s; and 72°C, 120 s for 35 cycles with a final extension at 72°C for 7 min. Reaction products were separated by electrophoresis through 1% Tris-acetic acid–EDTA agarose gels. After electrophoresis, PCR products were transferred to HybondN$^+$ (Amersham Pharmacia Biotech) in 0.4 M NaOH. Blots were hybridized using standard techniques with double-stranded DNA probes random prime labeled with [α-^{32}P]dCTP. Viral gene–specific DNA probes for labeling were obtained from cloned viral DNA fragments that were cleaved from plasmid vectors and purified before labeling. Murine β-actin blots were hybridized with a full-length chick β-actin probe. A series of PCR controls was incorporated as follows. To control for the carryover of viral DNA, PCR was performed on cDNA synthesized in the absence of RT. Controls for false positive signals included PCR analysis of cDNA from spleen and lung tissue from uninfected mice. Contamination was also controlled for with PCR performed in the absence of cDNA. These control reactions were negative in all cases. We determined the sensitivity of our PCR reactions by diluting a known number of copies of template tar-

Table I.   *Primers Used for PCR Analysis*

Gene	Primer	Genome coordinate*	Sequence	Product size
				bp
ORF50	Sense	68409	AAAAGTTCTGCATCCCAGACCC	293
	Antisense	68701	AGGGCTAATGGGTGAAAATGGC	
M2‡	Sense	4077	TAATAGGAAGACGTATCTCAGG	589
	Antisense	5875	CTGCTTCCTTAGCCAGTCTC	
M3	Sense	6566	TGGCACTCAAACTTGGTTGTGG	359
	Antisense	6925	TAACAGGCAGATTGCCATTCCC	
Murine β-actin‡	Sense	–	TGTGATGGTGGGAATGGGTCA	514
	Antisense	–	TTTGATGTCACGCACGATTTC	

*Genome coordinates as described by Virgin et al. (reference 34).
‡Primer sequences derived from different exons; therefore, spliced mRNA can be distinguished from unspliced genomic DNA.

get in a background of uninfected mouse cDNA, then performing the PCR reaction. The sensitivity limit was found to be one copy in all cases.

Results

Kinetics of Expression of Latency-associated Genes. In a previous report we identified two genes, M2 and M3, whose expression was associated with latent infection (10). To investigate whether these genes were expressed long-term during the latent infection, we isolated RNA from infected mouse tissues at various times after infection and performed RT-PCR to detect mRNA for M2 or M3. In addition, we used primers for the lytic cycle transactivator ORF50 and murine β-actin. It has been shown that ORF50 (a homologue of the EBV rightward transactivator) is expressed at all stages of the virus productive cycle and is responsible for reactivating virus from latency (20, 21). Therefore, analysis of ORF50 expression was performed to detect low-level productive virus replication in the samples. Expression of β-actin was monitored to control for RNA integrity and cDNA synthesis. We chose to examine two tissues that have been shown to harbor a latent infection: the spleen and the lungs (7).

As shown in Fig. 1, β-actin was expressed in all samples, confirming the integrity of the PCR templates in all cases. ORF50 gene expression was detected at days 7, 14, 21, and 32 after infection in all mice tested, consistent with previous data showing a low-level reactivation of virus in the
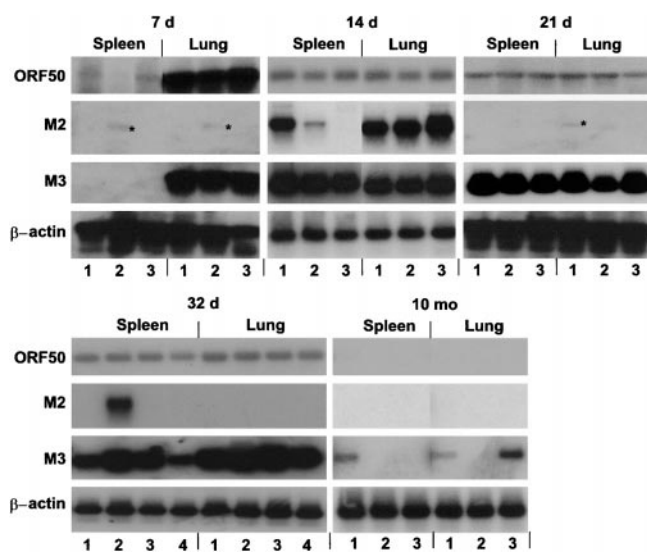

Figure 1. The kinetics of expression of latency associated genes in vivo. Mice were infected intranasally with MHV-68 for either 7, 14, 21, or 32 d, or 10 mo, as indicated. Spleens and lungs were then sampled and total RNA was analyzed by RT-PCR followed by Southern blotting and autoradiography. Primers and probes specific for ORF50, M2, M3, and β-actin were used as indicated. The numbers at the bottom of the figure refer to individual mice. The asterisks in two of the panels highlight the position of weak positive reactions. Several control reactions were performed that were negative in all cases (data not shown). The controls were as follows: PCR in the absence of RT, RT-PCR of tissue from uninfected mice, and PCR performed in the absence of cDNA.

spleen during the first month after infection (9). By 10 mo after infection we observed no ORF50 expression, indicating an undetectable level of productive virus replication at this time. RNA encoding M3 was detected at all times tested, although expression was sporadic at 10 mo after infection. In contrast, M2 expression followed a different pattern. It was detectable in only one of three spleens and one of three lungs at day 7 after infection; however, by 14 d there was expression in three of three lungs and two of three spleens. M2 expression declined by day 21 after infection (zero of three spleens and one of three lungs), and was detectable in only one of four spleens at day 32. No M2 expression was detectable at 10 mo after infection in either spleens or lungs. Therefore, these data showed that (a) there was a difference in the kinetics of M2 and M3 expression in vivo during latent infection, and (b) M2 expression appeared to be transient and no longer expressed in the majority of mice by day 21 after infection.

The M2$_{91-99}$/K^d–specific CD8$^+$ T Cell Response Is Induced during the Establishment of Latency. We have shown previously that the M2 protein is a target for the CD8$^+$ T cell response, and we mapped the M2$_{91-99}$/K^d epitope that is recognized (10). As we established that M2 expression was transient, we wished to determine the longevity of the M2$_{91-99}$/K^d–specific CD8$^+$ T cell response. We sampled spleen cells from mice at different times after intranasal infection with MHV-68 and measured the frequency of cells that synthesized IFN-γ in response to M2$_{91-99}$ peptide. As shown in Fig. 2 A, the response was not detectable until 16 d after infection, when it rose rapidly to ∼3.5% of CD8$^+$ T cells. However, the response was short lived and declined by day 21 after infection. It was possible that the downregulation in this response was not due to a loss of M2$_{91-99}$/K^d–specific CD8$^+$ T cells but rather to these cells failing to produce IFN-γ. To resolve this issue, we synthesized a tetrameric K^d reagent folded with M2$_{91-99}$ peptide that specifically bound M2$_{91-99}$/K^d–specific CD8$^+$ T cells. This reagent did not bind naive CD8$^+$ T cells or a CD8$^+$ T cell line of an irrelevant specificity (data not shown). Detection of M2$_{91-99}$/K^d–specific CD8$^+$ T cells using the tetrameric reagent showed similar kinetics to the IFN-γ staining (Fig. 2 C), demonstrating that this response is detectable only during a very narrow time frame in the spleen, between days 16 and 20 after infection. This is coincident with the decline in numbers of latently infected cells in the spleen. In contrast to lytic antigen–specific epitopes in C57BL/6 mice, we did not detect significant numbers (<1% of CD8$^+$ T cells) of M2$_{91-99}$/K^d–specific CD8$^+$ T cells in the bronchoalveolar lavage during the acute respiratory infection using the tetrameric reagent (data not shown).

To determine whether the kinetics of the M2$_{91-99}$/K^d–specific CD8$^+$ T cell response were merely a reflection of the CD8$^+$ T cell response to this virus as a whole, we also measured IFN-γ production from spleen cells stimulated with PMA and ionomycin. These reagents induce IFN-γ production from all activated T cells, and therefore allowed us to measure the proportion CD8$^+$ T cells that were acti-
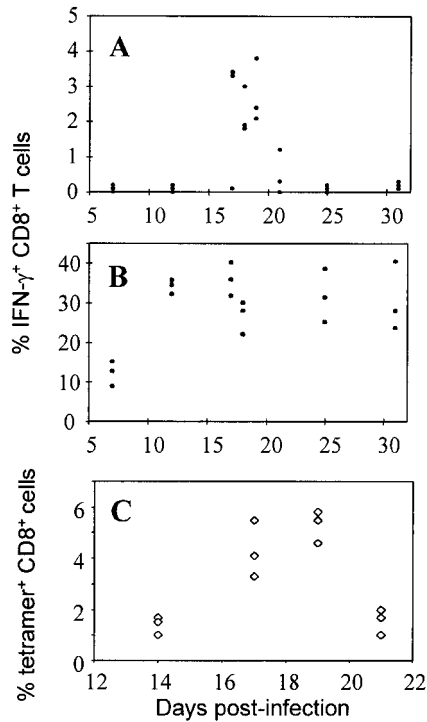
Figure 2. The kinetics of the $M2_{91-99}/K^d$–specific CD8$^+$ T cell response. Spleen cells from BALB/c mice infected intranasally with MHV-68 were stimulated in vitro with either $M2_{91-99}$ peptide (A) or PMA and ionomycin (B). The data represent the proportion of CD8$^+$ T cells that produced IFN-γ expressed as a percentage of total CD8$^+$ T cells. Each point represents one individual mouse, and the data presented represent pooled data from five independent infections. (C) The staining of spleen cells with an $M2_{91-99}/K^d$–specific tetrameric reagent. The data represent the proportion of tetramer-positive CD8$^+$ T cells as a percentage of total CD8$^+$ T cells, and each point represents an individual mouse. Note the different x-axis scale relative to A and B. An irrelevant tetramer (Flu $HA_{518-528}/K^d$) stained <0.5% of CD8$^+$ cells.

vated. As shown in Fig. 2 B, this analysis revealed strikingly different kinetics. A very high proportion of the CD8$^+$ T cell compartment was activated by day 14 after infection, and the response remained high for >30 d after infection. These results are consistent with previous reports detailing the mononucleosis-like syndrome that develops in response to MHV-68 infection (22, 23). Therefore, the $M2_{91-99}/K^d$–specific CD8$^+$ T cell response was unusual in that it emerged relatively late (day 16) then declined rapidly only 4–5 d later to a level below the detection limit of these assays. Thus, the kinetics of this response were consistent with $M2_{91-99}/K^d$–specific CD8$^+$ T cells contributing to the

reduction in titer of latently infected cells after day 14 after infection.

Memory Is Established to the $M2_{91-99}/K^d$ Epitope. The kinetics of the $M2_{91-99}/K^d$–specific CD8$^+$ T cell response suggested that it was an effector response in the spleen. We thus addressed the question of whether memory was established to this epitope after the decline in numbers of $M2_{91-99}/K^d$–specific CD8$^+$ T cells in the spleen. Therefore, we restimulated spleen cells from infected mice in vitro with the latently infected S11 cell line and then performed a ^{51}Cr-release assay to test for cytotoxic activity. Cultures established from mice infected for 20, 47, or 68 d after infection all specifically killed targets pulsed with the $M2_{91-99}$ peptide but not unpulsed cells (Fig. 3). This demonstrated that $M2_{91-99}/K^d$–specific CD8$^+$ T cells persist in the infected mouse after the decline in numbers at day 21 after infection, and that a low-level memory is established to the $M2_{91-99}/K^d$ epitope. In addition, it shows that these memory cells can be reactivated in vitro and display effector function. To accurately quantitate the level of memory that was established to this epitope, we used the IFN-γ ELISPOT technique using spleen cells from mice infected with MHV-68 for 95 d. This revealed that the frequency of $M2_{91-99}/K^d$–specific cells among CD8$^+$ T cells was 1/3,300 ($n = 4$, range 1/2,300–1/4,400).

Efficient Induction of the $M2_{91-99}/K^d$–specific CD8$^+$ T Cell Response Requires B Cells. B lymphocytes have an important role in MHV-68 pathogenesis. They are a major target cell for viral latency (24, 25) and in addition are able to suppress the extent of reactivation from latency (6, 7), probably through the production of a neutralizing antibody. Therefore, we wished to investigate whether B cells were necessary for the priming of the $M2_{91-99}/K^d$–specific CD8$^+$ T cell response. To study this we infected μMT mice on a BALB/c background and monitored their ability to mount a response to the $M2_{91-99}/K^d$ epitope.

In initial experiments we observed a smaller population of $M2_{91-99}/K^d$ tetramer–positive cells in the spleens of μMT mice compared with BALB/c mice at day 17 after intranasal infection (data not shown); however, the sensitivity limit of tetramer staining was relatively low (~0.5%). Therefore, we chose to restimulate spleen cells from infected mice in vitro with the latently infected S11 cell line, then stain them with the $M2_{91-99}/K^d$ tetramer. The expansion in the number of $M2_{91-99}/K^d$–specific T cells allowed us to detect even a small response against this epitope. Spleens were taken at days 23–25 after infection because we wished to avoid any activation-induced cell death that
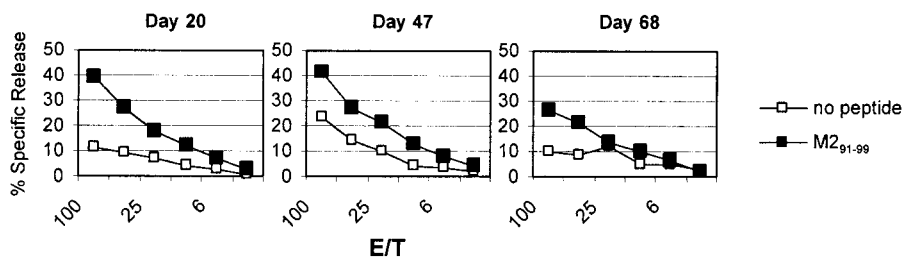

Figure 3. Memory is established to the $M2_{91-99}/K^d$ epitope. Spleen cells from BALB/c mice infected intranasally with MHV-68 were restimulated in vitro with irradiated S11 cells and IL-2 for 7 d. These cells were then tested for $M2_{91-99}K^d$–specific cytotoxicity using BALB/c 3T3 target cells pulsed with $M2_{91-99}$ peptide or no peptide.

may occur if the restimulation was performed during the putative effector phase of the response, between 16 and 20 d after infection. To measure cell proliferation, spleen cells were labeled with CFSE, a fluorescent dye that is progressively diluted with each cell division. As shown in Fig. 4, a substantial number of $M2_{91-99}/K^d$ tetramer–positive cells was present using spleen cells taken from intranasally infected BALB/c mice. However, a much smaller tetramer–positive population (10-fold less) was observed using spleen cells from intranasally infected μMT mice, demonstrating that these mice made a very weak CD8$^+$ T cell response to this epitope. This indicated that B cells are needed for efficient priming of the $M2_{91-99}/K^d$–specific CD8$^+$ T cell response.

We and others have shown that after intranasal infection of μMT mice, there was little or no latent virus detected in the spleen (6, 24). In contrast, a latent infection is established in the spleen after intraperitoneal infection (26). These data can be reconciled by our previous studies showing that B cells are important in the traffic of virus between the lung and spleen during latency (7). We concluded that one role of B cells after intranasal infection may be to traffic the virus to the spleen, where it can infect more B cells and also other cells such as macrophages (5), dendritic cells (8), and lung epithelial cells (7). In contrast, intraperitoneal infection leads to a direct infection of the spleen, bypassing the need for B cells. Therefore, we tested whether intraperitoneal infection of μMT mice restored their ability to prime the $M2_{91-99}/K^d$–specific CD8$^+$ T cell response. Infection by this route primed a response of similar magnitude to that observed using the intranasal route in BALB/c mice (Fig. 4); however, there was still a marked deficit in this response in μMT mice. These data show that in the absence of B cells the $M2_{91-99}/K^d$–specific

CD8$^+$ T cell response is not primed efficiently, even when the latent infection is established efficiently in non–B cells.

An $M2_{91-99}/K^d$–specific CD8$^+$ T Cell Line Can Reduce the Number of Latently Infected Cells. The kinetics of the $M2_{91-99}/K^d$–specific immune response were consistent with a role for these cells in the control of the initial "burst" of latently infected cells. After intranasal infection, free virus is cleared from the lungs by day 7–10 after infection, then the titer of latent virus begins to increase in the spleen. This peaks at day 14 after infection, at ~1 infective center per 10^4 spleen cells, then declines over the subsequent 2 wk to ~1 infective center per 10^7 spleen cells (4). Therefore, it seemed likely that the $M2_{91-99}/K^d$–specific CD8$^+$ T cell response was induced by the large increase in latently infected cells around day 14 after infection and subsequently killed many of these cells between days 16 and 20 after infection, contributing to the decline in latent virus titer.

Therefore, we took an adoptive transfer approach to determine whether the $M2_{91-99}/K^d$–specific CD8$^+$ T cell response has the ability to influence the load of latently infected cells during the early stages of latency. As detailed in a previous report, we generated an $M2_{91-99}/K^d$–specific CD8$^+$ T cell line from infected BALB/c mice, which killed latently infected S11 cells, target cells pulsed with $M2_{91-99}$ peptide, but not unpulsed target cells (10). We then transferred either 10^7 $M2_{91-99}/K^d$–specific CD8$^+$ T cell line cells (7 d after their last stimulation with antigen) or medium alone into BALB/c mice and infected the mice intranasally with MHV-68 on the same day. Spleens were removed at various times after infection, and we measured the numbers of latently infected cells. As shown in Table II, recipients of the $M2_{91-99}/K^d$–specific CD8$^+$ T cell line exhibited a 30–80-fold reduction in the number of latently infect cells at day 14 after infection compared with control
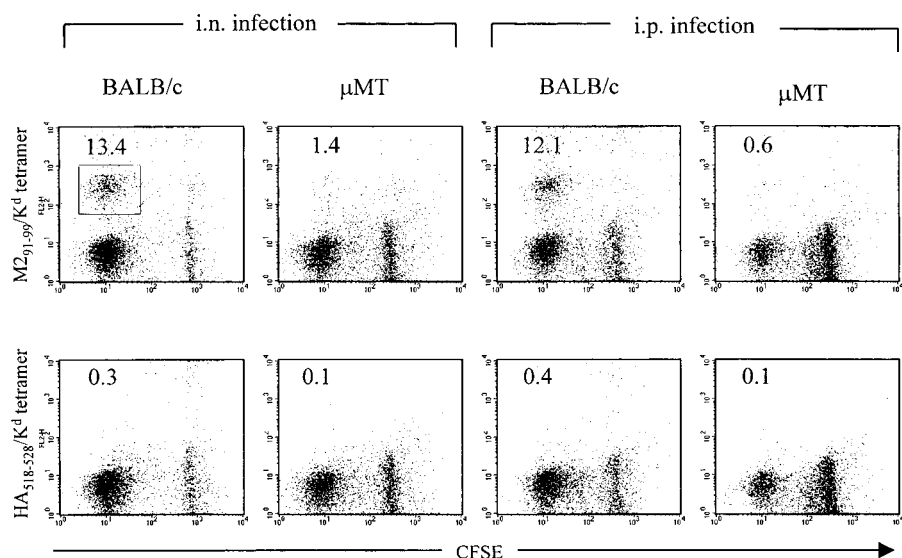

Figure 4. The $M2_{91-99}/K^d$–specific CD8$^+$ T cell response is not primed efficiently in μMT mice. BALB/c or μMT mice were infected either intranasally (i.n.) with 400 PFU or intraperitoneally (i.p.) with 10^6 PFU MHV-68, then spleens were removed at 23–25 d after infection. Spleen cell suspensions were labeled with the fluorescent dye CFSE then restimulated in vitro with irradiated S11 cells in the presence of 10 U/ml human recombinant IL-2 for 4 d. Cultures were then stained with anti-CD8 and either $M2_{91-99}/K^d$ tetramer or a control influenza $HA_{518-528}/K^d$ tetramer. The data show CFSE and tetramer staining gated on live CD8$^+$ cells. The numbers show the percentage of tetramer-positive CFSE^{lo} cells among CD8$^+$ cells (boxed). For intranasal infection, the ranges were 7.6–13.4% for BALB/c mice and 0.6–1.5% for μMT mice; for intraperitoneal infection, the ranges were 5.4–15.1% for BALB/c mice and 0.6–1.0% for μMT mice. Similar data were obtained on days 17–126 after infection. In general, three to four mice were used per experimental group.

Table II. *The Effect of Adoptive Transfer of an M2$_{91-99}$/K^d–specific CD8$^+$ T Cell Line on the Titer of Latent Virus in the Spleen*

Experiment	Time after infection	Treatment[*]	Latent virus titer[‡]	P value[§]	Fold reduction
	d				
1	7	–	19.0 (19.1)		
	7	Anti-M2	4.7 (3.0)	0.268	4.0
	14	–	3,816.7 (1,147.1)		
	14	Anti-M2	46.7 (32.5)	0.0047	81.7
2	14	–	4,812.5 (4,411.6)		
	14	Anti-M2	63.0 (58.0)	0.075	76.4
3	14	–	2,525.0 (1,533.2)		
	14	Anti-M2	81.6 (40.5)	0.019	30.9
	21	–	155.3 (104.8)		
	21	Anti-M2	346.7 (195.0)	0.155	<1
4	21	–	99.8 (68.5)		
	21	Anti-M2	79.8 (15.8)	0.650	1.25
5	21	–	112.3 (159.9)		
	21	Anti-M2	110.3 (86.0)	0.986	1.02
	28	–	35.0 (20.1)		
	28	Anti-M2	27.2 (32.9)	0.742	1.29

[*]Mice received either medium alone (–) or an M2$_{91-99}$/K^d–specific CD8$^+$ T cell line (anti-M2).
[‡]Shown are mean values with SD in parentheses; three to four mice were used in each group.
[§]P values were determined using the Student's *t* test; values <0.05 represent statistical significance.

mice. To determine the antigen specificity of this effect, we performed an additional experiment using a T cell line specific for the influenza HA$_{518-528}$/K^d epitope. Adoptive transfer of this line had no effect on the latent virus titer in the spleen at 14 d after infection (data not shown).

The reduction in latent virus at this time cannot be accounted for by a reduction in the initial viral load during the acute lung infection, as we observed no significant difference in lung virus titers between the two groups on day 7 after infection (Fig. 5). The reduction in latently infected cells was only observed at 14 d after infection, when peak levels of latent virus are detected after intranasal infection, whereas at days 21 or 28 after infection there was no significant difference between control and recipient groups (Table II). This shows that CD8$^+$ T cells specific for the M2$_{91-99}$/K^d epitope can contribute to the control of latently infected cells during the establishment of latency.
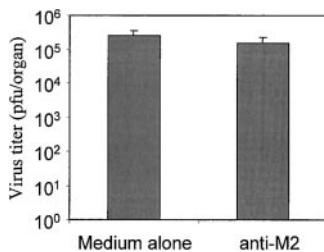

Figure 5. Adoptive transfer of an M2$_{91-99}$/K^d–specific CD8$^+$ T cell line has no effect on the acute lung infection. BALB/c mice received either 10^7 M2$_{91-99}$/K^d–specific CD8$^+$ T cell line cells or medium alone intravenously and were then infected intranasally with MHV-68. At 7 d after infection, lungs were removed and the titer of virus was assayed. The results show the mean titer of three animals per group, and error bars show 1 SD. Data are representative of three experiments.

Discussion

We present a profile of the CD8$^+$ T cell response to a latency-associated gammaherpesvirus antigen in the MHV-68 system. The response to the M2$_{91-99}$/K^d epitope followed unusual kinetics, being present for only 4–5 d before declining to a low level (<0.5% of CD8$^+$ T cells). Thus, the M2$_{91-99}$/K^d–specific response appears to be induced by the rapid rise in latently infected cells that peaks at ~14 d after infection. The peak of the M2$_{91-99}$/K^d–specific T cell response coincides with the reduction in the number of latently infected cells from the spleen (4). This contrasts with the CD8$^+$ T cell response to lytic cycle epitopes in C57BL/6 mice, which appears much earlier in the spleen (day 12) and declines slower, around day 30–40 after infection (27). In addition, we have shown that the adoptive transfer of an M2$_{91-99}$/K^d–specific CD8$^+$ T cell line can reduce the titer of latently infected cells at 14 d after infection. Taken together, these data suggest that the M2$_{91-99}$/K^d–specific T cell response contributes to the control of latently infected cells during the first 2–3 wk after infection. These are the first studies to show unambiguously that latent antigen-specific CD8$^+$ T cells have the ability to control a latent gammaherpesvirus infection.

The reason for the rapid decline in the M2$_{91-99}$/K^d–specific response is likely due to the absence of antigen, since, as shown in Fig. 1, transcripts for M2 had declined dramatically by 32 d after infection. In contrast, expression of both ORF50 (a lytic cycle product) and M3 (a latency-associated product) persisted through this time point. This pattern of

expression therefore correlates with the decline in the M2 response and the longer-lived persistence of the response to lytic cycle antigens. It is possible that the virus downregulates expression of M2 to evade the immune response, and that this forms part of a switch in the program of latency. An analogous phenomenon is believed to occur in EBV infection. When B cells are first infected, the virus is thought to enter the latency III or "growth program": it expresses several immunogenic proteins, and there is extensive proliferation of latently infected cells. The immune response then kills many infected cells, and the remaining latently infected B cells enter a more restricted program of gene expression. Recent studies in the MHV-68 system support this idea, showing that there was a disparity between the number of virus genome-positive cells and the number of cells capable of reactivation (6). The proportion of cells capable of reactivation decreased with time after infection, suggesting that there is a temporal shift in the state of latency, moving towards a latent state that does not reactivate in vitro. This change in the character of latency may restrict the expression of M2. Our data showing that M2 and M3 genes are not downregulated with the same kinetics support the idea of multiple states of latency. An alternative explanation for the switch to a more restricted program of MHV-68 latency is that $M2_{91-99}/K^d$–specific response eliminates all cells expressing M2 and therefore selects for a subpopulation of cells that is already in a different latent gene expression program.

Our data show that B cell–deficient mice do not mount an efficient CD8$^+$ T cell response against M2. This implies that B cells are the major antigen-presenting cell type presenting the $M2_{91-99}/K^d$ epitope. The most straightforward explanation for this is that a latent infection of B cells is required for the expression of M2 and correct presentation to T cells. We do not present evidence that directly shows the transcription of the M2 gene in different cell types; however, our data imply that other latently infected cell types such as macrophages (5) and dendritic cells (8) either (a) do not express M2, (b) do not process it in the same way, or (c) express such low levels that it leads to poor immunological priming. A report detailing the detection of M2 mRNA from intraperitoneally infected μMT mice (11) implies the protein is made in the absence of B cells, indicating that (b) or (c) above may be the more likely explanation.

There is a great deal of interest in developing vaccines against the human gammaherpesviruses. Most EBV vaccine strategies to date have focused on blocking the primary infection by the generation of antibody response to glycoprotein 340/220, the virus ligand for the cellular receptor CD21. An additional suggested approach is to vaccinate a CTL response to lytic cycle proteins (28). The disadvantage with both of these approaches is that an immune response directed towards lytic cycle proteins cannot recognize latently infected cells, so that once latency is established the virus has effectively escaped from the immune response. This has been shown to be true for MHV-68, in which vaccination with immunogenic lytic cycle proteins can re-

duce the lung virus titer and splenomegaly, but the virus still establishes a latent infection efficiently (29, 30).

An alternative approach is to vaccinate against latency-associated antigens (31), which will target latently infected cells directly and may lead to a lower latent virus load. This approach will not prevent initial infection; however, the majority of serious diseases associated with human gammaherpesviruses are a consequence of long-term latent infection. Indeed, the risk of developing certain EBV-associated malignancies has been correlated with the load of latent virus (32, 33). Given the potency of the $M2_{91-99}/K^d$–specific CD8$^+$ T cell line at reducing the day 14 latent virus titer, M2 is a promising vaccine candidate in the MHV-68 model system. Although M2 is only expressed transiently in the spleen, it may nevertheless be expressed by all latently infected cells during the early phase of latency, thus making them targets for the CD8$^+$ T cell response. Therefore, it is conceivable that if there is a sufficiently rapid and vigorous $M2_{91-99}/K^d$–specific CD8$^+$ T cell response in vaccinated animals, then it may be able to destroy a significant number of latently infected cells and perhaps reduce the long-term burden of latent virus. We observed only a transient decrease in the number of latently infected cells in our adoptive transfer system, but we do not know how long these cells survive in vivo, or the numbers of cells that persist in the spleen after transfer. Given the limitations of this adoptive transfer system, we are currently addressing whether active vaccination against M2 can provide a longer-lasting reduction in latently infected cell numbers.

In conclusion, we have presented the first description of the CD8$^+$ T cell response to a latency-associated antigen in the MHV-68 model system. This underlines the utility of the MHV-68 model as a very powerful tool that allows us to discover general mechanisms by which this class of virus is controlled in the host, and should illuminate the pathogenesis of gammaherpesvirus infections of importance for veterinary or human medicine. This report should lead to a better understanding of the role latent antigen-specific T cells play in the immune response to gammaherpesviruses, and experimentally determine whether vaccination with latent antigens is a feasible strategy for combating this significant class of pathogen.

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