A Secreted Chemokine Binding Protein Encoded by Murine Gammaherpesvirus-68 Is Necessary for the Establishment of a Normal Latent Load

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Abstract

Herpesviruses encode a variety of proteins with the potential to disrupt chemokine signaling, and hence immune organization. However, little is known of how these might function in vivo. The B cell–tropic murine gammaherpesvirus-68 (MHV-68) is related to the Kaposi's sarcoma–associated herpesvirus (KSHV), but whereas KSHV expresses small chemokine homologues, MHV-68 encodes a broad spectrum chemokine binding protein (M3). Here we have analyzed the effect on viral pathogenesis of a targeted disruption of the M3 gene. After intranasal infection, an M3 deficiency had surprisingly little effect on lytic cycle replication in the respiratory tract or the initial spread of virus to lymphoid tissues. However, the amplification of latently infected B cells in the spleen that normally drives MHV-68–induced infectious mononucleosis failed to occur. Thus, there was a marked reduction in latent virus recoverable by in vitro reactivation, latency-associated viral tRNA transcripts detectable by in situ hybridization, total viral DNA load, and virus-driven B cell activation. In vivo CD8+ T cell depletion largely reversed this deficiency, suggesting that the chemokine neutralization afforded by M3 may function to block effective CD8+ T cell recruitment into lymphoid tissue during the expansion of latently infected B cell numbers. In the absence of M3, MHV-68 was unable to establish a normal latent load.

Key words: latency • herpesvirus • immune evasion • CD8+ T lymphocyte • pathogenesis

Introduction

The traffic of leukocytes through lymphoid organs where they are primed, and from blood vessels into sites of viral infection and inflammation where they can be effective, is crucial to normal immune function. Chemokines are chemotactic cytokines that play a central role in these processes, through their interactions with the G protein–coupled receptors expressed by all lymphoid cells (1, 2). Chemokines also control the appropriate migration of antigen-presenting cells. Consistent with an important role for chemokines and chemokine receptors in antiviral defense, large DNA viruses have developed a variety of strategies to disrupt chemokine signaling (3, 4). Those described include the expression of secreted chemokine binding proteins that block the interactions of chemokines with cellular receptors or glycosaminoglycans (5–9), chemokine-like proteins that can either block or activate specific signaling pathways (10), and chemokine receptor homologues that may adsorb chemokines locally or may subvert normal signaling in infected cells (11). But despite a large body of in vitro data, the in vivo effects of viral chemokine blockade on host immunity are largely unknown. So far, only the disruption of a secreted 35-kD chemokine binding poxvirus protein, vCKBPII, has been analyzed in this regard (5, 8, 12): vCKBPII-deficient mutants cause increased inflammation at the primary site of infection, but show no evidence of attenuation relative to wild-type (WT)* virus (13, 14).

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Abbreviations used in this paper: BHK, baby hamster kidney; MHV-68, murine gammaherpesvirus-68; MLN, mediastinal lymph node; pi, postinfection; RT, reverse transcription; vtRNA, viral transfer RNA; WT, wild-type.

Gammaherpesviruses characteristically establish latency in lymphocytes and thus have a particularly intimate association with the host immune response. Murine gammaherpesvirus-68 (MHV-68) is a natural pathogen of murid rodents (15) with a close genetic homology to Kaposi's sarcoma–associated herpesvirus (KSHV) and EBV (16–18). MHV-68 infection of conventional mice has proved useful in analyzing the roles of different viral genes (19–22) and immune effector mechanisms (23–27) in gammaherpesvirus pathogenesis. After intranasal infection, MHV-68 replicates transiently in respiratory epithelial cells and spreads to lymphoid tissue where latency is established in B lymphocytes, macrophages, and dendritic cells (28–31). The seeding of virus to lymphoid tissue is followed by a massive amplification of latently infected B cells in germinal centers (32, 33), accompanied by antigen nonspecific B cell activation (34, 35) and an infectious mononucleosis illness analogous to adolescent EBV infection (36). During this amplification, lytic viral gene transcripts and infectious virus remain essentially undetectable in lymphoid tissue, but abundant viral transfer RNA-like (vtRNA) transcripts can be identified in splenic germinal centers by in situ hybridization (32, 33). When latent virus first reaches the spleen, transcripts corresponding to the MHV-68 M3 gene are detectable in parafollicular areas (33), and reverse transcription (RT)-PCR analyses have supported the idea that the M3 genomic region is transcribed during latency (37). The M3 gene encodes a 44-kD secreted protein, which is translated from an abundant 1.4-kb early-late lytic transcript (38). More recently the M3 gene has been shown to encode a broad spectrum chemokine binding protein that neutralizes cellular responses to chemokines in vitro (7, 9). Crucially, an M3-deficient mutant virus was found to lack detectable secreted chemokine-binding activity (7). Here we have analyzed the in vivo pathogenesis of a virus deficient in M3 in order to determine the role of chemokine blockade in gammaherpesvirus pathogenesis and latency.

Materials and Methods

Viruses and Cell Culture. Virus working stocks were prepared by infection of baby hamster kidney (BHK-21) cells grown in Glasgow's modified Eagle's medium containing 10% newborn calf serum, 10% tryptose phosphate broth, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete-GMEM). Cells were infected at low multiplicity (0.001 PFU/cell) and harvested 3–4 d after infection as described previously (21). Infectious virus titers were determined by suspension assay using BHK-21 cells in complete-GMEM (21). After incubation at 37°C in 5% CO_2 for 4 d, monolayers were fixed in 10% formal saline, counterstained with toluidine blue, and plaque numbers enumerated using a plate microscope.

Recombinant Viruses. Recombinant viruses containing a disruption of the M3 gene were generated as described previously (7). In brief, M3LacZ and an independently derived mutant, designated M3LacZi, were generated by cotransfection of virion DNA with linearized pM3MV10. This plasmid contains a 2,602-bp fragment of MHV-68 (genomic coordinates 5,347–7,929) with a cassette containing LacZ under control of the human cy-

tomegalovirus immediate early promoter inserted at a HincII site (genomic coordinate 7,157), disrupting the M3 open reading frame (ORF). The mutants, M3LacZ (7) and M3LacZi (this report), were generated from independent transfections. To generate an M3 revertant, M3LacZ virion DNA was cotransfected with pM3 which contains an intact M3 gene and flanking sequences (nucleotide coordinates 5,347–7,929). Recombinant viruses were identified by X-Gal staining, plaque purified three times, and their genetic structures confirmed by Southern blot hybridization.

In Vivo Infections. Female BALB/c mice (Charles River Laboratories) were inoculated intranasally at 5–6 wk of age with 4×10^3–10^4 PFU of virus in 20 μl phosphate-buffered saline while under halothane anesthesia. At various times after infection mice were killed by intraperitoneal injection of sodium pentobarbitone and lungs, spleen, and mediastinal lymph nodes (MLNs) removed. For infectious virus assays, tissue homogenates were frozen and thawed twice before suspension assay with BHK-21 cells. Infectious center assays (21) were performed on dissociated spleens and lymph nodes. For this assay, homogenized spleens and MLNs were passed through a 140-μm filter to remove stromal debris. Single cell suspensions were plated with BHK-21 cells, incubated for 5 d, fixed with 10% formal saline, and counterstained with toluidine blue. Plaques were counted with a plate microscope. All animal studies were conducted under approval of UK Home Office Project Licence no. 80/1378.

CD8+ T Cell Depletion. Mice were injected intravenously 2 d before infection with 0.1 ml of ascitic fluid containing mAbs to CD8α (2.43; a gift from Dr. P.C. Doherty, St. Jude's Children's Research Hospital, Memphis, TN) and every second day from the day of virus infection by intraperitoneal injection of 0.1 ml anti-CD8α ascites. Depletion was >99% effective, as determined by flow cytometry of spleen cells.

Flow Cytometry. Single cell suspensions were washed in PBS/0.01% azide/0.1% BSA, blocked with 10% normal mouse serum, and stained with CD69 FITC, anti-CD4 tricolor, or anti-CD8 tricolor (Caltag), and one of CD62L-PE, CD19 PE, or DX5-PE (Becton Dickinson). After a 30-min incubation on ice, the cells were washed and analyzed on a FACSort™ with LYSYS™ II software (Becton Dickinson). Data were analyzed with FCSPress 1.0J software (www.fcspress.com).

RT-PCR Analyses of Virus Transcription. mRNA was prepared from virus infected BHK cells using a Direct mRNA kit (Sigma-Aldrich). All RNA samples were DNASe-treated overnight and phenol/chloroform extracted. Samples of RNA were reverse transcribed for 1 h at 42°C. PCR was carried out with Taq polymerase (Roche) according to the manufacturer's instructions. The M2 forward primer corresponds to 5′-GGCTG-GATATAGACTGGTTCA-3′ (nucleotides 4,212 to 4,232) and reverse primer to 5′-TGTTACAGACTCTCACGCACA-3′ (nucleotides 5,819 to 5,840). These M2-specific primers span the M2 intron generating a predicted product size of 499 bp from mRNA and 1,784 bp from viral DNA. The M4 forward primer corresponds to 5′-GCCATAACATACTGGGCAGAAATAC-3′ (nucleotides 9,054 to 9,079) and reverse primer to 5′-CAT-GAATGACAATCTCTGGTACTGG-3′ (nucleotides 9,477 to 9,501) generating a predicted product size of 447 bp. PCR reactions were carried out as follows: 1 cycle at 95°C for 5 min, 30 cycles at 95°C for 1 min, 65°C (54°C for M2) for 1 min, 72°C for 1 min followed by a final extension at 72°C for 10 min. PCR products were analyzed on 1% agarose gels.

In Situ Hybridization. Digoxigenin (Boehringer)-labeled riboprobes corresponding to MHV-68 vtRNAs 1–4 were gener-

ated by T7 transcription of pEH1.4 (32). In situ hybridization was performed as described previously (39). In brief, 5-μm paraffin-embedded sections were dewaxed in xylene, rehydrated through graded ethanol solutions, treated with 100 μg/ml proteinase K for 10 min at 37°C, and acetylated with 0.25% vol/vol acetic anhydride-0.1 M triethanolamine. Sections were hybridized with labeled riboprobes in 50% formamide, 1× SSC overnight at 55°C. The stringent wash (0.1× SSC, 30% formamide, 10 mM Tris, pH 7.5) was carried out at 58°C. Hybridized probe was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer) according to the manufacturer's instructions.

Southern Blot Hybridization Analysis. Spleens were homogenized in complete-GMEM and passed through 140-μm filters to remove stromal tissue debris. Aliquots of 10^7 cells were pelleted, frozen, and later resuspended in 0.5 ml of TE (10 mM Tris, 50 mM EDTA, pH 8), lysed by the addition of SDS to a final concentration of 0.5%, and incubated with proteinase K (50 μg/ml) at 37°C overnight. DNA was purified by phenol/chloroform extraction and ethanol precipitation. 1 and 10 copy/cell reconstructions were prepared by the addition of known amounts of a cloned PstI 1.2-kb repeat fragment (40) to 5 μg of purified BHK-21 cell DNA. For Southern blot analyses, 5 μg of DNA was digested with PstI and electrophoresed through a 0.8% Tris acetate agarose gel. DNA was then transferred to a nitrocellulose membrane and probed with an [α-^{32}P]dCTP random prime-labeled 1.2-kb Pst repeat fragment. Hybridizations were performed at 65°C in 4× SSC, 3× Denhardt's solution, 0.5% SDS, 0.2 mg/ml sonicated salmon sperm DNA, 0.001 M phosphate buffer, and 10% dextran sulfate. Membranes were washed at 65°C three times with 2× SSC, 0.1% SDS followed by two stringent washes in 0.2× SSC, 0.1% SDS at 65°C for 15 min. Filters were then exposed to X-ray film for 7 d before development.

Results

Acute Phase Replication Kinetics of M3-deficient Mutants. The construction of an M3-deficient virus, M3LacZ, that lacks secreted chemokine binding activity has been described (7). To determine the role of M3 in pathogenesis we generated an independent mutant, M3LacZi, and a revertant of M3LacZ, designated M3R. All viruses were free of unrelated genomic rearrangements as determined by restriction enzyme analyses and Southern blot hybridization (data not shown). RT-PCR analyses of infected cell RNA demonstrated that insertion of the HCMV IE LacZ cassette into the M3 gene had no detectable affect on the transcription of the flanking M2 and M4 genes (Fig. 1). Single and multistep growth curves revealed no obvious requirement for M3 in lytic replication in vitro (Fig. 2, a and b). Similarly, no significant deficit in lytic phase replication was observed in vivo after intranasal infection of mice with either M3LacZ and M3LacZi, in comparison to WT MHV-68 from 3 to 7 d after infection (Fig. 3). However, lower levels of virus from the lungs of animals infected with the M3-deficient mutants was evident at 9 d after infection, suggesting that an M3 deficit results in enhanced clearance of lytic virus at late times after infection. Nonetheless, consistent with the relatively normal kinetics of lytic phase respiratory infection observed, flow cytometric analyses of cells
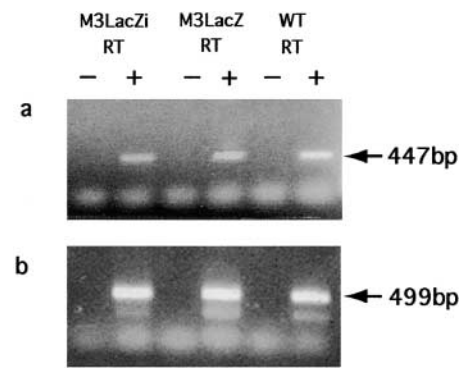

Figure 1. RT-PCR analyses of M2 and M4 transcription during lytic infection. RT-PCR analysis of M4 (a) and M2 (b) transcription in M3 LacZ, M3 LacZi, or WT-infected BHK cells. These data demonstrate that insertion of the CMV LacZ cassette into the M3 gene has no detectable affect on the transcription of the neighboring M2 and M4 gene.

recovered from lungs by bronchioalveolar lavage showed no evidence that an M3 deficit led to an enhanced infiltration of NK cells (DX5$^+$) 3 d after infection or of activated (CD62L^{lo}) CD4$^+$ or CD8$^+$ T cells 9 and 13 d after infec-
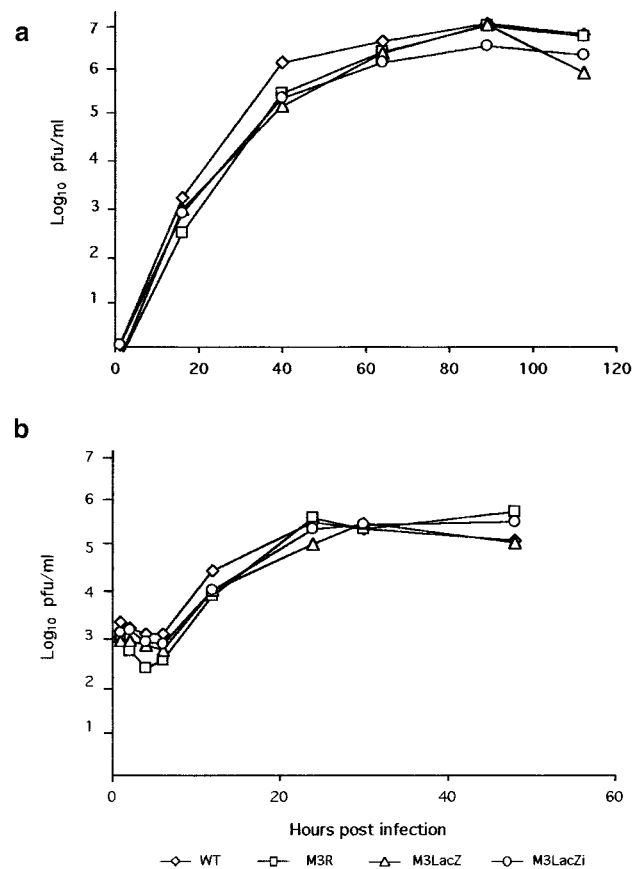
Figure 2. M3-deficient viruses exhibit normal growth kinetics in vitro. (a) Multistep (0.01 PFU/cell) and (b) single-step (5 PFU/cell) growth curves of M3-deficient and WT MHV-68 were performed to determine any growth deficits in vitro. Infected BHK cell monolayers were harvested at the times indicated and virus titers were determined by suspension assay.
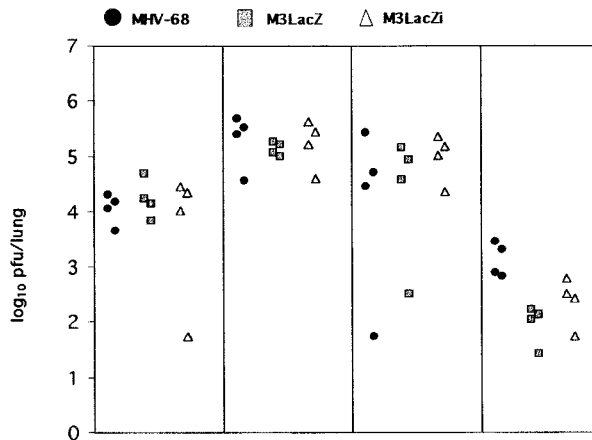
Figure 3. The M3 gene is not required for the initial phase of acute lung infection. After intranasal inoculation with 10^4 PFU of WT MHV-68, M3LacZ, or M3LacZi, four animals were sampled per virus 3, 5, 7, and 9 d after infection. Titers were determined by suspension assay. Each point represents the titer of an individual mouse.

tion (data not shown). Thus, we conclude that M3 plays no essential role in protecting MHV-68 against the immune response during acute, lytic phase replication in the lung.

M3-deficient Mutants Are Defective for the Establishment of Latency. After epithelial infection, MHV-68 establishes a latent infection in lymphoid tissue, colonizing germinal centers (32, 33) and driving antigen–nonspecific B cell activation (34, 35). This is associated with an amplification of latent virus and marks the onset of an infectious mononucleosis–like illness that lasts several weeks before slowly resolving. Previous studies have shown that inhibiting MHV-68 replication in the lung, through vaccination against a lytic phase CD8$^+$ T cell epitope, reduces the initial seeding of virus to the draining MLNs but not the subsequent course of latent virus amplification in either the MLNs or spleen (41). Thus, lytic and latent virus amplification appear to be distinct processes. The M3-deficient MHV-68 mutants replicated relatively normally in the lung and infectious center assays showed little difference between WT and M3-deficient viruses in the extent of MLN colonization 7 d postinfection (pi; Table I, top). However, the subsequent amplification of M3-deficient latent virus was grossly impaired (Table I, top). Restoring a functional M3 gene to M3LacZ (M3R) reversed the deficit and led to normal amplification of latent virus in lymphoid tissues (Table I, bottom).

On the basis that the level of recovery of virus by explant culture is indicative of the level of virus latency, M3 expression appeared unnecessary for the initial seeding of virus to (mediastinal) lymphoid tissue, but essential for its subsequent amplification in the spleen. Monitoring virus-driven B cell activation gave a broadly equivalent picture. At 7 d after infection, B cells (CD19$^+$) recovered from the MLNs were 62.8% activated (CD69$^+$) with WT MHV-68, 45.0% with M3LacZ, 38.4% with M3LacZi, and 54.4% with M3R (samples were pooled from five mice in each case). Thus, while slightly greater activation was observed

Table I.

		MHV-68			M3LacZ			M3LacZi		
Day pi		7	13	21	7	13	21	7	13	21
Spleen	1	<1[a]	1,860	<1	2	<1	<1	<1	<1	<1
	2	3	390	17	<1	<1	<1	<1	<1	<1
	3	13	830	4	<1	<1	<1	<1	<1	<1
	4	7	140	<1	<1	<1	<1	1	<1	<1
	5	5	2,470	17	<1	<1	<1	3	<1	<1
	6	13	880	12	<1	<1	<1	2	<1	<1
MLN		57	2,340	<10	17	<10	<10	64	<10	<10

		MHV-68		M3LacZ		M3LacZi		M3R	
Day pi		9	13	9	13	9	13	9	13
Spleen	1	15	50	3	<1	<1	<1	6	20
	2	24	530	<1	<1	<1	<1	34	20
	3	3	220	<1	<1	<1	<1	4	<1
	4	10	60	<1	<1	<1	<1	<1	100
	5	5	960	<1	<1	<1	<1	15	220
	6	10	600	<1	<1	<1	<1	<1	270
MLN		510	1,390	<10	<10	<10	<10	860	810

Mice were infected intranasally with 4×10^3 PFU of M3LacZ, M3LacZi, or WT MHV-68 (top), or M3LacZ, M3LacZi, WT MHV-68, or M3R (bottom). Spleens were harvested at 7, 13, and 21 d pi (top), 9 and 13 d pi (bottom). MLN samples were pooled from six mice per group prior to assay. The latent virus load was determined in each instance by infectious center assay. Preformed infectious virus was not detected at any time point. At day 13, *t* test showed no significant difference in virus titer between WT MHV-68 and M3R–infected mice ($P > 0.08$).
[a]Infectious centers per 10^7 cells.

with the M3$^+$ viruses, in all cases the level far exceeded that of naive control mice (<3% CD69$^+$), consistent with a fairly normal seeding of M3-deficient virus to the MLNs. In contrast, the levels of splenic B cell activation 13 d pi
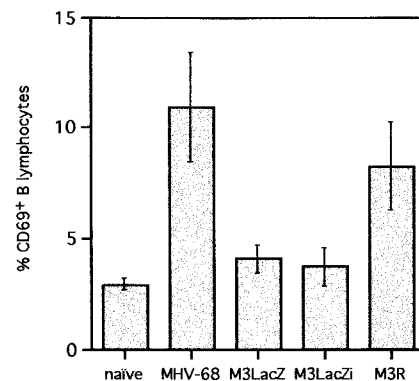

Figure 4. An M3 deficit leads to diminished virus-driven B cell activation. Spleens cells from animals ($n = 6$) infected with the indicated viruses were sampled at 13 d pi and analyzed by flow cytometry to determine the proportion of activated (CD69$^+$) cells in the total CD19$^+$ B cell population. Mean ± SD values from each group of animals are shown.

were considerably less with M3-deficient viruses than with M3+ controls (Fig. 4).

Quantification of Splenic Viral Load by In Situ Hybridization Detection of Viral tRNAs. We next sought to analyze splenic viral colonization by in situ hybridization after intranasal infection of mice with either M3-deficient or WT MHV-68. During MHV-68–induced infectious mononucleosis, the virus expresses high levels of small tRNA-like transcripts in latently infected lymphoid tissue, analogous to the EBV-encoded small RNAs (EBERs) of EBV and URNAs of *Herpesvirus saimiri* (21, 32). Although these vtRNAs are also detectable in sites of lytic infection, they provide a useful means to monitor viral latency in lymphoid germinal centers, where in contrast to epithelial sites no other viral transcripts are detectable (33). To follow the establishment and maintenance of viral latency within the spleen, mice were infected with either WT MHV-68 or M3LacZ, and spleens removed from three animals per group at days 5, 7, 13, and 21 pi were processed for in situ hybridization using a riboprobe specific for vtRNAs 1–4. M3-deficient virus showed evidence of initial lymphoid colonization (day 7), but entry into germinal centers was diminished and the amplification of vtRNA-positive cells seen with WT MHV-68 at day 13 pi failed to occur (Fig. 5). Table II shows the total number and percentage of vtRNA-positive of splenic follicles over time. At 7 d pi little difference in
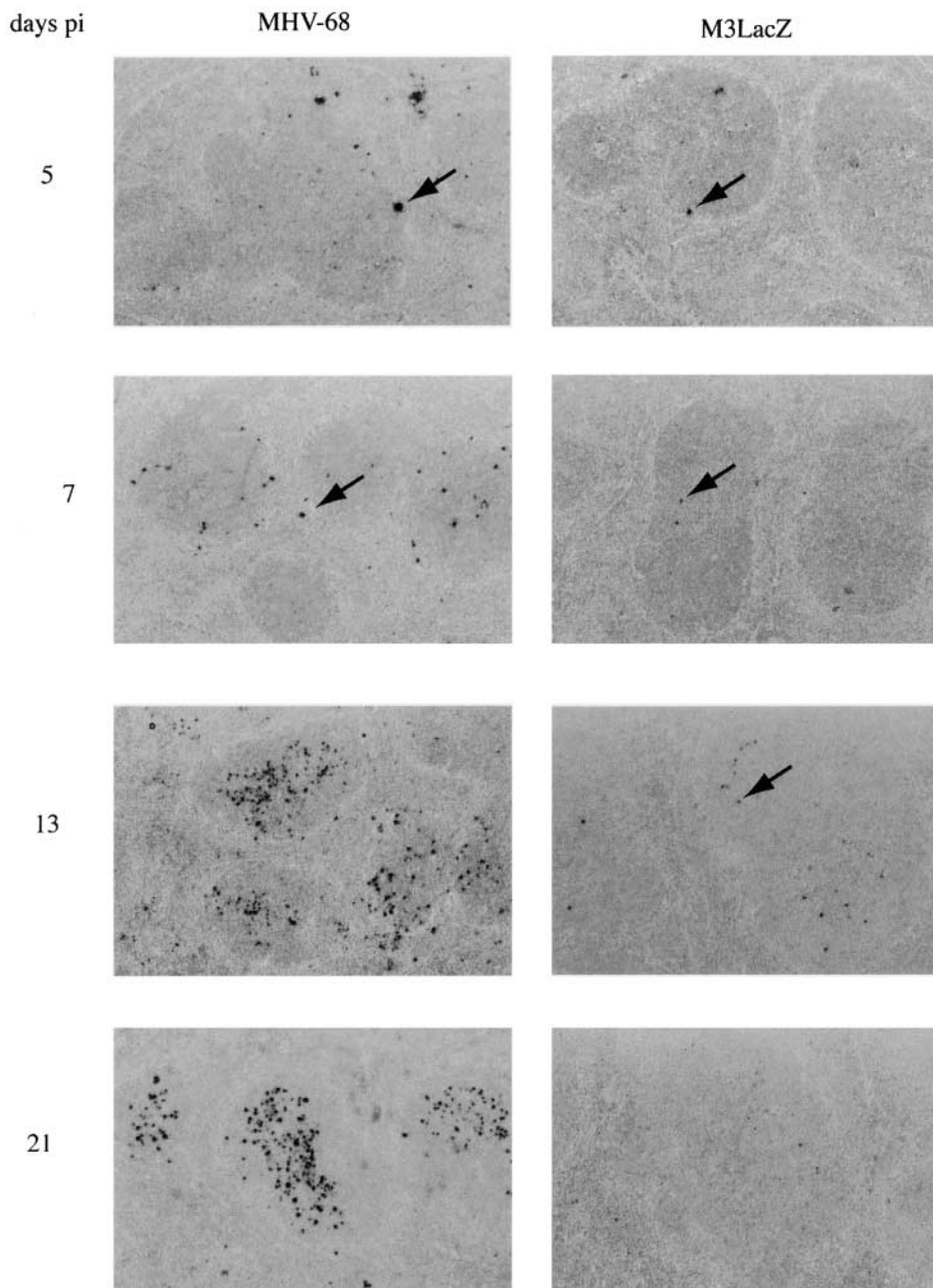
Figure 5. In situ hybridization analyses indicates clearance of M3-deficient virus from the spleen. Spleens from mice infected with WT MHV-68 or M3LacZ were sectioned and probed for vtRNA expression. Shown is a representative panel of spleen sections analyzed for vtRNA expression from each group of animals sampled at 5, 7, 13, and 21 d pi. Arrows indicate vtRNA-positive cells.

Table II.

Day pi	Mouse	MHV-68			M3LacZ		
		Total number of follicles	Number of vtRNA-positive follicles	Mean % of total vtRNA positive	Total number of follicles	Number of vtRNA-positive follicles	Mean % of total vtRNA positive
5	1	75	19		67	0	
	2	36	11	21	83	2	2
	3	73	6		69	3	
7	1	79	14		80	11	
	2	50	16	26	66	7	15
	3	79	22		62	13	
13	1	83	63		83	18	
	2	78	56	70	92	5	13
	3	56	35		102	13	
21	1	87	83		52	0	
	2	111	84	76	58	5	3
	3	91	51		50	0	

Quantitation of viral load by in situ hybridization detection of vtRNAs at 5, 7, 13, and 21 d pi. Similar results to M3LacZ were obtained with M3LacZi (data not shown).

the number of vtRNA–positive splenic follicles was apparent between WT MHV-68 and M3LacZ-infected mice. At later time points (13 and 21 d pi) there was a pronounced decrease in the number of splenic follicles positive for vtRNA expression in animals infected with M3LacZ (equivalent data, not shown, were obtained with M3LacZi) in comparison to WT virus. Similarly, the number of vtRNA-positive cells within individual splenic follicles was little different between WT MHV-68 and M3LacZ-infected mice at 7 d pi, but by 13 d pi, when a massive expansion of vtRNA-positive cells in germinal centers was observed in mice infected with WT MHV-68, the number of vtRNA-positive cells was markedly reduced with the M3LacZ mutant (Table III). This emphasizes that it was the expansion of the latently infected population in germinal centers that was most obviously lacking in the absence of M3. Finally, Southern blot hybridization of viral DNA from spleens sampled at 13 d pi showed a much lower level of M3-deficient virus than WT MHV-68 (Fig. 6, a and b).

Clearance of M3-deficient Virus from the Spleen Is Immune Mediated. The decline in latent load after fairly normal initial seeding suggested that immune clearance of M3-deficient virus from lymphoid tissue was occurring. Thus, we wished to determine whether CD8^{+} T cell depletion could rescue the M3-deficient virus phenotype. Mice were depleted of CD8^{+} T cells from −2 to 12 d pi and latent virus in lymphoid tissue was quantitated by infectious center assay (Fig. 7 a). CD8-depleted mice ($n = 8$) infected with WT-MHV-68 showed a mean 35-fold increase in recover-

Table III.

| Day pi	Mouse	Mean vtRNA^{+} cells/follicle	
		MHV-68	M3LacZ
5	1	11	1.6
	2	6	1.4
	3	1.5	0
7	1	4.6	3.7
	2	3.5	2.2
	3	3.5	1.9
13	1	47	5
	2	17	4.8
	3	12	3.5
21	1	27	1.4
	2	24	0
	3	29	0

Quantitation of viral load by in situ hybridization detection of vtRNAs at 5, 7, 13, and 21 d pi. The mean number of vtRNA-positive cells within individual follicles from mice infected with the indicated virus are shown. Equivalent results to M3LacZ were obtained with M3LacZi (data not shown).
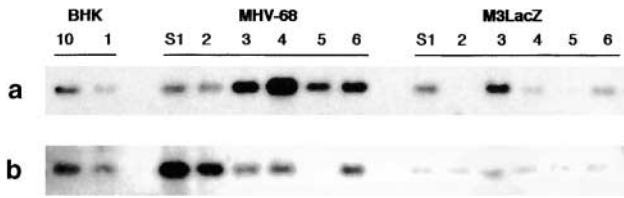
Figure 6. Southern blot analyses of viral DNA load in the spleen. Mice were infected with 4×10^3 PFU of WT MHV-68 or M3LacZ and spleens were removed at 13 d pi. Total spleen DNA was extracted and subjected to Southern blot analyses to determine the viral DNA load. For each splenic DNA sample, 5 μg of high molecular weight DNA was digested with PstI. Restriction-cleaved DNA was transferred to nitrocellulose and probed with a radiolabeled 1.2 kb Pst repeat fragment. 1 and 10 copy/cell reconstructions in uninfected BHK cell DNA are indicated. a and b show two independent experiments.

able splenic virus relative to the undepleted infected controls. In contrast, animals ($n = 6$) infected with either M3LacZ or M3LacZi showed a mean 2,243- and 2,021-fold increase in virus reactivation, respectively. In neither case could the increase in recoverable virus consequent on CD8$^+$ T cell depletion be accounted for by an increase in the amount of preformed infectious virus (Fig. 7 b). Thus, the mean number of infectious centers in WT MHV-68 infected CD8-depleted animals was 19,900/10^7 splenocytes, whereas the mean level of infectious virus detected was 105 PFU/10^7 disrupted splenocytes, that is 0.5% of the plaque numbers generated in the infectious center assay. With the M3-deficient mutants, M3LacZ and M3LacZi, infectious virus accounted for ∼0.3 and 3% of plaques generated in the infectious center assay, respectively. We next determined the affect of CD8 depletion on acute phase virus replication in order to ascertain whether there is any obvious correlation between the extent of virus load in the lung and levels of latent virus in the spleen at 9 and 13 after infection. WT and revertant infectious virus titers in the lungs of both depleted and undepleted mice were relatively high at 9 d after infection (Fig. 7 c), whereas only low levels of latent virus were detected in the spleens of these animals (Fig. 7 d). By day 13, the amount of infectious virus
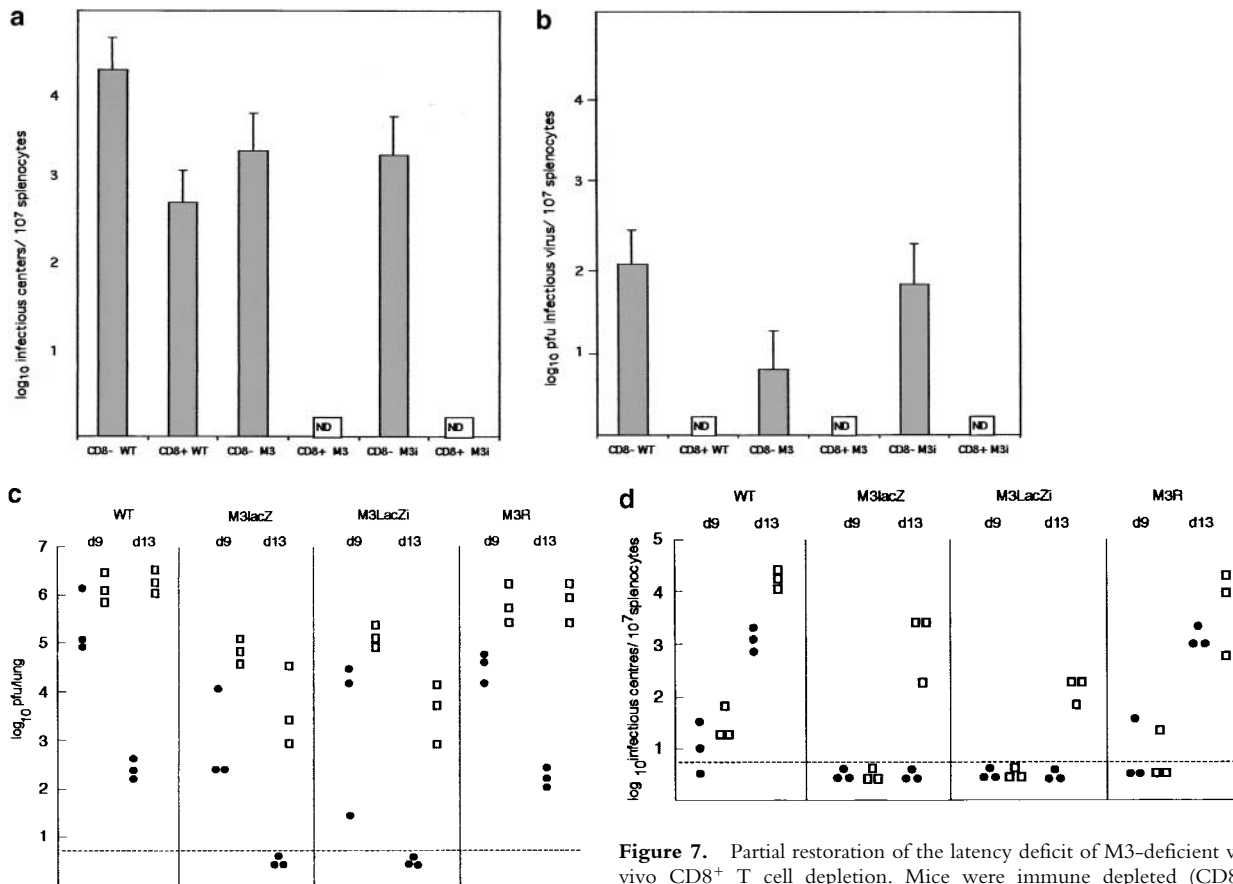

Figure 7. Partial restoration of the latency deficit of M3-deficient viruses by in vivo CD8$^+$ T cell depletion. Mice were immune depleted (CD8−) or not (CD8+) with anti-CD8 mAb starting 2 d before infection with either WT MHV-68 ($n = 8$), M3LacZ (M3; $n = 6$), or M3LacZi (M3i; $n = 6$). Spleens from all mice were harvested at 12 d pi. Infectious center assays were performed with (a) freshly isolated, or (b) freeze-thawed and sonicated spleen cells to distinguish total from preformed infectious virus. Mean ± SEM titers are shown. ND, virus not detected. Note that the log$_{10}$ scale indicates that infectious virus never exceeded 5% of the total recoverable by infectious center assay, implying that even after CD8$^+$ T cell depletion, the vast majority of infected cells harbored latent virus. The differences between CD8− WT and CD8− M3 and CD8− M3i samples were not statistically significant ($P > 0.05$ by t test). Individual lung virus titers from mice either CD8-depleted (□) or not (●) infected with either WT MHV-68, M3LacZ, M3 LacZi, or M3R are shown (c). Latent virus was determined by infectious center assay from the spleens of animals sampled at 9 and 13 d after infection (d). Data points shown below the horizontal dashed line represent tissue samples containing no detectable virus.

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was largely unchanged in the lungs of CD8-depleted mice and considerably reduced in the case of WT and M3R–infected immunocompetent controls. However, in both groups of animals the amount of latent virus had increased considerably. Thus, it would appear that the amplification of latent virus in the spleen was not influenced greatly by the absolute level of infectious virus in the lung. This observation is consistent with previous studies showing relatively normal amplification of latent virus in lymphoid tissue despite an almost complete absence of lytic replication in the lung (26). After CD8 depletion, the M3-deficient viruses showed a decrease in infectious virus titers in the lung but an increased level of latent virus at day 13 relative to the day 9 time point (Fig. 7, c and d). Thus, here too there was evidence of amplification of latent virus, independent to the course of infection in the lung. The lower titers of the M3-deficient viruses in the lungs at day 9 and 13 were consistent with the somewhat more rapid clearance observed previously (Fig. 3).

CD8$^+$ T cell depletion before infection also restored to WT levels the level of splenic B cell activation by M3LacZ virus (Fig. 8). As the immune depletion resulted in a partial restoration of the capacity of M3-deficient mutants to establish latency in the spleen and drive B cell activation, we were interested to determine whether the pattern of vtRNA expression within splenic germinal centers was also restored. To this end, CD8$^+$ T cell–depleted animals were infected with either WT MHV-68 or the M3LacZ mutant and the number of vtRNA-positive splenic follicles evalu-
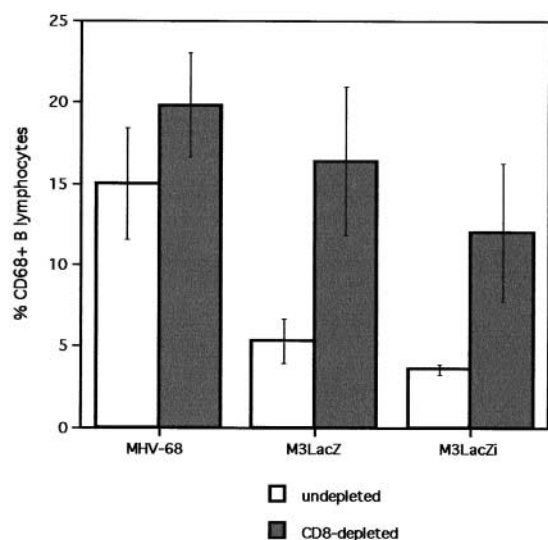
Figure 8. Effect of CD8 depletion on B cell activation mediated by M3-deficient mutants. Dissociated splenocytes from either CD8-depleted or normal animals ($n = 6$) infected with WT MHV-68, M3LacZ, or M3LacZi were sampled at 13 d pi and analyzed by flow cytometry to determine the proportion of activated (CD69$^+$) cells in the total CD19$^+$ B cell population. Columns represent the mean $\pm$ SD of the individual mice within each group. Comparison by t test showed that the difference between WT MHV-68 and M3LacZi groups after CD8$^+$ T cell depletion, although considerably reduced, remained statistically significant ($P < 0.002$); the difference between WT MHV-68 and M3LacZ groups after CD8$^+$ T cell depletion was not significant ($P = 0.12$).

ated at 11 d pi. Consistent with the partial restoration of latency evaluated by explant cocultivation assay (Fig. 7 a), the mean percentage of splenic follicles containing vtRNA-positive cells was 63% in animals infected with M3LacZ and 81% with WT MHV-68. This contrasts with a maximum of 22% of splenic follicles being vtRNA-positive in undepleted mice 7 or 13 d pi with M3-deficient virus (Table II). The restoration of vtRNA-positive follicle numbers by CD8$^+$ T cell depletion correlated with a relatively normal histological distribution of vtRNA-positive cells within individual follicles (Fig. 9). Enumeration of the mean number of vtRNA positive cells/follicle at 11 d pi demonstrated that WT-infected CD8-depleted animals contained 35.3 positive cells/follicle ($n = 3$) while animals infected with the M3-deficient mutant (M3LacZ) contained 15 positive cells/follicle. Thus, after CD8 depletion, the partial restoration of latency establishment by M3-deficient MHV-68 apparent by infectious center assay is mirrored by an expansion of vtRNA-positive cells within the spleen and relatively normal levels of B cell activation.

Discussion

The 44-kD MHV-68 M3 gene product has been shown to encode a potent, broad range chemokine binding protein capable of inhibiting chemokine signaling (7, 9). To determine the role of M3 in viral pathogenesis we have utilized two independently derived viral insertion mutants in which the M3 ORF was disrupted with a LacZ expression cassette. The key observations from these phenotypic studies are that M3-mediated chemokine blockade makes little contribution to initial lytic phase viral replication in the lung, but that it is crucial for the amplification of latent virus in lymphoid tissue.

The fact that acute phase viral replication does not correlate with the efficiency of latency establishment (41) highlights the importance of the latent amplification process in gammaherpesvirus colonization of the host. Here, despite relatively normal lytic replication of M3-deficient mutants in the lung, the amplification of latently infected cells in the lymphoid tissue was grossly defective. The extent of initial seeding to the MLNs does depend on lytic replication in the lung (41), and little difference was observed between WT MHV-68 and the M3-deficient mutants in the extent of MLN latency and B cell activation 7 d pi. This was in complete contrast to the dramatic reduction in amplification of latent M3-deficient virus in the spleen at 13 d pi. There was broad agreement in this finding between four different measures: infectious center assay, vtRNA in situ hybridization, Southern blot quantitation of viral DNA, and the level of B cell activation. Quantitative differences between the different assays in the precise degree of deficit observed due to M3 disruption emphasize that not all cell types and not all forms of viral latency may have been equally affected. Nevertheless, the total latent virus load was considerably reduced by every measure. Probably all forms of viral latency were quantitatively deficient to some degree in the absence of M3.
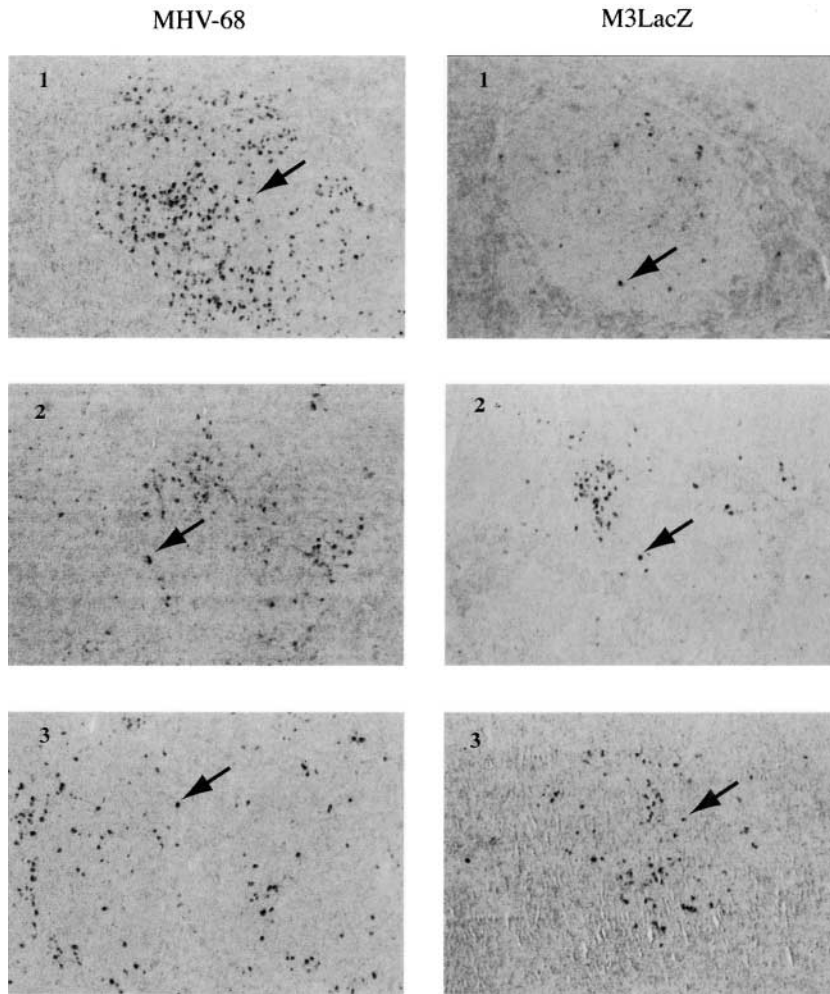
Figure 9. A restoration of M3-deficient virus phenotype in CD8^{+} T cell–depleted animals. CD8^{+} T cell–depleted mice were infected with WT MHV-68 or M3LacZ and spleens were removed for in situ hybridization analysis of vtRNA expression at 11 d pi. Three representative spleen sections are shown for WT MHV-68 or M3LacZ-infected mice. Comparison with the time course illustrated in Fig. 5 illustrates the extent of phenotypic reversion of the M3 deficit achieved by immune depletion. Equivalent results to M3LacZ were obtained with M3LacZi (data not shown).

M3-deficient virus was not essential to establish latency at the level of the individual cell, as there was normal initial seeding of latent virus to the MLNs, and CD8^{+} T cell depletion led to fairly normal latent viral loads. The deficit in latency was instead at the level of the host, reflecting a requirement for immune evasion to increase the number of latently infected cells. Intriguingly, disruption of the homologous M1 gene of MHV-68 via insertion of a CMV LacZ cassette has no obvious effect on latency establishment (19, 21). This suggests that these genes have diverged to fulfil very different roles. We found normal transcription of the M2 and M4 genes after infection with the M3 mutants, as judged by RT-PCR analyses, indicating that insertional mutagenesis of M3 had no detectable effect on transcription of these neighboring genes. Thus, the observed phenotype of the M3-deficient mutants is most likely due to a deficiency in secreted chemokine binding activity and our conclusions are based on the assumption that no other viral functions have been compromised by insertion of the LacZ reporter gene cassette at the M3 locus. We also consider it unlikely that an immune response to β–galactosidase is responsible for the in vivo clearance of the M3LacZ insertion mutants, as earlier work has shown that insertion of the same LacZ cassette in place of the M1 gene, or insertion of this reporter gene cassette into the tRNA 3 locus, has no detectable affect on the latency competence of theses recombinants (21).

The partial reversal in the capacity of M3 mutant viruses to establish latency in the spleen after in vivo CD8^{+} T cell depletion suggests that M3 chemokine blockade protects WT MHV-68–infected splenocytes against elimination by this subset. Although it has been reported that depletion with anti-CD8 antibody may lead to detrimental CD4-T cell responses (42), we consider such detrimental affects to be unlikely in the context of MHV-68 infection. Previous studies have shown that anti-CD8 treatment has no detectable affect on the frequency of CD8^{+} dendritic cells, which may play a role in driving CD4 responses (42), and CD4 T cell immunity appears unaffected after CD8 depletion of MHV-68 infected animals (43). It has also been established that CD4/CD8 double depletion has considerably greater effect than CD8 depletion alone, indicating that CD4^{+} T cell immunity and CD4-dependent virus-specific antibody are not diminished by anti-CD8 treatment (23, 26). Furthermore, the phenotypic reversion achieved by CD8^{+} T cell depletion, namely amplification of latent MHV-68 in lymphoid tissue, is CD4 dependent and does not occur

when the CD4$^+$ T cell response is impaired (30, 44). Nonetheless, as the effect was incomplete, other effectors such as CD4$^+$ T cells and NK cells may also contribute and such effectors may play a more important role in the enhanced clearance of M3-deficient virus from the lungs of infected mice, as there was little evidence of CD8$^+$ T cell depletion reversing the deficit in lytic replication. The implication is that M3 expression inhibits a range of cellular immune effectors to prevent the elimination of latent MHV-68 from lymphoid tissue. The fact that the CD4$^+$ T cell subset also drives latency expansion through the provision of B cell help underlines the potential complexity of lymphocyte interactions within MHV-68–infected lymphoid tissue. In situ hybridization studies have demonstrated that at least quantitatively, M3 transcription occurs primarily in the paracortical areas of splenic follicles. This is maximal early in lymphoid colonization, with M3 transcription downregulated during the expansion of vtRNA$^+$ cells in germinal centers (33). Paradoxically, a lack of M3 expression had little affect on the number of tRNA$^+$ cells in paracortical areas, but a profound effect on vtRNA expression within germinal centers. It may be that M3 protein secreted in paracortical areas of lymphoid follicles allows cells expressing an immunogenic latency program to enter germinal centers where a relatively physiological amplification of activated B cells can occur. A recent report found latent MHV-68 in three spleen cell types — B cells, macrophages, and dendritic cells — after intranasal infection (28). Certainly removing the protection afforded by M3 abrogated the generation of vtRNA-positive germinal center cells. But whether M3 is actually expressed by virus-infected B cells in germinal centers remains to be seen. Analyzing viral gene transcription within these different cell types could be one way forward in understanding exactly how MHV-68 has adapted to life in a chemokine-rich world.

Given the abundant expression of M3 during lytic cycle infection (33, 38, 45), it was surprising that an M3 deficit had little effect on the initial stages of MHV-68 replication in the lung. However, the consistent detection of enhanced clearance of M3-deficient mutants at late times after infection suggests that chemokine neutralization afforded by M3 has a role in optimizing the late stages of acute phase replication. It may be that tissue damage, at least during the initial stages of acute phase replication in the lung, results in such an abundance of chemokines that M3-mediated blockade is overwhelmed. Lytic replication is notably lacking in MHV-68–infected lymphoid tissue, and the lack of tissue damage and consequent innate immune activation in this site may be crucial for successful immune evasion by M3. We have recently described an MHV-68 protein, K3, that blocks antigen presentation to CD8$^+$ T cells by downregulating MHC class I–restricted antigen presentation (46). K3 may be insufficient to escape CD8$^+$ T cell recognition of virus-infected B cells, which in contrast to fibroblasts have high levels of constitutive antigen presentation. In addition, unlike M3, K3 expression is probably restricted to lytic replication and thus may not prevent the recognition of latency-related CTL targets. It is possible that M3 has a greater role during

reactivation from latency. At least initially, this should not generate as much tissue damage as primary infection.

Herpesviruses are generally considered to establish latency as a default program, when immediate early gene expression critical for the initiation of lytic cycle replication is lacking (47). To our knowledge our phenotypic characterization of an M3-deficient virus represents the first description of a viral gene essential for the normal establishment of latency. One implication is that a strategy of immune evasion is critical for the programmed entry of the viral genome into latency. A variety of herpesviruses cause an acute mononucleosis syndrome, probably driven by viral amplification; the KSHV chemokine antagonist, vMIP-II (48, 49), suggests that this virus at least has evolved an independent approach to the same goal as MHV-68. Chemokine blockade may indeed prove to be a common requirement for effective host colonization by lymphotropic herpesviruses. That this might provide a potential target for effective antiviral therapy is reason enough to further investigate M3.

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