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The Absence of M1 Leads to Increased Establishment of Murine Gammaherpesvirus 68 Latency in IgD-Negative B Cells

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The secreted M1 protein of murine gammaherpesvirus 68 (MHV68) promotes effector Vß4⁺ CD8⁺ T cell expansion to impact virus control and immune-mediated pathologies in C57BL/6 mice, but not BALB/c mice. We report a striking increase in the number of genome-positive, IgD⁻ B cells during chronic infection of both mouse strains. This suggests a novel role for M1 in influencing long-term maintenance in a major latency reservoir irrespective of the degree of Vß4⁺ CD8⁺ T cell expansion.

The complex interplay of the virus and host during a chronic herpesvirus infection is often revealed only by analyses of target latency reservoirs in the context of the infected host. The infection of laboratory mice with the genetically tractable murine gammaherpesvirus 68 (MHV68) has provided a pathogenesis model to identify viral and host factors that control latency- and infection-associated pathologies (1–3). Many of these viral factors have homologs in the other gammaherpesviruses, yet others are seemingly unique to MHV68 (4). While the specific molecular mechanisms of some virus-host interactions have diverged as the gammaherpesviruses coevolved with their hosts, unique viral molecules may highlight conserved strategies of immune modulation critical to chronic infections (1, 5). For instance, Epstein-Barr virus (EBV) carries a gene encoding viral interleukin 10 (vIL-10), while MHV68 uses a strategy of upregulating IL-10 via the unique M2 protein to drive B cell proliferation (6, 7).

M1 was first identified as an open reading frame (ORF) that is unique to MHV68, encoding a protein with limited homology to cellular and poxvirus serpins (4). Early functional analyses established M1 as a candidate latency-associated gene product critical to chronic infections (1, 2, 21). This is consistent with earlier reports that M1 is dispensable for acute replication in the spleen following infection with wild-type (WT) MHV68 (8) or control WT MHV68 (γHV68/MHV68 WUMS; ATCC VR1465). Mice were housed in static, microisolator cages with autoclaved water and food in the Yerkes Vivarium or the Whitehead Vivarium of Emory University in accordance with federal and university guidelines. All protocols for animal studies were approved by the Institutional Animal Care and Use Committee of Emory University. As measured by plaque assay of spleen homogenates on NIH 3T12 fibroblast cells as previously described (20), neither M1 null virus had a defect in acute replication in the spleens of infected mice compared to the marker rescue repair virus M1.MR or WT virus at 9 days postinfection (dpi) (Fig. 1A). This is consistent with earlier reports that M1 is dispensable for acute virus replication (8, 21).

M1 transcripts are detected in splenocytes (22, 23), yet the role of M1 in the establishment of latency in the spleen early

Since B cells are required for inducing the Vß4⁺ T cell response (14), M1 might be secreted from infected B cells to promote T cell effector responses. However, the roles of M1 in B cell latency and establishment at early and late times during chronic infection have not been reported. Here, we describe studies aimed at determining the impact of the loss of M1 on the ability of MHV68 to establish latency in B cells and access the major long-term latency reservoir, IgD⁻ B cells (15–17).

The absence of M1 impairs reactivation from the spleen. While the frequency of latently infected B cells observed in the spleen following infection with wild-type (WT) MHV68 is largely independent of the inoculating dose, a 2-log-unit decrease in the amount of virus input can lead to substantially different outcomes upon infection with some mutant viruses (18, 19). To provide a stringent assessment of M1 function during infection, C57BL/6J mice were infected with a low-dose inoculum of 100 PFU by the intraperitoneal route by using M1 null recombinant viruses containing either a 511-bp deletion of the M1 genomic region (M1Δ511) (8) or a stop insertion early in the M1 coding sequence (M1stop) (9) or by using the control M1 marker rescue (M1.MR) virus (8) or control WT MHV68 (γHV68/MHV68 WUMS; ATCC VR1465).
FIG 1 M1 enhances reactivation from the splenocytes of infected C57BL/6J mice. (A) Acute replication in the spleens of C57BL/6 mice 9 days after intraperitoneal (i.p.) inoculation with 100 PFU of the indicated M1 null virus (M1Δ or M1stop) or control virus (M1.MR or WT MHV68). The spleens were harvested from the mice and disrupted, and the titers of the virus were determined on NIH 3T12 fibroblasts. Data are shown as log_{10} titer. Each symbol represents the value for an individual mouse, and the black horizontal bar indicates the geometric mean titer for the group of mice. The limit of detection of this assay is log_{10} 1.7 of 50 PFU/ml of sample homogenate and is shown by the broken line. No significant differences were identified. (B) Frequency of splenocytes harboring viral genomes as an indicator of latency 16 days after i.p. infection. Limiting dilution viral genome PCR analysis was used to determine the frequency of latency in single-cell suspensions of bulk, nonsorted splenocytes and CD19+ B cells from mice infected with 100 PFU of M1Δ (bulk, −1/610; CD19+, −1/805; purity, 98.2% ± 0.8%) and M1.MR (bulk, −1/596; CD19+, −1/507; purity, 98.7% ± 0.3%). (C and D) Frequency of splenocytes reactivating virus 16 dpi. Limiting dilution reactivation analysis was used to determine the frequency of ex vivo reactivation in splenocytes from mice 16 days after i.p. infection with M1Δ (−1/22,320) and M1.MR (−1/5,098) or with M1.stop (−1/7,547) and WT MHV68 (−1/1,684). (E) Frequency of splenocytes harboring viral genomes 43 to 45 dpi. Single-cell suspensions of splenocytes were analyzed by limiting dilution viral genome PCR analysis to determine the frequency of latency in single-cell suspensions of splenocytes from mice infected with M1Δ (−1/2,863), M1.stop (−1/1,646), M1Δ.MR virus (−1/1,723), and WT MHV68 (−1/1,979). (F) Frequency of splenocytes reactivating virus 45 dpi. For both limiting dilution assays, curve fit lines were derived from nonlinear regression analysis, and symbols represent the mean percentage of wells positive for virus (viral DNA or CPE) ± standard error of the mean (SEM). The dotted line represents 63.2%, from which the frequency of viral-genome-positive cells or the frequency of cells reactivating virus was calculated based on the Poisson distribution. For panels C, D, and F, mechanically disrupted cells were plated in parallel, and no significant levels of preformed infectious virus were detected. Latency data for 16 dpi in panel E are the results from two independent experiments with spleen cells pooled from seven or eight mice per group. Reactivation data for 16 dpi in panel C are the results from two (WT and M1.stop) and three (M1Δ and M1.MR) independent experiments with spleen cells pooled from five to eight mice per group. Latency data for 6 weeks in panel E represents one (WT and M1.stop) and two (M1Δ and M1.MR) independent experiments with spleen cells pooled from four to seven mice per experimental group. Reactivation data for 6 weeks in panel F represent a single experiment with spleen cells pooled from four to seven mice per group.
after infection has not been reported. The establishment of latency at the peak of splenic expansion 16 dpi was determined using a single-copy-sensitive, quantitative limiting dilution PCR assay of intact viral-genome-positive splenocytes as previously described (24). The frequency of splenocytes harboring MHV68 was determined by Poisson distribution analysis of the percentage of reactions positive for the genome with decreasing numbers of input cells. The frequency of splenocytes from mice infected with M1Δ was nearly identical to the frequency of MHV68-positive splenocytes in mice infected with M1.MR at 16 dpi (Fig. 1B).

The B cell compartment is the primary reservoir of MHV68 latency upon dissemination to the secondary lymphoid tissues. To verify that the latency compartment was not grossly skewed in the absence of M1, we examined latency in B cells that were isolated from bulk splenocyte suspensions by fluorescence-activated cell sorting (FACS) as previously described (15, 25). Stained cell populations were acquired using a FACSVantage (BD Biosciences) or MoFlo cytometer (Beckman Coulter, Brea, CA) and analyzed using FlowJo software (Treestar, Ashland, OR). The percentage of CD19+ B cells was higher in M1Δ-infected mice (54.7% ± 4.4%) (P = 0.011) than in MR-infected mice (45% ± 5.2%), but this increase was offset by fewer cells per spleen in the M1Δ-infected mice (Table 1). CD19+ B cells were isolated to greater than 95% purity and subjected to limiting dilution PCR. The CD19+ B cell fraction accounted for a large portion of the viral-genome-positive cells in the bulk splenocyte analysis, and there was no significant difference in the frequency of B cells that harbor MHV68 isolated from M1Δ versus M1.MR-infected mice (Fig. 1B). These data indicate that M1 is not essential for the establishment of latency in splenic B cells, consistent with the previous analysis of a mutant that deleted the tRNA-like transcript and M1 (21).

Reactivation from latency at 16 dpi was also examined in the spleens of the infected animals using a limiting dilution explant reactivation assay of intact, live splenocytes as previously described (26). To detect preformed infectious virus, parallel samples of mechanically disrupted cells were plated onto mouse embryo fibroblast (MEF) monolayers. Significant levels of preformed infectious virus were not detected in the disrupted samples, confirming that the cytopathic effect (CPE) observed reflects reactivation from latently infected splenocytes. Notably, there was a 4-fold decrease in virus reactivation from splenocytes recovered from mice infected with M1Δ and M1.stop in comparison to splenocytes from M1.MR (n = 3, P = 0.05) or WT (n = 2) control virus-infected mice, respectively (Fig. 1C and D). This leads to a 6-fold decrease in the total number of reactivating cells in M1Δ-infected animals compared to M1.MR-infected animals (P = 0.04). The reactivation defect was also apparent when purified CD19+ B cells from the M1Δ-infected animals (below the limit of detection) were compared to those isolated from the M1.MR-infected animals (~1/18,991) (data not shown). The establishment of latency and reactivation of virus were also examined in mice 6 weeks after infection, at which time no significant differences were noted (Fig. 1E and F). Virus reactivation from splenocytes 6 weeks postinfection with 100 PFU of M1Δ is consistent with the lack of a significant reactivation phenotype previously reported for M1.lacZ-infected animals 6 weeks postinfection with a higher dose (1,000,000 PFU) of virus (3). This is in sharp contrast to the hyperreactivation phenotype observed in the macrophage latency reservoir observed from 6 weeks onward in mice infected with 100 PFU (9) or 1,000,000 PFU of M1Δ (8). Taken together, M1 influences the maintenance of latency in a cell-type-dependent manner.

The absence of M1 alters long-term carriage of MHV68 in IgD− B cells. MHV68 is detected in multiple B cell subsets, including immature and transitional B cells and mature B cells in both the marginal zone and germinal center of the follicles at the peak of splenic latency between 14 and 18 dpi (17, 23, 27, 28). By 3 months after infection, IgD-negative class-switched memory B cells are the major reservoir for latency (15, 16). M1 transcripts are detected in infected spleens during chronic infection (23), and the maintenance of the M1-driven effector Vβ4+ CD8+ T cells throughout infection suggests that M1 may have long-term effects on the latency reservoir (9). Therefore, we next examined how the loss of M1 impacts MHV68 latency in the IgD− B cell subset late during chronic infection.

Murine B cells were first enriched to >93% purity by depletion of non-B cells using the B cell isolation kit (Miltenyi Biotec, Cologne, Germany) and autoMACS (Miltenyi Biotec) per the manufacturer’s recommendations. Following magnetic-bead separation, the B cell subset of interest was isolated by flow cytometry; the results of a typical isolation of the CD19+ IgD− and CD19+ IgD+ subset of B cells are shown in Fig. 2A. CD19+ IgD− B cells and CD19+ IgD+ B cells were sorted to near purity from the spleens of M1Δ− and M1.MR-infected mice. As previously reported for latency studies in long-term infected mice (15, 16), upon limiting dilution PCR analysis of the CD19+ IgD− and CD19+ IgD+ populations, there was a significant bias for detecting the MHV68 genome in the CD19+ IgD− B cells. The frequency of viral-genome-positive IgD− B cells was approximately 1/1,000 cells in mice infected with the M1.MR control virus at late time points ranging from 3 to 6 months (Fig. 2B and D), while the frequency of viral-genome-positive IgD+ B cells was below the limit of accurate quantitation (Fig. 2C and E). Strikingly, there was a significant 5-fold increase (P = 0.03 by a paired, two-tailed t test) in the frequency of latency establishment in the IgD− B cell compartment of M1Δ-infected mice (~1/326) compared to the M1.MR-infected mice at 3 to 5 months after infection (~1/1,655) (Fig. 2B). This hyperestablishment phenotype in the IgD− B cell compartment was maintained 6 months after infection (Fig. 2D and E). Given the increase in the total number of B cells at late time points, the increased frequency of latency led to an 8-fold larger number of IgD− B cells that harbor MHV68 per animal late during infection in the absence of M1 (Table 1). Thus, the absence of M1 has a dramatic influence on the number of IgD− B cells that harbor latent MHV68 during chronic infection.

Evidence for persistent replication in the lungs in the absence of M1. In the absence of M1, MHV68 is defective in driving Vβ4+ CD8+ effector T cell expansion in C57BL/6 mice, and there is a concomitant failure to control virus reactivation from peritoneal macrophages. The latter is likely directly attributable to a decrease in a CD8+ T cell effector function(s) such as the production of IFN-γ which is known to control MHV68 reactivation from this reservoir (8, 9, 29). Importantly, previous MHV68 pathogenesis studies in immunodeficient mice have provided evidence that ongoing replication in the lung is associated with heightened latency in splenocytes (20, 25), suggesting that persistent replication in distal reservoirs may lead to continual seeding of lymphoid tissues. Given
the increased frequency of MHV68 in the IgD⁺ B cell reservoir, we examined whether persistent virus replication could be detected in the lungs of mice infected with M1 null MHV68 late after infection.

Preformed infectious virus in the lungs was measured by a modified form of the limiting dilution ex vivo reactivation assay, as previously described (20). As expected in WT MHV68-infected animals late during infection, no virus was detected from the lungs of mice harvested at 9 months after intraperitoneal infection with 100 PFU of M1.MR virus (Fig. 3). However, there was a striking, high level of infectious virus detected in the lungs of 6 out of 10 M1Δ-infected animals even at high dilutions of lung lysates (50% of the wells exhibited CPE at a 1:250 dilution). Thus, loss of M1 leads not only to increased virus reactivation from peritoneal macrophages but also leads to persistent virus replication or frequent recrudescence in the lungs of a significant percentage of mice. The fact that virus was not detected in all M1Δ-infected mice raises the possibility that this replication is episodic and linked to an increased frequency of virus reactivation from peritoneal macrophages but also identifies a form of the limiting dilution that is strongly linked to the increased virus reactivation from peritoneal macrophages but also identifies a form of the limiting dilution analysis of unsorted or isolated cell population (numbers are reciprocals). ND, not determined; BLD, below limit of detection.

### Table 1: Frequency of cell populations harboring viral genomes

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mouse strain</th>
<th>Time after infection</th>
<th>Cell population</th>
<th>% Parental cell population</th>
<th>Total no. of cells</th>
<th>Frequency of viral-genome-positive cells (1 in)²</th>
<th>Total no. of genome-positive cells³</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1Δ511</td>
<td>C57BL/6J</td>
<td>16 days</td>
<td>Unsorted</td>
<td>54.7 (4.4)*</td>
<td>1.23E+08 (3.39 e6)</td>
<td>610</td>
<td>201,639</td>
</tr>
<tr>
<td>M1-MR</td>
<td>C57BL/6J</td>
<td>16 days</td>
<td>Unsorted</td>
<td>45 (5.2)*</td>
<td>1.78E+08 (5.58 e7)</td>
<td>596</td>
<td>298,658</td>
</tr>
<tr>
<td>M1Δ511</td>
<td>C57BL/6J</td>
<td>6 wks</td>
<td>Unsorted</td>
<td>7.04E+07 (2.74 e7)</td>
<td>2,863</td>
<td></td>
<td>24,590</td>
</tr>
<tr>
<td>M1-MR</td>
<td>C57BL/6J</td>
<td>6 wks</td>
<td>Unsorted</td>
<td>6.35E+07 (2.47 e7)</td>
<td>1,723</td>
<td></td>
<td>36,854</td>
</tr>
<tr>
<td>M1Δ511</td>
<td>C57BL/6J</td>
<td>3–5 mos</td>
<td>Unsorted</td>
<td>1.24E+08 (6.09 e7)</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M1-MR</td>
<td>C57BL/6J</td>
<td>3–5 mos</td>
<td>Unsorted</td>
<td>1.22E+08 (7.83 e7)</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M1Δ511</td>
<td>C57BL/6J</td>
<td>6 mos</td>
<td>Unsorted</td>
<td>5.55E+07 (5.03 e5)</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M1-MR</td>
<td>C57BL/6J</td>
<td>6 mos</td>
<td>Unsorted</td>
<td>4.47E+07 (2.33 e6)*</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M1Δ511</td>
<td>BALB/cJ</td>
<td>4 mos</td>
<td>Unsorted</td>
<td>1.6E+08 (5.7 e7)</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M1-MR</td>
<td>BALB/cJ</td>
<td>4 mos</td>
<td>Unsorted</td>
<td>1.24E+08 (3.3 e7)</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

² Mean percentage of parental cell population in CD19⁺ B cells of unsorted, bulk splenocytes and IgD⁺ or IgD - CD19⁺ B cells. Standard deviations are shown in the parentheses. For experiments in C57BL/6 mice, data were determined as follows. For the experimental data at 16 dpi, the data are the means of two or three independent experiments with splenocytes pooled from 7 or 8 mice per experimental group. For the experimental data at 6 weeks postinfection, the data are the means of three independent experiments with splenocytes pooled from 4 to 7 mice per experimental group. For the experimental data at 3 to 6 mos postinfection, the data are the means of three independent experiments with splenocytes pooled from 9 to 15 mice per experimental group. For experiments in BALB/c mice 4 months postinfection, the data are from two independent experiments with splenocytes pooled from eight or nine mice per experimental group.
³ Cell numbers were derived from the calculated total number of cells per organ and from the percentage of total cells that each subset represents, as calculated from flow cytometry gating. *, significant difference (P < 0.05 in t test) between M1 mutant and control virus.
⁴ Frequency of genome-positive cells determined by limiting dilution analysis of unsorted or isolated cell population (numbers are reciprocals). ND, not determined; BLD, below limit of detection.
The absence of M1 leads to an increase in latency in IgD⁺ class-switched B cells in C57BL/6J mice. (A) Flow cytometric separation of CD19⁺ IgD⁺ B cells from other B cell subsets in the spleens of mice at late time points after i.p. viral infection. The values in the graphs of unsorted FACS analysis (presort) are the averages of the 4- and 5-month experiments. The values in the graphs of postseparation FACS analysis (postsort) are the mean purities of three experiments ranging 3 to 5 months after infection. Comparable CD19⁺ purities were obtained for the IgD⁺ (99.0% ± 0.3% for M1Δ and 99.6% ± 0.6% for M1.MR) and the IgD⁺ (97.5% ± 1.2% for M1Δ and 97.5% ± 1.2% for M1.MR) for the three experiments at 6 months after infection. (B and C) Frequency of splenocytes harboring viral genomes 3 to 5 months after i.p. viral infection. Limiting dilution viral genome PCR analysis was utilized to determine the frequency of latency from CD19⁺ IgD⁺ splenocytes from mice 3 to 5 months postinfection with M1Δ and M1.MR (1/1326 and 1/1655, respectively; P = 0.03). The frequency of CD19⁺ IgD⁺ B cells positive for MHV68 was below the threshold of accurate quantitation. (D and E) Frequency of splenocytes harboring viral genomes 3 to 5 months after i.p. infection. Limiting dilution viral genome PCR analysis was utilized to determine the frequency of latency from CD19⁺ IgD⁺ splenocytes from mice 6 months postinfection with M1Δ and M1.MR (1/1183 and 1/955, respectively; P = 0.08). The frequency of CD19⁺ IgD⁺ B cells positive for MHV68 was below the threshold of accurate quantitation. Curve fit lines were derived from nonlinear regression analysis, and symbols represent the mean percentages of wells positive for viral DNA ± standard errors of the means (SEMs) (error bars). The dotted line represents 63.2%, from which the frequency of viral-genome-positive cells was calculated based on the Poisson distribution. The data shown represent independent experiments ranging from 3 to 5 months (109, 129, and 150 dpi) and 6 months (195, 200, and 200 dpi) with spleen cells pooled from 9 to 15 mice per experimental group.
going replication in the lungs may reseed B cells to drive higher latency establishment during chronic infection.

Role for M1 in restricting latency in mice lacking a Vβ4+ CD8+ T cell expansion. To test whether the higher latency establishment of M1Δ in IgD+ B cells is dependent upon an effector T cell response to control reactivation from the peritoneum and lungs, we examined viral latency in BALB/c mice, a strain that lacks a potent Vβ4+ CD8+ T cell expansion in response to MHV68 infection (9). IgD+ and IgD+ B cells were sorted to near purity in BALB/c mice 4 months after infection with M1Δ or M1.MR (Fig. 4A). As found for 3- to 6-month infections of C57BL/6 mice, the frequency of IgD+ B cell latency and number of genome-positive IgD+ B cells was increased 8-fold in the absence of M1 in BALB/c mice 4 months after infection with M1Δ or M1.MR (Fig. 4B). MHV68 was not detected in IgD+ B cells (Fig. 4C). This strain-independent phenotype of enhanced establishment of latency in a major reservoir of latency late during

FIG 3 Persistent virus replication in the lungs of C57Bl6/J mice late after infection in the absence of M1. Lung tissue was isolated from mice 9 months after i.p. viral infection with 100 PFU of M1Δ or M1Δ.MR. Bars for M1Δ or M1Δ.MR virus represent the mean percentage (± SEM) of 16 wells positive for cytopathic effect (CPE) upon plating 12 twofold dilutions (1:10 to 1:20,500) of mechanically disrupted lung tissue on an indicator MEF monolayer (the value for the last dilution is not visible) (n.d., not detected). Two sets of pooled five mice were analyzed for M1Δ, and two sets with three and five pooled mice were analyzed for M1.MR.

FIG 4 The absence of M1 leads to an increase in latency in IgD+ class-switched B cells in BALB/cJ mice. (A) Flow cytometric separation of CD19+ IgD+ B cells from other B cell subsets in the spleens of mice 4 months after i.p. viral infection. The purities of unsorted and sorted splenocytes are indicated in each quadrant of the plotted data. (B) Frequency of splenocytes harboring viral genomes. Limiting dilution viral genome PCR analysis was utilized to determine the frequency of latency from CD19+ IgD+ splenocytes from mice 4 months postinfection with M1Δ and M1.MR (~1/272 and ~1/2,143, respectively). (C) CD19+ IgD+ B cells were not found to harbor MHV68. Curve fit lines were derived from nonlinear regression analysis, and symbols represent the mean percentage of wells positive for viral DNA ± standard error of the mean (SEM). The dotted line represents 63.2%, from which the frequency of viral-genome-positive cells was calculated based on the Poisson distribution. The data shown represent two independent experiments with spleen cells pooled from eight or nine mice per experimental group.
infection reveals a novel role for the M1 latency-associated gene of MHV68.

**Summary.** In the absence of M1, the virus life cycle is significantly altered, as evidenced by heightened latency in the class-switched IgD− B cell reservoir and persistent virus replication in the lungs at late times during chronic infection. The increased persistence of virus replication in the lungs of C57BL/6 mice is consistent with previous reports of M1 as an immunomodulatory protein with important consequences for MHV68 pathogenesis in vivo. The ability of M1 to drive Vβ4+ CD8+ T cell expansion via a superantigen-like function and associated CD8+ T cell effector functions such as IFN-γ cytokine production is likely critical for control of reactivation from the peritoneal macrophage compartment in immunocompetent mice and protection from reactivation-associated disease in the IFN-γR−/− fibrosis model (8, 9, 30). Additional data supporting this model is the absence of the M1Δ hyperreactivation phenotype in the peritoneal compartment of BALB/c mice, a strain that lacks the hallmark Vβ4+ CD8+ T cell expansion that is typical of C57BL/6 mice upon infection with WT MHV68 (3).

The increase of latency in the IgD− B cells of both C57BL/6 and BALB/c mouse strains in the absence of M1 suggests that this function is independent of the Vβ4+ CD8+ T cell expansion, calling into question a mechanism whereby virus infection of IgD− B cells is attributable to increased seeding from the peritoneal macrophages upon the loss of the M1/Vβ4+ CD8+ T cell/IFN-γ-immune modulation axis. The loss of M1 may lead to an as yet unidentified disruption in the immune response in addition to the loss of Vβ4+ CD8+ IFN-γ-producing T cells that impacts B cell maturation. Alternatively, M1 may have a direct B cell intrinsic function for maintaining latency. For instance, the MHV68 M2 latency-associated gene alters B cell biology to induce the production of cytokines that promote B cell proliferation. M2 also drives plasma cell differentiation to promote reactivation (7, 31, 32).

The IgD− B cells examined in this study likely represent class-switched memory B cells, based on similar frequencies of latency in the CD19+ IgD− IgG/A/E− class-switched subsets previously reported for animals infected with WT virus (15). However, the increase in genome-positive cells in the absence of M1 could represent an amalgam of multiple B cell types that lack IgD−, including germinal center and memory B cells (15–17), T1 transitional B cells (33), and plasma cells (31). Recent advances in marking infected B cells in vivo will enable B cells infected with WT or mutant viruses to be profiled by surface markers and isolated for analysis of the viral gene expression program (17, 27, 28, 31).

To the best of our knowledge, the downmodulation of latency by M1 is an unexpected and rare, if not unique, observation for a latency-associated herpesvirus gene. The increase in latency in the IgD− reservoir of mice infected with MHV68 in the absence of M1 is consistent with the report of heightened severity of atypical lymphoid hyperplasia in BALB β2-microglobulin knockout mice infected with M1Δ compared to WT and mutant viruses lacking other latency genes (34). The significant alterations in virus latency and persistence upon loss of the M1 latency-associated gene suggest that successful maintenance of chronic infection by a viral latency determinant may prevent virus-associated pathologies, including lymphoproliferation.

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