A Replication-Defective Gammaherpesvirus Efficiently Establishes Long-Term Latency in Macrophages but Not in B Cells In Vivo

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Murine gammaherpesvirus 68 (γHV68 or MHV68) is genetically related to the human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV), providing a useful system for in vivo studies of the virus-host relationship. To begin to address fundamental questions about the mechanisms of the establishment of gammaherpesvirus latency, we previously generated a replication-defective γHV68 lacking the expression of the single-stranded DNA binding protein encoded by orf6. In work presented here, we demonstrate that this mutant virus established a long-term infection in vivo that was molecularly identical to wild-type virus latency. Thus, despite the absence of an acute phase of lytic replication, the mutant virus established a chronic infection in which the viral genome (i) was maintained as an episome and (ii) expressed latency-associated, but not lytic replication-associated, genes. Macrophages purified from mice infected with the replication-defective virus harbored viral genome at a frequency that was nearly identical to that of wild-type γHV68; however, the frequency of B cells harboring viral genome was greatly reduced in the absence of lytic replication. Thus, this replication-defective gammaherpesvirus efficiently established in vivo infection in macrophages that was molecularly indistinguishable from wild-type virus latency. These data point to a critical role for lytic replication or reactivation in the establishment or maintenance of latent infection in B cells.

The gammaherpesviruses establish lifelong infections in their hosts and are associated with the development of numerous types of malignancies. For example, the human gammaherpesvirus Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV) establish asymptomatic chronic infections in most hosts, but they also are associated with the development of Burkitt’s lymphoma, nasopharyngeal carcinoma, Kaposi’s sarcoma, and primary effusion lymphoma. Thus, it is important to understand the critical viral and host determinants that regulate chronic gammaherpesvirus infections.

Sustainable lifelong gammaherpesvirus infection is a function of the delicate counterpoise between (i) the pathogenic and immune evasive strategies of the virus and (ii) the myriad of antiviral tactics employed by the host immune response. Among the multiple pathogenic strategies employed by gammaherpesviruses, the establishment of latency is perhaps the most critical. However, the mechanisms underlying the establishment and subsequent maintenance of latency are not well understood. Several studies have demonstrated roles for specific viral gene products in the regulation of latent infection, but the dynamic interrelatedness of the viral processes of acute lytic replication, latency, reactivation from latency, and persistent replication (7, 12, 31, 32) confound the interpretation of data from such experiments. A potentially more definitive approach is the generation of recombinant viruses that are blocked in their ability to undergo complete lytic replication and reactivation. Such attenuated viruses represent powerful tools to dissect the contribution of individual viral processes and viral factors to latent infection. For example, we and others have demonstrated that, contrary to the most widely held paradigm, lytic replication is not required for the establishment of a chronic infection in vivo. We have previously generated and characterized a mutant γHV68 (γHV68ΔssDNABP) that is unable to undergo the processes of lytic replication and reactivation due to a mutation in the single-stranded DNA binding protein (ssDNABP) encoded by orf6 (31). Despite this restriction, the virus establishes long-term infection even in fully immunocompetent animals, as defined by the stable carriage of the viral genome at 42 days postinoculation (31). Similarly, other groups demonstrated that γHV68 mutated in a protein of unknown function (encoded by orf31) establishes infection in splenocytes following high-dose intraperitoneal inoculation (4) and that γHV68 mutated in a key transactivator of lytic replication (encoded by orf50) establishes infection at a low level in lung B cells following low-dose intranasal administration (18). These studies have used replication-defective viruses to demonstrate that gammaherpesviruses can establish long-term infection in vivo in the absence of lytic replication.

Although the stable carriage of viral genome in host cells is consistent with the conclusion that a replication-defective virus can establish latency in vivo, a careful examination of the molecular state of the viral genome during infection is required for definitive proof, as wild-type gammaherpesviruses are maintained as an episome and express a restricted subset of genes during latency (reviewed in reference 12). Also in question is whether the attenuation of the gene expression pro-
grams required for lytic replication and reactivation alters the establishment of latency in specific cellular compartments that are associated with wild-type virus infection. Similarly to the case for EBV, it is believed that B cells are the primary reservoir for long-term latency of γHV68 infection (6, 27, 35). In the early phases of infection (16 days postinfection), naive, germinal-center, and memory B cells all harbor latent virus (6, 35), although at later stages (3 to 6 months postinfection) latent virus is restricted primarily to isotype-switched memory B cells (5, 35). Like EBV (16, 21, 24) and KSHV (17, 20), γHV68 also latently infects other leukocytes, including macrophages (34) and dendritic cells (3), but the role that the macrophage and dendritic cell latency reservoirs play in the maintenance of chronic infection and viral pathogenesis is not yet understood. The use of replication-defective viruses offers the potential to dissect the role of these individual latency reservoirs in the maintenance of long-term infection.

In the work described here, we sought to determine whether the stable association of the γHV68 ΔssDNABP genome with host cells represented a state of infection that was molecularly similar to latency. Hallmarks of gammaherpesvirus latency include the absence of the production of infectious virus, the maintenance of episomal viral genome in infected cells, and the expression of a restricted subset of viral genes. We demonstrate that the γHV68 ΔssDNABP genome was maintained as an episomal state in infected cells, and that the viral genome was transcriptionally active within regions known to be operational during latent, but not lytic, infection. We further demonstrate that long-term γHV68 ΔssDNABP infection was efficiently established in macrophages but not B cells, pointing to a critical role for lytic replication or reactivation in the maintenance of latent infection in B cells.

MATERIALS AND METHODS

Mice, virus, and cells. C57BL/6j (B6) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were housed and bred in a pathogen-free facility at the Louisiana State University Health Sciences Center in Shreveport in accordance with all federal and university guidelines. Wild-type γHV68 strain WUMS originally was obtained from the American Type Culture Collection (ATCC VR1465). The working γHV68 virus stock was generated on NIH 3T12 cells. γHV68 ΔssDNABP was generated by using bacterial artificial chromosome (BAC) technology and allelic exchange to create a specific mutation in strain WUMS. The stable association of the ssDNABP was generated by using bacterial artificial chromosome (BAC) technology and allelic exchange to create a specific mutation in strain WUMS originally was obtained from the American Type Culture Collection (ATCC VR1465). The working γHV68 virus stock was generated on NIH 3T12 cells. γHV68 ΔssDNABP was generated by using bacterial artificial chromosome (BAC) technology and allelic exchange to create a specific mutation in strain WUMS.

Limiting-dilution nested PCR. The frequency of peritoneal cells harboring wild-type γHV68 or γHV68 ΔssDNABP genomes was determined using a single-copy-sensitive nested PCR analysis of serial dilutions of peritoneal cells, as previously described (30). Briefly, cells were resuspended in isotonic buffer and
subjected to threefold serial dilutions in a background of uninfected RAW 264.7 cells, maintaining a total of 10^4 cells per well. Cells were plated in 12 replicates for each cell dilution. After being plated, cells were subjected to lysis by proteinase K at 56°C for 8 h. Following enzyme inactivation at 95°C, samples were subjected to nested PCR using primers specific for γHV68 orf72 (30). Positive controls of 10, 1, and 0.1 copies of viral DNA and negative controls of uninfected RAW 264.7 cells alone were included on each plate. Reaction products were separated using 2.5% UltraPure agarose (Invitrogen) gels and visualized by ethidium bromide staining.

Statistical analyses. All data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). The frequencies of genome-positive cells were statistically analyzed using the paired Student’s t test. The frequencies of viral genome-positive cells were determined from a nonlinear regression analysis of sigmoidal dose-response best-fit curve data. Based on a Poisson distribution, the frequency at which at least one event is present in a given population occurs at the point at which the regression analysis line intersects 63.2%.

RESULTS

The replication-defective gammaherpesvirus genome is maintained as an episome during long-term in vivo infection. During latent gammaherpesvirus infection, the viral genome circularizes and is maintained as an episome; in contrast, during lytic infection the genome is replicated and packaged in a linear form. Thus, the presence of circular versus linear genomes in cells infected with a herpesvirus correlates with latent or lytic infection, respectively. To determine whether a replication-defective gammaherpesvirus could establish true latent infection in the absence of lytic replication, we directly examined the molecular state of the viral genome during long-term in vivo infection. Gardella gel analysis has been used previously to separate circular and linear herpesvirus genomes from infected cells in vitro (8). A PCR- and Southern blotting-based modification of this procedure greatly enhanced the sensitivity of Gardella analysis, facilitating the visualization of genomes from in vivo samples (1). Using this assay, we determined whether the γHV68ΔssDNABP genome was maintained in cells in an episomal form in vivo during long-term infection, as would be expected during latency. Wild-type B6 mice were infected i.p. with γHV68 or γHV68ΔssDNABP, and 42 to 45 days postinfection peritoneal cells were harvested for analysis. Cells from each sample group were loaded into individual agarose plugs and run on a horizontal agarose gel. Each lane of the gel then was cut into 12 sections, viral DNA was amplified by PCR, and PCR products were subjected to Southern blot analysis to detect viral DNA (Fig. 1A). In samples from mice infected with wild-type γHV68, viral DNA was detected primarily in gel sections 3 and 4, which is consistent with the expected migration of episomal DNA in a Gardella gel. Similarly, in samples from mice infected with γHV68ΔssDNABP, viral DNA was detected primarily in sections 3 and 4. In samples from both γHV68- and γHV68ΔssDNABP-infected mice, an extremely low quantity of signal was detected in sections that correspond with the expected migration of linear DNA. These signals may be indicative of episome breaks that occurred during sample processing, as we have previously noted in control samples (not shown); alternatively, these signals may represent an extremely low level of linear genome present in some infected cells. No signal was detected in samples from peritoneal cells harvested from mock-infected mice. After the in vitro infection of control NIH 3T12 fibroblasts with wild-type γHV68, an infection that results in lytic replication (33), viral DNA was detected in sections 9 and 10, consistent with the expected migration of linear DNA (Fig. 1B).

To quantify the relative number of viral genomes in each Gardella section, we modified this approach to include the use of quantitative real-time PCR analysis. For these experiments, DNA isolated from Gardella gel fractions was amplified using primers specific for γHV68 orf73 (Fig. 1C). Again, both viral genomes were detected almost completely in a circular form in infected cells. As noted above, the low quantity of linear signal detected in peritoneal cells from both γHV68- and γHV68ΔssDNABP-infected mice may be indicative of episome breaks that occurred during sample processing. Thus, these data demonstrate that a gammaherpesvirus deficient in replication es-

![FIG. 1. Replication-defective viral genome was maintained in an episomal state in peritoneal cells during chronic infection. (A) Gardella PCR Southern analysis of in vivo samples. Peritoneal cells were harvested from B6 mice 42 to 45 days after infection with γHV68 or γHV68ΔssDNABP. Harvested cells were subjected to Gardella gel electrophoresis, individual gel lanes were divided into 12 equal sections, and DNA from each section was transferred to PC mixes containing primers specific for γHV68 orf73. PCR products were visualized by Southern blotting using a probe specific for γHV68 orf73. M, marker. Results are representative of three independent experiments. (B) Gardella PCR Southern analysis of lytically infected cells. For a positive control of lytic infection, NIH 3T12 fibroblasts were infected with wild-type γHV68 for 18 h and subjected to Gardella PCR Southern blot analysis, as described above. Results are representative of three independent experiments. (C) Gardella real-time PCR analysis of in vivo samples. Peritoneal cells from γHV68- or γHV68ΔssDNABP-infected mice were subjected to Gardella gel electrophoresis, individual gel lanes were divided into 12 equal sections, and DNA from each section was transferred to real-time PCR mixes containing primers specific for γHV68 orf73. Data points represent the means from three independent experiments (five pooled mice per sample group per experiment) ± standard errors.](http://jvi.asm.org/content/101/7/8502.long)
transcripts were normalized to the levels of cellular GAPDH in reference 12). Although latency gene programs for one hallmark of gammaherpesvirus latency is the expression of a restricted subset of viral genes in infected cells (reviewed for sequences of the viral genomes that encode M2, M11, orf73, orf74, K3, orf50, and M9. For each sample, the relative levels of viral gene transcripts were normalized to the levels of cellular GAPDH transcripts. For each experiment, each reaction was performed in triplicate. Data represent the means from three independent experiments (five pooled mice per sample group per experiment) ± standard errors. (Inset) NIH 3T12 cells were infected with γHV68 for 18 h, and the relative levels of viral gene transcripts were quantified as described for peritoneal cells.

established a long-term infection in peritoneal cells in which the viral genome was maintained as an episome.

The replication-defective gammaherpesvirus genome is transcriptionally active during long-term in vivo infection.

One hallmark of gammaherpesvirus latency is the expression of a restricted subset of viral genes in infected cells (reviewed in reference 12). Although latency gene programs for γHV68 have not yet been fully defined, previous work has demonstrated regions of the γHV68 genome that are transcriptionally active during chronic infection (10, 22, 25, 33), including sequences within M2, M11, orf73, orf74, and K3. To determine whether the episomal configuration of the viral genome conformed with a transcriptional profile of latency, we used quantitative real-time RT-PCR analysis to detect viral transcripts corresponding to various regions of the viral genome (Fig. 2). In samples from mice infected with wild-type γHV68, we detected transcripts corresponding to regions of the genome that are known to be active during latent infection, including transcripts corresponding to M2, M11, and orf73. In contrast, transcripts corresponding to regions of the genome known to be active during lytic replication, including the immediate-early gene orf50 (encoding the latent to lytic transactivator Rta [13, 36]) and the late gene M9 (one of the most abundantly expressed genes during lytic replication [2, 15, 22, 33]), were not detected. Similarly, in samples harvested from mice infected with γHV68ΔssDNABP, amplification products corresponding to M2, M11, and orf73, but not orf50 and M9, were detected at 45 days postinfection. In contrast, in control experiments to detect viral gene expression during productive replication (Fig. 2, inset), extremely high levels of M9 were detected and moderate levels of several latency-associated genes were detected, as has been previously described (2). Notably, little or no signal was detected in RNA samples that were processed in the absence of RT, demonstrating that the detection of amplimers was not due to DNA contamination. Thus, the γHV68ΔssDNABP genome was transcriptionally active during long-term in vivo infection and displayed a pattern of gene expression identical to the pattern observed during wild-type γHV68 latency.

The replication-defective gammaherpesvirus establishes long-term infection in macrophages but not B cells. The primary reservoirs of γHV68 latency include B cells (27), macrophages (34), and dendritic cells (3). As the peritoneum is a rich source of both B cells and macrophages, we sought to determine whether γHV68ΔssDNABP efficiently established long-term infection in both of these cellular reservoirs. We first examined whether γHV68ΔssDNABP infection altered the cellular composition of the peritoneal cavity, as has been reported for wild-type γHV68 (34). Forty-five days after γHV68ΔssDNABP infection, the number of peritoneal cells (4.62 × 10⁶ cells/mouse) was increased 31% (Fig. 3A) compared to that of mock-infected animals (3.52 × 10⁶ cells/mouse) was increased 31% (Fig. 3A) compared to that of mock-infected animals (3.52 × 10⁶ cells/mouse), slightly less than the 40% increase in cells observed after wild-type γHV68 infection (4.94 × 10⁶ cells/mouse). A flow-cytometric analysis of these peritoneal cell compositions (Fig. 3B) indicated that in naïve or mock-infected mice, 36.5% of the cells were B cells (CD19⁺/CD11b⁻/CD11c⁻) and 41.7% were macrophages (CD19⁻/CD11b⁺/CD11c⁻). Infection with wild-type γHV68 resulted in a significant decrease in the percentage of B cells (to 17.0%) but little change in the percentage of macrophages (44.8%), results that are consistent with a previous report (34). In contrast to infection with wild-type virus, infection with γHV68ΔssDNABP resulted in little change to either cell population (37.2% B cells, 40.7% macrophages).
Consistent with these findings, the Wright stain analysis of peritoneal cell cytopsins indicated little change in overall macrophage or lymphocyte populations (Fig. 3C and data not shown). Thus, infection with γHV68.ΔssDNABP does not significantly alter the cellular composition of the peritoneal cavity and, unlike wild-type γHV68, does not result in a reduction of B cells at this site.

We next sought to determine whether B cells or macrophages harbored viral genome after long-term infection. Forty-five days after infection with wild-type γHV68 or γHV68. ΔssDNABP, peritoneal cells were harvested and subjected to three-color flow-cytometric sorting to isolate CD19⁺ CD11b⁻⁻ CD11c⁻ B cells and CD19⁻ CD11b⁺ CD11c⁻ macrophages. After isolation, the frequency of cells harboring viral genomes in each population was quantified using limiting-dilution nested PCR analysis (Fig. 4). Following wild-type γHV68 infection, a high frequency of both CD19⁺ CD11b⁻⁻ CD11c⁻ macrophages and CD19⁻ CD11b⁺ CD11c⁻ B cells harbored the viral genome (1/690 and 1/520, respectively). A similar frequency of macrophages harbored the viral genome (1/860) after γHV68.ΔssDNABP infection. In contrast, though the frequency of B cells harboring the γHV68. ΔssDNABP genome (~1/84,500) was reduced approximately 290-fold compared to that of wild-type virus. In mock-sorted infected control samples, the frequency of total peritoneal cells harboring the γHV68.ΔssDNABP genome (1/2,690) was three-fold lower than the frequency of cells harboring the wild-type γHV68 genome (1/890), which is consistent with our previously published results on bulk peritoneal cells populations (31). The lack of B-cell infection by γHV68.ΔssDNABP was not due to the incorporation of the BAC cassette in this virus, since wild-type γHV68 containing the BAC cassette established normal infections in B cells (Fig. 5). Furthermore, Cre recombinase-mediated removal of the BAC sequence from γHV68. ΔssDNABP did not enhance B-cell infection (data not shown). Taken together, these results demonstrate that γHV68. ΔssDNABP efficiently established infection in macrophages but not B cells.

To confirm that macrophages indeed harbor the γHV68. ΔssDNABP genome, we performed similar experiments using four-color flow cytometric sorting using F4/80 as a definitive marker for tissue macrophages and Gr1 as a marker for neutrophils. Using this strategy, peritoneal cells were sorted 45 days postinfection into CD19⁺ CD11b⁻⁻ CD11c⁻, and macrophages were identified as CD19⁻ CD11b⁻⁻ CD11c⁻, and macrophages were identified as CD19⁻ CD11b⁻⁻ CD11c⁻, and macrophages were identified as CD19⁻ CD11b⁻⁻ CD11c⁻, and macrophages were identified as CD19⁻ CD11b⁻⁻ CD11c⁻, and macrophages were identified as CD19⁻ CD11b⁻⁻ CD11c⁻. Data from four to six independent experiments (5 pooled mice per mock group or 13 to 15 pooled mice per virus-infected group per experiment) ± standard errors.

**FIG. 3.** Cellular composition of the peritoneal cavity was not altered by replication-defective virus infection. (A) Total numbers of peritoneal cells harvested from naïve or mock-infected mice ( naïve/mock) or mice infected with γHV68 or γHV68.ΔssDNABP for 42 to 45 days. Data represent the means ± standard errors from four to six independent experiments. *, *P = 0.01* compared to results for mock infection. **P = 0.003** compared to results for mock infection. (B) Flow-cytometric analysis of the percentage of B cells and macrophages in the peritoneum 42 to 45 days postinfection. Cells were stained with anti-CD19 (PerCP/Cy5.5), anti-CD11b (R-PE), and anti-CD11c (PE-Cy7). B cells were identified as CD19⁺ CD11b⁻⁻ CD11c⁻, and macrophages were identified as CD19⁻ CD11b⁻⁻ CD11c⁻. Data represent the means from four to six independent experiments (5 pooled mice per mock group or 13 to 15 pooled mice per virus-infected group per experiment) ± standard errors. ***, *P = 0.0007** compared to results for mock infection. (C) Wright staining of cytospin preparations from peritoneal cells. Closed arrows indicate lymphocytes. Open arrows indicate macrophages. Magnification, ×630. Data are representative of three independent experiments.
when the results are translated into absolute cell numbers (Table 1), taking into account the total cell numbers in the peritoneal cavity (derived from data shown in Fig. 3A), the percentage of each cell type in the overall population (derived from data shown in Fig. 3B), and the frequency of cells harboring viral genome (derived from data shown in Fig. 4 and 6). While the absolute number of macrophages harboring viral genomes in each population is similar, the number of B cells harboring the \( \gamma \)HV68.\( \Delta \)ssDNABP genome is reduced more than 75-fold (78-fold in three-color sorting experiments and 145-fold in four-color experiments). Taken together, these data demonstrate that this replication-defective gammaherpesvirus can establish long-term infection in macrophages at a level similar to that of wild-type virus, but that the replication-defective virus is significantly impaired in its ability to establish long-term infection in B cells.

To confirm that the \( \gamma \)HV68.\( \Delta \)ssDNABP infection of macrophages is consistent with the establishment of latency, we examined the state of the viral genome in the sorted macrophage populations 45 days postinfection using real-time Gardella PCR analysis (Fig. 7). Similarly to the bulk peritoneal cell population, in sorted macrophages the \( \gamma \)HV68 and \( \gamma \)HV68.\( \Delta \)ssDNABP genomes were detected at positions that were consistent with the positions observed for episomes. Thus, \( \gamma \)HV68.\( \Delta \)ssDNABP establishes long-term infection in macrophages and maintains the viral genome in a molecular state that is identical to that observed during wild-type virus latency.

**DISCUSSION**

We have previously shown that a murine gammaherpesvirus deficient in the expression of the ssDNABP is replication defective yet establishes long-term infection in peritoneal cells at a level nearly equivalent to that of wild-type virus (31). However, these results did not conclusively demonstrate whether this infection represented a true form of latency. In the work described here, we extended the previous findings by demonstrating that long-term \( \gamma \)HV68.\( \Delta \)ssDNABP infection indeed displayed two important hallmarks of latency: (i) maintenance of the genome as an episome and (ii) transcriptional activity at regions of the genome known to be active during wild-type virus latency. Thus, these data clearly demonstrated that a replication-defective gammaherpesvirus established latency in vivo and that acute replication was not an absolute requirement for the naissance of latent infection. Notably, lytic replication (or specific viral proteins that are expressed during lytic replication) was differentially required for the establishment and/or maintenance of latent infection in different cell types. Although \( \gamma \)HV68.\( \Delta \)ssDNABP established latency in macro-
phages at a frequency equivalent to that of wild-type γHV68, it was severely impaired in the establishment of latency in B cells. Thus, these data point to a fundamental difference in the mechanism by which γHV68 latency is established in macrophages versus B cells.

Little is known about the physiology of macrophage infection by gammaherpesviruses, including the mechanisms used by these viruses to access and maintain latency in this cellular compartment. On the other hand, a plethora of detailed analyses of EBV-infected human peripheral blood cells and tumor cells has laid the groundwork for models that address the establishment of latency in the B-cell compartment. The current paradigm is that EBV commandeers normal B-cell signaling pathways to activate the differentiation program of naïve B cells, thus driving the establishment of latency in a pool of long-lived circulating memory B cells (28). In support of a related mechanism for γHV68, B-cell proliferation is required for the efficient establishment of latency in the memory B-cell compartment (19), although it is not yet clear whether the virus itself intrinsically drives the proliferation of infected cells or whether the virus instead preferentially infects cells that are driven to proliferate by virus- or host-derived extrinsic factors. In support of the latter hypothesis, γHV68-driven B-cell proliferation is not restricted to infected cells (19), and several reports have described a polyclonal activation of B cells during γHV68 infection (23, 26). Although we have not yet examined B-cell proliferation in γHV68ΔssDNABP-infected animals, it...
is noteworthy that the percentage of B cells in the peritoneum is significantly reduced following \( \gamma \)HV68, but not \( \gamma \)HV68. \( \Delta \)ssDNABP, infection, pointing to a pathophysiological B-cell response that is lacking in the absence of lytic replication. In accordance with the EBV paradigm, it is conceivable that the virus enters a subset of naive or germinal-center B cells but that the pool of infected B cells does not undergo proliferation and expansion, presumably as a direct consequence of the inability of the virus to express protein products that are critical to activate the infected cells. It will be of great interest to determine what specific role the virus has in driving B-cell proliferation, whether the mechanism is intrinsic or extrinsic, and whether this activation mechanism is utilized by the virus in order to gain access to the B-cell compartment in vivo.

Regardless of these experimental outcomes, our data implicate a critical role for specific viral factors produced during lytic replication/reactivation in the establishment of \( \gamma \)HV68 latency in B cells. Although it is formally possible that the ssDNABP itself directly plays a crucial role in B-cell latency establishment, we hypothesize that the critical factor(s) is expressed downstream of the ssDNABP in the highly coordinated lytic gene expression cascade. In support of the hypothesis that specific viral factors, rather than infectious virions per se, are critical for B-cell latency establishment, a recent report demonstrated that a replication-defective \( \gamma \)HV68 that is mutated in the late gene \( orf31 \) establishes normal latency in B cells (11). This observation contrasts with our results that were derived by using an early gene replication-defective \( \gamma \)HV68 mutant, and it appears to rule out a role for the production of progeny virions in facilitating the establishment of latency in B cells. Moreover, these results suggest that a viral factor produced in the lytic replication cascade downstream of ssDNABP expression facilitates the establishment of infection in B cells.

An alternative, but not mutually exclusive, hypothesis to explain our results is that \( \gamma \)HV68,\( \Delta \)ssDNABP infects B cells at early times, but that cells harboring viral genomes are not maintained because they undergo natural turnover or virus-induced cell death. A loss of virus from natural turnover is unlikely within this time frame, because the half-life of most mature peripheral B cells is believed to be significantly longer than the course of these experiments (9). At this time, we cannot rule out the virus-induced death of infected B cells, as this would likely occur at a level below our limit of detection. However, if cell death was the primary obstacle to maintaining long-term latency in B cells, we would expect to observe a reduction in infected B cells over time; this is not the case, though, as the frequency of infected B cells does not change significantly over the course of infection (data not shown).

Although no detailed study of the peritoneal B-cell subsets infected by wild-type \( \gamma \)HV68 has been reported, it should be noted that the B-cell population at this site contains a high number of B1 cells, which are believed to be a self-renewing population of innate-like B cells (14). Thus, the paradigms that apply to the infection of mature B2 cells in secondary lymphoid organs may not necessarily apply to some peritoneal B cells. Nonetheless, our data are consistent with the conclusion that long-term latent \( \gamma \)HV68 infection of peritoneal B cells requires the production of proteins that are expressed during lytic replication or reactivation. Of note, another study has demonstrated the infection of lung B cells following the administration of \( \gamma \)HV68 deficient in the expression of critical immediate-early transactivator encoded by \( orf50 \) (18), and a second study has demonstrated the infection of splenocytes (a primary reservoir of B cells) following the administration of \( \gamma \)HV68 deficient in the late gene \( orf31 \) (4). Thus, it will be of great interest to determine whether the inability of \( \gamma \)HV68, \( \Delta \)ssDNABP to establish latency in peritoneal B cells is a function of the peritoneal microenvironment, the specific B-cell subsets that reside in the peritoneal cavity, or the genes expressed by this specific virus during in vivo infection.

The results presented here demonstrate that a gammaherpesvirus can efficiently establish long-term latent infection in vivo in the absence of lytic replication. The maintenance of this infection was attributable primarily to the establishment of latency in macrophages, as the infection of B cells was extremely limited. Thus, this work outlines a fundamental role for viral lytic replication factors in the establishment of B-cell latency. The use of this and other replication-defective mutants of \( \gamma \)HV68 should greatly facilitate future studies directed toward defining the mechanisms used by gammaherpesviruses to establish chronic infection in B cells.

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