γ-Herpesvirus Latency Is Preferentially Maintained in Splenic Germinal Center and Memory B Cells

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Abstract

The γ-herpesviruses are oncogenic B cell lymphotrophic viruses that establish life-long latency in the host. Murine γ-herpesvirus 68 (MHV-68) infection of mice represents a unique system for analyzing γ-herpesvirus latency in splenic B cells at different stages of infection. After intranasal infection with MHV-68 we analyzed the establishment of latency 14 days after infection, and the maintenance of latency 3 months after infection in different purified subpopulations of B cells in the spleen. The data show that MHV-68 latency is mainly established in germinal center B cells and that long-term latency is preferentially maintained in two different subsets of isotype-switched B cells, germinal center and memory B cells. Cell cycle analysis indicates that MHV-68 is located in both cycling and resting isotype-switched B cells. Analysis of viral gene expression showed that both lytic and latent viral transcripts were differentially expressed in germinal center and memory B cells during long-term latency. Together, these observations suggested that γ-herpesviruses exploit the B cell life cycle in the spleen.

Key words: murine herpesvirus 68 • gammaherpesvirinae • viral latency • B lymphocytes • germinal centers

Introduction

The human γ-herpesviruses, EBV and Kaposi's sarcoma-associated herpesvirus (KSHV),* are persistent oncogenic viruses that are maintained in a quiescent state in immunocompetent individuals. Immunosuppression, however, is frequently associated with virus reactivation and the development of malignancies. It is therefore important to understand how these B lymphotrophic viruses establish and maintain a persistent infection in the immunocompetent host. Initial infection by EBV polyclonally activates B cells, yet long-term latency is maintained in resting B cells. It has been suggested that the virus exploits normal B cell biology by gaining entry into the long-lived, quiescent memory B cell compartment through germinal center reactions (1–4). Due to the generally asymptomatic characteristics of the initial infection in man, and the stringent species specificity of the virus precluding the use of small animal models, the establishment of EBV latency during the early stages of infection cannot be directly assessed in vivo. Little is known about the establishment and maintenance of KSHV latency.

Murine γ-herpesvirus 68 (MHV-68), a γ2-herpesvirus, is structurally and biologically similar to the human γ-herpesviruses and provides an important experimental in vivo model of γ-herpesvirus infection in the natural host (5–9). Intranasal infection of mice with MHV-68 causes an acute respiratory infection that is rapidly resolved, followed by the establishment of splenic latency, which peaks 14 d after infection (10). Analogous to the human γ-herpesviruses, B cells are polyclonally activated and a major reservoir of latent MHV-68 (11–14). B cell activation and the development and maintenance of germinal center and memory B cells can readily be studied in the murine spleen.

In these studies, we have phenotypically characterized viral reservoirs in splenic B cells during the establishment and maintenance phases of latency, 14 d and 3 mo after intranasal infection. The data show that viral latency is established at extremely high frequencies in germinal center B cells and is maintained long-term in two subsets of class-switched B cells, memory and germinal center B cells. Analysis of viral gene transcription shows the expression of multiple viral transcripts in memory and germinal center B cells during latent infection, consistent with a complex interaction between the virus and the host. Our data analyzing long-term MHV-68 latency in the murine spleen suggest that the overall strategy for maintaining latency by taking advantage of the normal B cell biology and

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*Abbreviations used in this paper: CPE, cytopathic effect; KSHV, Kaposi's sarcoma-associated herpesvirus; LDA, limiting dilution analysis; MHV-68, murine γ-herpesvirus 68; ORF, open reading frame; PNA, peanut agglutinin.

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gaining entry into the memory B cell pool is shared by the γ-herpesviruses.

Materials and Methods

Virus and Mice. MHV-68, clone WUMS (15), was propagated and titered on monolayers of NIH-3T3 fibroblasts (CRL1568; American Type Culture Collection). C57BL/6J (B6) mice were purchased from Taconic Farms and housed in BL3 containment. The Institutional Animal Care and Use Committee at the Trudeau Institute approved all studies described here. Mice were anesthetized with 2,2,2 tribromoethanol and inoculated intranasally with 400 PFU of MHV-68 in 30 μl PBS.

Cell Staining and Purification. Isolation of spleen cell populations was done using a FACSVantage™ SE/Diva sorter (Becton Dickinson) and antibodies were obtained from BD Biosciences. Pooled splenocytes from five to seven mice were used in all experiments. To isolate B cells, spleen cells were incubated with Fc-block (2.4G2) and then stained with CD11c (HL3), CD11b (M1/70), CD3 (17A2), and B220 (RA3–6B2). B cells were sorted as $B220^+$ and negative for the other markers. To isolate activated and resting B cells 14 d after infection, T cells were magnetically depleted with beads (Dynal) after incubation with anti–Thy-1.2 supernatant (AT83). Remaining cells were incubated with Fc-block and stained with peanut agglutinin (PNA; Sigma-Aldrich), CD11b, CD11c, CD3, and B220. Activated B cells were sorted as $B220^+$ PNA^{high} and resting B cells as $B220^+$ PNA^{low}, and negative for all other markers (14). To isolate naive germinal center and memory B cells 3 mo after infection, spleen cells were incubated with Fc-block and stained with IgD (11-26c.2a), IgM (R6-60.2), CD38 (90), CD5 (53-7.3), and a cocktail of antibodies to detect isotype switching (IgA [C10-1], IgG_1 [A85-1], IgG_{2ab} [R2-40], and IgG_3 [R40-82]). $CD5^+$ cells were gated out to avoid T and B-1 cell contamination. Naive B cells were sorted as non-isotype-switched IgD/M^+, germinal center B cells as isotype-switched IgD/M^-CD38^{low}, and memory B cells as isotype-switched IgD/M^-CD38^{high}. Anti-Fas (Jo2) was used to characterize germinal center B cells.

Cell cycle analysis was performed on isotype-switched B cells ($CD19^+$ IgA^+ IgG_1^+ IgG_{2ab}^+ IgG_3^+ IgD/M^-) 3 mo after infection. After surface staining, cells were permeabilized with 0.05% saponin and DNA was stained by adding 10 μg/ml Hoechst 33342 (Sigma-Aldrich) to the buffer 30 min before the sorting. Cells were sorted after the exclusion of doublets and the postsorting purity of the G_0+G_1 population was 99%. The cell cycle analysis was performed using FloJo® (Tree Star, Inc.) and according to the mathematical model of Watson et al. (16) without applying any parameter constraints.

Limiting Dilution-nested PCR. The frequency of cells containing the MHV-68 genome was determined by a combination of limiting dilution analysis (LDA) and nested PCR (17, 18). Sorted cells were serially diluted in uninfected NIH-3T3 fibroblasts in 96-well plates, lysed, and DNA amplified by PCR as previously described (19) using primers specific for MHV-68 ORF50. A 2-μl aliquot of the product was then reamplified using nested primers and the final product was analyzed by ethidium bromide staining of DNA after electrophoresis in a 3% agarose gel. This procedure was able to consistently detect a single copy of the target sequence. 12 replicates were assessed for each cell dilution and linear regression analysis was performed to determine the reciprocal frequency (95% degree of confidence) of cells positive for MHV-68 DNA. As controls of nested PCR, 10^4 NIH-3T3 cells per well with and without plasmid DNA containing the MHV-68 ORF 50 gene were included in each 96-well plate. At least three independent experiments were used to determine the mean reciprocal frequency and standard deviation of MHV-68 DNA^+ cells in each sorted cell population.

Virus LDA. LDA of infectious virus titer was performed as previously described (14) with minor modifications. In brief, the presence of lytic virus was determined by analysis of the total cell lysate of the FACS®-purified spleen populations after a single cycle of freeze/thawing. Serial dilutions of the cell equivalents were plated onto murine embryonic fibroblast monolayers (1.5×10^4/well) in flat-bottom 96-well plates. 24 wells were plated for each dilution and the number of wells exhibiting cytopathic effect (CPE) was counted after 3 wk of culture. Control experiments have shown that the limit of detection of the infectious virus-LDA assay is 1 PFU in 10^6 cells. One cycle of freeze/thawing has a <20% reduction in lytic virus titers (20). Therefore, our limit of detection is 1.2 PFU in 10^6 cells.

Gene Expression Analysis. The gene expression analysis was adapted from human and murine γ-herpesvirus gene expression studies (19, 21). Purified cell populations were aliquoted in 10^5 cells/aliquot and total RNA free of DNA was isolated by extraction with RNAqueous-4PCR (Ambion) according to the manufacturer's guidelines. cDNA was synthesized from total RNA using M-MLV RT (GIBCO BRL) and random hexamers (Roche). For each experiment, we combined cDNA from three independent cell purifications, each one containing seven mice, to increase the likelihood of detecting transcripts that may be sporadically expressed. This allowed RT-PCR to be performed for all genes from one cDNA pool. 10-μl aliquots of cDNA, each one containing cDNA from ~12 latently infected cells, were then amplified by PCR in a 25-μl reaction containing primers (6.25 μmol each) specific for MHV-68 open reading frames (ORFs) M1, M2, M3, M9, M11, 50, 72, 73, 74, and K3 (19), 1 U TaKaRa Ex Taq (Takara Biomedicals), 2.5 μl 10× Ex Taq buffer (Takara Biomedicals), and 3 μl dNTP mixture (Takara Biomedicals). Amplification was for 45 cycles (94°C for 60 s; 67°C for 60 s; and 72°C for 30 s) followed by a 7-min extension at 72°C. A 2-μl aliquot was then reamplified using a set of gene-specific nested primers (19) for 30 cycles. Negative control reactions for each sample lacked RT. Reactions were included to amplify the mouse β actin mRNA as a control for RNA quality and they were performed for 30 cycles. Amplified DNA was then analyzed by gel electrophoresis. The sensitivity of our RT-PCR assay is such that we can detect M2 expression in a single latently MHV-68–infected S11 B cell diluted in 10^5 noninfected 3T3 cells (unpublished data). The high frequencies of latently infected cells in the purified populations of B cells (see Table II) were well within the predicted sensitivity of the nested RT-PCR assays for viral gene transcription. Each RT-PCR reaction was run in duplicate for each experimental sample and the experiment was done twice accounting for a total of 42 spleens analyzed from 6 FACS® sortings.

Results

Latency Is Established Preferentially in Splenic Germinal Center B Cells. Previous determination of the frequency of latently infected subsets of spleen cells was performed at the peak of latency using an in vitro reactivation assay that depends on the ability of latently infected cells to react and form viral plaques on a susceptible cell monolayer (14).

MHV-68 latency peaks 14 d after infection in the spleen and rapidly falls to levels at the limit of detection (10), precluding frequency analysis of long-term latency. Recently a more sensitive assay has been developed, which is based on the frequency of cells harboring viral genome determined by PCR analysis of cells plated in limiting dilution (LDA-PCR; reference 17). Although both lytically and latently infected cells harbor viral genome, in the absence of preformed infectious virus the presence of viral DNA indicates latent virus (17, 18). In this study, we took advantage of this sensitive assay to quantitatively monitor long-term latency in different cell subsets in the spleen. Spleens were magnetically depleted of T cells 14 d after infection. Splenic B cells ($B220^+$ $CD11c^-$ $CD11b^-$ $CD3^-$) were separated by FACS® sorting into highly purified phenotypic subsets representing activated/germinal center cells ($B220^+$ PNA^{high}) and resting B cells ($B220^+$ PNA^{low}; Fig. 1, A–C). Initially, the frequency of latency in the purified B cell populations harboring viral genome and preformed infectious virus was analyzed at day 14, the peak of viral latency. The data were plotted as percentage of wells positive for PCR or CPE (Fig. 2) and linear regression analysis was used to determine the frequency of cells containing viral DNA (Table I). The results showed an absence of infectious lytic MHV-68 in both resting and germinal center B cells, eliminating the possibility that genome-positive cells reflected infectious virus (Fig. 2). The data (Table I) show that an extremely high frequency (1 in 8) of germinal center B cells harbored latent virus at the peak of latency (14 d after infection), compared with a frequency of 1 in 2,700 for resting B cells. Although the purity of the resting B cells was >98%, the high frequency of latency among the 0.03% of contaminating germinal center B cells accounted for ~10% of the latently infected resting B cells. The observation that latency is preferentially in the activated/germinal center B cells is consistent with our previous analysis using the reactivation-based assay (14), but the frequency obtained is 100-fold higher. This quantitative difference may reflect the relative inability of B cells, especially activated B cells, to survive during the culture period required for virus reactivation. For example, it has been shown that 40–50% of germinal center B cells undergo apoptosis within 5 h of culture (22). The difference in frequency may also reflect qualitative differences in latently infected B cells, as suggested by our previous observation that not all cells latently infected with MHV-68 can be reactivated in culture (18).

MHV-68 Maintains Long-Term Latency in Isotype-switched B Cells. Next, we measured the frequency of latency in splenic B cells during long-term infection. Analysis of purified splenic B cells 3 mo after infection (Fig. 3) using LDA-PCR showed that viral DNA was detectable, and infectious virus-LDA showed that B cells lacked infectious virus (Fig. 4 A). The data in Table II show that latency among total B cells was significantly diminished relative to the peak of latency but was maintained at readily detectable frequencies (1 in 27,000). In addition, the absolute numbers of latently infected B cells dropped dramatically from $\sim 3 \times 10^6$/spleen at the peak of latency to $<10^3$/spleen 3 mo after infection (Table II). The finding that MHV-68 latency is maintained at a relatively low frequency in splenic B cells during long-term infection raised the possibility that latency might be preferentially associated with distinct phenotypic populations of B cells. Therefore, we analyzed latency among different subsets of splenic B cells 3 mo after infection. Using FACS® sorting, splenic B cells were purified as naive (IgD/M^+) and isotype-switched (IgA^+ $IgG_1{}^+$ $IgG_{2ab}{}^+$ $IgG_3{}^+$) B cells as shown in Fig. 3, A–C. At this time point, activated/germinal center B cells could not be sorted on the basis of PNA binding because of their low frequency and low level of PNA

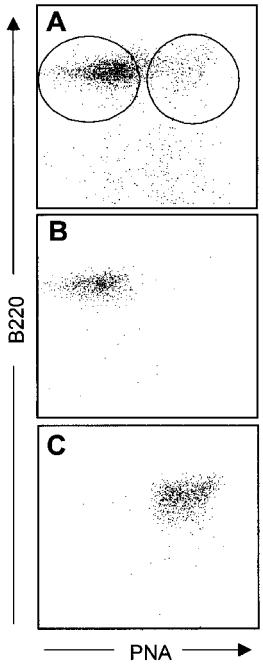

Figure 1. Flow cytometry analysis of MHV-68–infected mice spleen B cells 14 d after infection. (A) Germinal center and resting B cells were purified. $CD3^+$, $CD11b^+$, and $CD11c^+$ cells were gated out and resting B cells were then sorted as $B220^+$ PNA^{low} and germinal center B cells as $B220^+$ PNA^{high}. (B) Resting B cells ($B220^+$ PNA^{low}) isolated 14 d after infection were recovered with a mean purity of 98.15% and the contaminating fraction was 1.5% non-$B220^+$ cells and 0.03% $B220^+$ PNA^{high} cells. (C) Germinal center B cells ($B220^+$ PNA^{high}) isolated 14 d after infection were recovered with a mean purity of 98.64% and the contaminating fraction was 0.47% non-$B220^+$ cells and 0.83% $B220^+$ PNA^{low} cells.

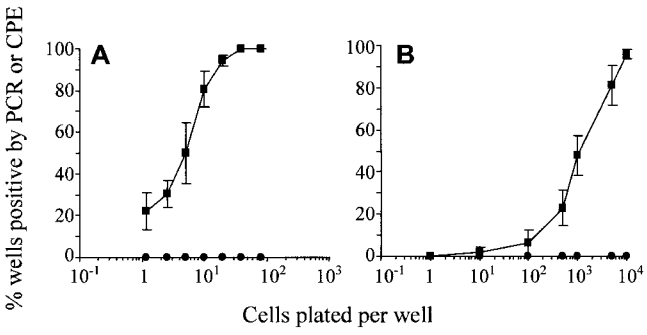

Figure 2. MHV-68 establishes latency in the spleen mainly in germinal center B cells. Spleen B cells 14 d after infection were sorted as (A) germinal center B cells and (B) resting B cells. The purified cells were analyzed by two different methods to determine the frequency of cells carrying MHV-68. The frequency of genome-positive cells was determined by LDA-PCR assay (■). The frequency of infectious virus was determined by infectious virus-LDA (●). The percentages of CPE or PCR reactions that scored positive as a function of the number of cells analyzed are shown. Data represent the average of three to four experiments and the error bars represent SD.

Table I. *MHV-68 Latency within Splenic B Cells During the Establishment of Latency (14 d after Infection) Is Mainly Established in Germinal Center B Cells*

Cells	Reciprocal frequency of genome-positive cells (SD)[a]	Percent of total spleen[b]	Total number of cells[c]	Latently infected cells[d]
Resting B cells[e]	2,700 (1,600)	45	9×10^7	3.3×10^{4}[f]
Germinal center B cells[g]	8 (5)	10.8	2.2×10^7	2.7×10^6

Data shown are the mean of three to four independent experiments, each analyzing pooled spleens from five to seven mice. Standard deviation values are shown between brackets.
[a]Frequencies ± 95% confidence limits were determined by linear regression analysis of LDA-PCR data.
[b]Percentage of each subset of total spleen cells was determined by FACS® analysis.
[c]Total number of cell subset per spleen based on an estimate of 2×10^8 total cells/spleen 14 d after infection.
[d]Number of latently infected cells based on the frequency of viral genome-positive cells within each cell type and its estimated total number per spleen.
[e]Resting B cells were sorted as $B220^+$ PNA^{low} with 98.15% purity. The contaminating fraction was 1.5% non-B cells and 0.03% germinal center B cells.
[f]The 0.03% contamination with germinal center B cells, due to their high level of infection (one in eight), could account for one genome positive cell in every 3,413 purified resting B cells. Therefore, they could contribute as many as 3.2×10^3 cells to the total number (3.3×10^4) of latently infected resting B cells.
[g]Germinal center B cells were sorted as $B220^+$ PNA^{high} with 98.64% purity. The contaminating fraction was 0.47% non-B cells and 0.83% resting B cells.

binding. Therefore, we took advantage of the observation that CD38 can be used to distinguish memory and germinal center class-switched B cells (23–25), and further separated isotype-switched B cells by sorting, based on CD38 expression, into memory B cells ($CD38^{high}$) and germinal center B cells ($CD38^{low}$; Fig. 3, D and E). An additional key difference in the phenotype of germinal center and memory B cells is the expression level of two activation markers, PNA and Fas (23). We corroborated the identity of the subsets of cells as germinal center and memory B cells by showing that isotype-switched $CD38^{low}$ germinal center B cells and isotype-switched $CD38^{high}$ memory B

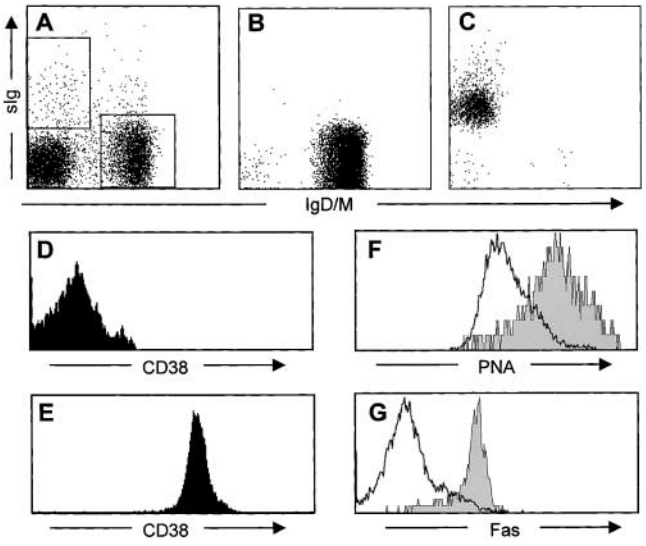

Figure 3. Flow cytometry analysis of MHV-68–infected spleen cells 3 mo after infection. (A) Spleen B cells were purified as isotype-switched cells (IgA^+, IgG_1^+, IgG_{2ab}^+, IgG_3^+) and naive cells (IgM/D^+). (B) Naive B cells (IgM/D^+) were obtained with a mean purity of 99% and the contaminating fraction was 0.8% non-B cells and 0.02% isotype-switched B cells. (C) Isotype-switched B cells (IgA^+, IgG_1^+, IgG_{2ab}^+, IgG_3^+) were further separated into memory and germinal center subpopulations on the basis of CD38 expression. (D) Germinal center B cells (isotype-switched $CD38^{low}$) were recovered with a mean purity of 96% and the contaminating fraction was 2.23% nonisotype-switched cells and 1.9% memory B cells. (E) Memory B cells (isotype-switched $CD38^{high}$) were recovered with a mean purity of 95% and the contaminating fraction was 2.23% nonisotype-switched cells and 2.29% germinal center B cells. (F) PNA expression on germinal center (isotype-switched $CD38^{low}$, gray curve) and memory (isotype-switched $CD38^{high}$, empty curve) B cells 3 mo after infection. (G) Fas expression on germinal center (isotype-switched $CD38^{low}$, gray curve) and memory (isotype-switched $CD38^{high}$, empty curve) B cells 3 mo after infection.

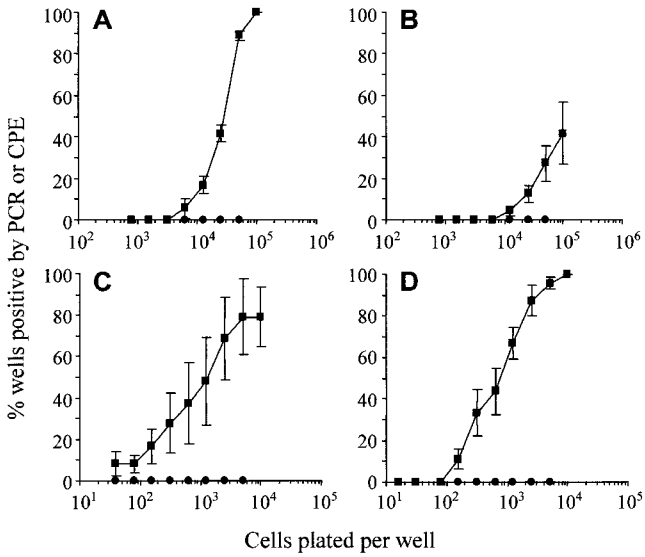

Figure 4. MHV-68 maintains latency in every subset of splenic B cells. Spleen B cells 3 mo after infection were sorted as (A) total B cells, (B) naive B cells, (C) memory B cells, and (D) germinal center B cells. The purified cells were analyzed by two different methods to determine the frequency of cells carrying MHV-68. The frequency of genome-positive cells was determined by LDA-PCR assay (■). The frequency of infectious virus was determined by infectious virus-LDA (●). The percentages of CPE or PCR reactions that scored positive as a function of the number of cells analyzed are shown. Data represent the average of three to four experiments and the error bars represent SD.

Table II. *MHV-68 Latency in the B Cell Compartment During Latent Infection (3 mo after Infection) Is Mainly Maintained in Memory and Germinal Center Cells*

Cells	Reciprocal frequency of genome-positive cells (SD)[a]	Percent of total spleen[b]	Total number of cells[c]	Latently infected cells[d]
Total B cells[e]	27,000 (4,600)	42	2.5×10^7	913
Naive B cells[f]	290,000 (150,000)	36	2.2×10^7	74[g]
Memory B cells[h]	3,100 (2,400)	0.99	5.9×10^5	189
Germinal center B cells[i]	1,100 (469)	0.44	2.6×10^5	233

Data shown are the mean of three to four independent experiments, each analyzing pooled spleens from five to seven mice. Standard deviation values are shown between brackets.

[a]Frequencies ± 95% confidence limits were determined by linear regression analysis of LDA-PCR data.

[b]Percentage of each subset of total spleen cells was determined by FACS® analysis.

[c]Total number of cell subset per spleen based on an estimate of 6×10^7 cells/spleen.

[d]Number of latently infected cells based on the frequency of viral genome-positive cells within each cell type and its estimated total number per spleen.

[e]B cells were sorted as $B220^+$ with 99.17% purity. The contaminating fraction was 0.75% non-B cells.

[f]Naive B cells were sorted as IgD/M^+ with 99% purity. The contaminating fraction was 0.8% non-B cells and 0.02% isotype-switched cells.

[g]The 0.02% contamination with isotype-switched B cells, due to their high level of infection (1 in 2,100), could account for 1 genome-positive cell in every 5,004 purified resting B cells. Therefore, they would make a contribution of one to two cells to the total number (74) of latently infected naive B cells.

[h]Memory B cells were sorted as isotype-switched (IgG_1^+, IgG_{2ab}^+, IgG_3^+, IgA^+) $CD38^{high}$ with 95% purity. The contaminating fraction was 2.23% nonisotype-switched B cells and 2.9% germinal center B cells.

[i]Germinal center B cells were sorted as isotype-switched (IgG_1^+, IgG_{2ab}^+, IgG_3^+, IgA^+) $CD38^{low}$ with 96% purity. The contaminating fraction was 2.23% nonisotype-switched B cells and 1.9% memory B cells.

cells expressed high and low levels, respectively, of both PNA and Fas (Fig. 3, F and G).

Analysis of highly purified phenotypic subsets of splenic B cells by LDA-PCR and infectious virus-LDA showed that viral genome was detectable at distinct levels in each population and that infectious virus was not detectable in any of the B cell subsets analyzed (Fig. 4, B–D). The absence of lytic virus in any of the populations analyzed is consistent with the interpretation that cells harboring viral DNA 3 mo after the initial infection are latently infected. As shown in Table II, latency was maintained at comparable frequencies in both memory and germinal center B cells (1 in 3,100 and 1 in 1,100, respectively). Although these combined populations represent a low percentage of spleen cells (~1.5%), they contain most of the latently infected cells 3 mo after infection. Despite the low frequency of latency in naive B cells (1 in 290,000), these cells also contribute to the latency pool as they represent most of the B cells in the spleen. The high purity of the sorted naive B cells, with only 0.02% contamination by isotype-switched B cells, suggested that the presence of viral genome in the naive B cells could not be attributed solely to the presence of contaminating isotype-switched B cells. It is not possible to determine whether these latently infected cells are a small percentage of truly naive B cells or are located exclusively in a small subpopulation of IgD^+ IgM^+ memory B cells (26, 27).

MHV-68 Maintains Long-Term Latency in Cycling and Resting Isotype-switched B Cells. As latency is harbored both in memory and germinal center B cells in the spleen, we predicted that both resting and cycling B cells harbored viral DNA during long-term latent infection. To address this possibility, we sorted isotype-switched splenic B cells during long-term infection based on their DNA content into G_0+G_1 and $S+G_2/M$, and performed LDA-PCR analysis on the populations obtained. As shown in Fig. 5, G_0+G_1 cells constituted 94.9% and $S+G_2/M$ cells constituted 5.3% of the isotype-switched B cell population. Both subsets of cells harbored virus and linear regression analysis determined that the frequencies of viral content were similar (1 in 3,400 in G_0+G_1 cells and 1 in 3,300 in $S+G_2/M$ cells; Fig. 5). Taking into account the percentage of cells in each phase of the cell cycle, their viral frequencies, and the total number of isotype-switched cells per spleen (Table II),

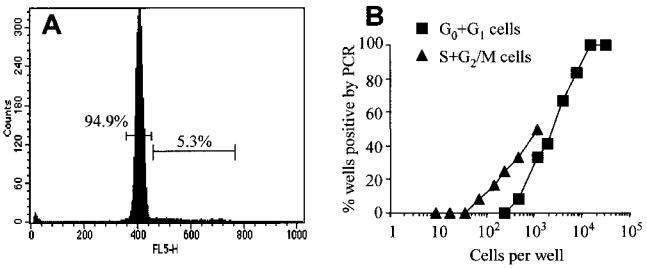

Figure 5. Detection of MHV-68–infected cells in purified G_0+G_1 and $S+G_2/M$ subpopulations of isotype-switched spleen B cells 3 mo after infection. (A) FACS® analysis of the DNA content of isotype-switched B cells gated as $CD19^+$ IgA^+ IgG_1^+ IgG_{2ab}^+ IgG_3^+ IgD/M^- before the fractionation into G_0+G_1 (94.9% of the population) and $S+G_2/M$ (5.3% of the population). The percentages are the average of two independent experiments. (B) The percentage of isotype-switched cells into G_0+G_1 and $S+G_2/M$ harboring viral genome was assessed by LDA-PCR assay. The analysis shown is representative of two independent experiments, each one pooling seven spleens 3 mo after MHV-68 infection.

there were 240 latently infected cells in the G_0+G_1 population and 14 in the $S+G_2/M$ population. Thus, MHV-68 is detected in cycling as well as resting isotype-switched B cells in the spleen during long-term latency.

MHV-68 Gene Expression During Long-Term Latency. An important step in understanding the virus–host interactions involved in maintaining viral latency in an immunocompetent host, is to identify patterns of viral gene expression. The multiple programs of EBV latency-associated gene expression are thought to reflect exploitation by this human γ-herpesvirus of the host B cell biology, in that patterns of latency gene expression reflect the differentiation stage of the infected B cell (21). However, little is known about MHV-68 gene expression in vivo during long-term latency and the analysis has never been performed focusing on B cells, the major viral reservoir in the spleen. Nested RT-PCR analysis was used to detect RNA encoded by regions of the MHV-68 genome that contain candidate latency-associated or lytic genes, described in Table III, using a previously characterized panel of primers (19). Analysis of cDNA from a latently infected B cell tumor line, S11 (28), confirmed that the sizes of the transcripts detected were correct (Fig. 6 A). Therefore, we analyzed viral transcripts from FACS®-purified germinal center and memory B cells from the spleens of MHV-68–infected mice 3 mo after infection. Unexpectedly, there was expression of a wide variety of viral transcripts in both resting memory and activated germinal center B cells. As shown in Fig. 6 B, memory B cells contained transcripts specific for 8 out of 10 ORFs tested, including M1, M2, M9, ORF 50, ORF 72, ORF 73, ORF 74, and K3, whereas germinal center B cells expressed a subset of these transcripts, including M1, M2, M9, and ORF 72. Interestingly, two genes associated with lytic virus, K3 and ORF50, were expressed in memory but not germinal center B cells and the expression of two genes thought to be latency associated, M3 and M11, was never detected in any of the samples analyzed. Bearing in mind that we are assessing viral transcripts rather than bona fide gene expression, and that we are not technically able to analyze transcripts on a per cell basis, the data are consistent with the conclusion that MHV-68 maintains transcript expression during long-term latent infection and suggest key differences in gene expression between different B cell subpopulations.

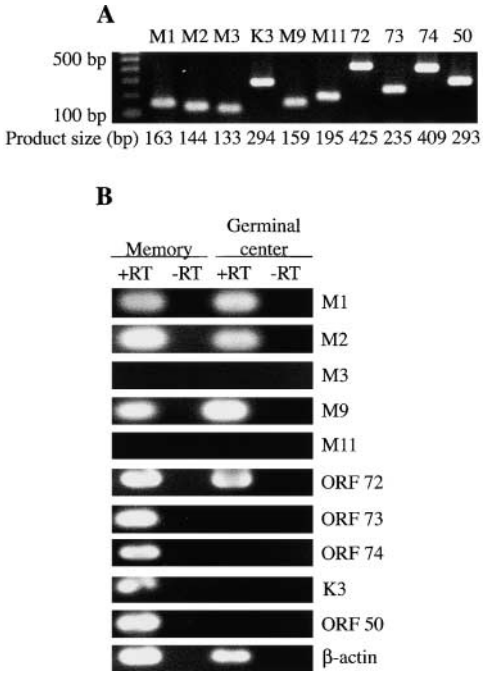

Figure 6. (A) Detection of MHV-68 gene expression in the tumor B cell line S11. Total RNA from S11 cells was subjected to RT-PCR as described in Materials and Methods. (B) Detection of MHV-68 gene expression in purified memory and germinal center B cells. Spleen cells were sorted and subjected to RT-PCR and agarose gel electrophoresis as described in Materials and Methods. Primers specific for MHV-68 ORFs M1, M2, M3, M9, M11, 72, 73, 74, K3, and 50 and for mouse β actin were used as indicated. Control reactions lacking RT are also shown.

Discussion

This study represents the first detailed characterization of splenic B cell subsets in which γ-herpesvirus latency is es-

Table III. *MHV-68 Lytic and Latency-associated Transcripts Analyzed*

Gene	Function and/or homology	References
M1	Serpin homologue (poxvirus), M3 homologue, suppresses reactivation from latency	59
M2	Differentially required for acute replication and establishment of latency	60, 61
M3	Chemokine scavenger	40, 46, 47
M9	Unknown	15
M11	Bcl-2 homologue (EBV, KSHV), apoptosis inhibitor	48, 62
ORF 72	Cyclin-D homologue (KSHV), transforming potential, controls reactivation from latency	63–65
ORF 73	Putative genome maintenance function based on homology to KSHV-LANA	15
ORF 74	G protein–coupled receptor; homology to IL-8R (KSHV), transforming potential	66
ORF 50	Lytic cycle activator, homologous to EBV-Rta transactivator	42–44
K3	Homologous to KSHV K3 and K5, inhibits class I antigen presentation	67, 68

tablished early after infection and maintained during long-term latency. During the establishment of viral latency in the spleen, MHV-68 is preferentially located in germinal center B cells. The major long-term reservoir of latent virus is isotype-switched B cells, with memory and germinal center B cells making equal contribution. Thus, splenic germinal center B cells have a key role both in the establishment and maintenance of viral latency. In addition, gene expression analysis shows that multiple viral transcripts are expressed in memory and germinal center B cells isolated ex vivo from long-term latently infected mice.

Remarkably, the data show that during the establishment phase of viral latency, one in eight germinal center B cells harbors MHV-68 virus. Although this finding might be incidental for MHV-68 biology, we favor the interpretation that this extremely high frequency suggests that MHV-68 exploits germinal center reactions during the establishment of latency in the host B cell compartment. Two different strategies are possible. First, the virus can directly activate naive B cells to become latently infected germinal center cells. Second, the virus can latently infect any B cell, with infection of germinal center B cells being much more efficient. In support of viral activation of naive B cells, our previous phenotypic analysis of nongerminal center B cells 14 d after infection shows them to be phenotypically heterogeneous, with a subset showing signs of early activation markers (14). Regardless of whether naive infected B cells progress through a germinal center reaction or whether germinal center B cells are directly infected with a high efficiency, the data suggest that the virus takes advantage of germinal center reactions to efficiently establish latency in the spleen. This important role of germinal center B cells in γ-herpesvirus latency is consistent with our previous analysis of $CD28^{-/-}$ mice, which are deficient in germinal center reactions. The data showed that although B cell latency is established in $CD28^{-/-}$ mice, the efficiency is reduced by ~10-fold (14). Despite this, latency is maintained at normal levels for ~90 d after infection (18) probably due to the fact that $CD28^{-/-}$ mice produce activated B cells after MHV-68 infection (14) and to their ability to generate isotype-switched memory B cells that have undergone affinity maturation in the absence of germinal centers by alternative mechanisms (29–31). Thus, although the virus may preferentially utilize germinal center cells, they are not absolutely required for the establishment of latency.

The observation that germinal center and resting memory B cells are major reservoirs of long-term MHV-68 latency in the spleen implies similarities and differences with the proposed model for EBV latency, in which EBV gains entry into the long-lived pool of resting memory B cells by usurping the normal mechanisms of B cell activation (1, 4). EBV encodes a viral gene, LMP-1, which directly mediates CD40 signaling to induce polyclonal B cell activation and differentiation (4, 32). This may allow EBV to enter memory B cells directly, by routes other than the germinal center reaction (33). MHV-68, on the other hand, doesn't have an LMP-1 homologue, and seems to be dependent upon $CD4^+$ T cells to induce B cell activation in vivo (34). This dependence on $CD4^+$ T cell help is consistent with the viral use of the germinal center reactions to establish latency (our results and reference 14). Thus, is it possible that γ1- (EBV) and γ2- (MHV-68) herpesviruses target many of the same cellular pathways although they use different strategies to achieve the same effects. KSHV, a γ2-herpesvirus, also establishes latency in B cells but the mechanism is currently unknown.

Similar to what has been shown for EBV in lymphoid organs (21, 35, 36), all the splenic B cell subsets analyzed harbor latent MHV-68. However, the initial EBV infection is usually asymptomatic and current understanding of EBV latency in vivo is mainly based on analysis of peripheral blood and tonsils either during infectious mononucleosis or in long-term asymptomatic individuals. At these time points, when latency has already been established, latent EBV is harbored preferentially in resting memory B cells in the peripheral blood (35, 37, 38), in naive, memory, and/or germinal center B cells in the tonsil (21, 33), and in IgD^+ and IgD^- B cells in the spleen (36). An important advantage of the mouse model is that the events that trigger the initial establishment of latency can be experimentally investigated.

A key finding of this study is that long-term latency in the spleen is harbored not only in resting memory B cells, but a comparable latent reservoir is also found in activated germinal center B cells long after the initial infection. Consistent with this, analysis of latency reservoirs in different stages of cell cycle confirms that both resting and cycling isotype-switched B cells harbor MHV-68. Not unexpectedly, these results differ from the previous cell cycle analysis of latently EBV-infected peripheral blood B cells showing that latency was harbored exclusively in resting cells (38).

The observation that long-term latency is harbored in germinal center, memory, and naive B cells in the spleen can be interpreted in two ways. One possibility is that $MHV\text{-}68^+$ germinal center cells in the spleen reflect ongoing viral reactivation, resulting in the reinfection of naive B cells and reinitiation of the germinal center reaction in the spleen. This has similarities with the observation that EBV in the tonsil of asymptomatic carriers is harbored in B cells with a naive (IgM^+ IgD^+) phenotype, as well as memory and germinal center B cells (21). As the tonsil is a known site of EBV reactivation, this has been interpreted as infection of naive B cells by reactivating virus. Arguing against this interpretation of the current data is the fact that the spleen is not thought to be a site of reactivating MHV-68. We and others have failed to show evidence for lytic virus in immunocompetent mice (this report and references 13, 14, and 17), although we cannot rule out the possibility that there are a few lytically infected cells below our limit of detection. An alternative explanation is that latent virus in activated germinal center B cells reflects virus-independent reactivation of latently infected memory B cells driven by antigen or bystander activation. In this case, the low frequency of latency among naive IgM^+ IgD^+ cells may actually reflect selective latent infection of the subset of IgD^+ IgM^+ B cells that are memory B cells (26, 27). As the role of viral reactivation and reinfection in the mainte-

nance of γ-herpesvirus latency has implications for therapeutic strategies, additional studies are necessary to resolve these fundamental issues.

It is also possible that the frequency of latency in different cell subsets 3 mo after infection is influenced by the half life of the cells. However, the half life of a follicular B cell in the spleen is 4.5 mo and is thought to be longer for marginal zone B cells (39). Therefore, our analysis 3 mo after infection should not be influenced by the life span of B cells.

This study represents the first analysis of MHV-68 gene expression in long-term latently infected B cells. The detection of transcripts putatively associated with latency (M2, M9, and ORF 73), transcripts corresponding to genes associated with viral reactivation from latency (ORF 50, 72, and M1), and lytic cycle transcripts (ORF 50 and K3), suggests a complex mechanism of regulation of MHV-68 latent infection in distinct splenic B cell subsets (refer to Table III for a brief description of the role of each gene). A previous study of MHV-68 gene expression during latency in B cell–deficient μMT mice (19) also detected expression of ORFs M1, M2, M9, 72, and 74 in spleen cells, which suggests an important role of those genes in splenic latency independently of the host cell population. The absence of M3 transcripts in B cells 3 mo after infection is consistent with other studies. M3 could not be detected by in situ hybridization of latently infected mouse spleens 21 d after infection (40). Also, the expression of M3 transcripts detected by RT-PCR was shown to be dramatically down-modulated between 32 d and 10 mo after infection (41).

Transcription of the lytic genes ORF 50 and K3 in memory B cells 3 mo after infection was unexpected as these genes have been associated with reactivation from latency and lytic cycle (19, 42–44) and we and others have consistently failed to detect lytic virus in the spleen this late after the infection of immunocompetent mice. In contrast, the recent detection of EBV lytic gene expression in B cells in the spleen suggests that the spleen, like the tonsil, is an active site of EBV infection (36). Thus, it is possible that B cells in the spleen are a site of MHV-68 reactivation below the limits of detection and we are measuring gene transcription by a low frequency of lytically infected cells. In this sense, we consistently fail to detect transcription of M3 and M11. M3 encodes a chemokine scavenger and is strongly expressed early in infection (40, 45–47). M11 is required for in vivo reactivation from latency (48). Therefore, our consistent failure to detect these transcripts correlates with the absence of viral reactivation and reinfection in the germinal center and memory B cells analyzed. Another possibility is that latently infected memory B cells are continuously trying to reactivate and thus express early lytic viral transcripts, but that viral reactivation is blocked before the formation of infectious particles by the host immune system. The idea that the virus is continually trying to reactivate is also supported by the fact that in the absence of both cellular and humoral immune surveillance (18, 49), or in class $II^{-/-}$ and IFN-$\gamma R^{-/-}$ immunodeficient mice (10, 50), recrudescent lytic virus is readily detected. In addition, continuous exposure to viral lytic antigens might explain the sustained activation and cycling phenotype of $CD4^+$ and $CD8^+$ T cells specific for MHV-68 lytic epitopes (51, 52) during long-term latent infection. Therefore, although we cannot exclude the possibility that our assays to detect lytic virus are not sensitive enough and/or we could be detecting aberrant transcripts, it is also possible that lytic viral genes are expressed but the virus is blocked from full reactivation and production of lytic virus in the spleen.

Although the expression of a viral transcript does not guarantee protein expression, the detection of multiple transcripts is in apparent contrast to the virtual shutdown of viral gene expression in peripheral blood memory B cells harboring latent EBV (3, 38, 53). However, recent analysis of EBV gene expression in tonsillar memory B cells and spleen B cells has shown more promiscuous gene expression than in peripheral blood memory B cells (21, 36, 54, 55). These data suggest that γ-herpesviruses gene expression in latently infected B cells varies with phenotype and anatomical site.

In conclusion, the data provide a compelling argument for γ-herpesvirus utilization of the splenic B cell biology during both the establishment and maintenance phases of latent infection. Both viral latency and gene expression results support a complex interaction between the virus and the B cell life cycle. It must be remembered, however, that B cells are not the only reservoir of γ_2-herpesvirus latency. We and others have shown that MHV-68 latency is established in cells other than B cells, including macrophages and dendritic cells (14, 56). Cell types other than B cells have also been shown to be latently infected with KSHV (57, 58). Thus, the different strategies for the establishment and maintenance of γ-herpesvirus latency in B cells are only one aspect of a very complex strategy for survival.

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