Use of a Virus-Encoded Enzymatic Marker Reveals that a Stable Fraction of Memory B Cells Expresses Latency-Associated Nuclear Antigen throughout Chronic Gammaherpesvirus Infection

Michael S. Nealy, Carrie B. Coleman, Haiyan Li, and Scott A. Tibbetts

Center for Molecular and Tumor Virology, Department of Microbiology and Immunology, and Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130

Received 8 December 2009/Accepted 12 May 2010

An integral feature of gammaherpesvirus infections is the ability to establish lifelong latency in B cells. During latency, the viral genome is maintained as an extrachromosomal episome, with stable maintenance in dividing cells mediated by the viral proteins Epstein-Barr nuclear antigen 1 (EBNA-1) for Epstein-Barr virus and latency-associated nuclear antigen (LANA) for Kaposi’s sarcoma-associated herpesvirus. It is believed that the expression of episome maintenance proteins is turned off in the predominant long-term latency reservoir of resting memory B cells, suggesting that chronic gammaherpesvirus infection is primarily dormant. However, the kinetics of LANA/EBNA-1 expression in individual B-cell subsets throughout a course of infection has not been examined. The infection of mice with murine gammaherpesvirus 68 (MHV68, γHV68) provides a model to determine the specific cellular and molecular events that occur in vivo during lifelong gammaherpesvirus latency. In work described here, we make use of a heterologously expressed enzymatic marker to define the types of B cells that express the LANA homolog (mLANA) during chronic MHV68 infection. Our data demonstrate that mLANA is expressed in a stable fraction of B cells throughout chronic infection, with a prominent peak at 28 days. The expression of mLANA was detected in naïve follicular B cells, germinal-center B cells, and memory B cells throughout infection, with germinal-center and memory B cells accounting for more than 80% of the mLANA-expressing cells during the maintenance phase of latency. These findings suggest that the maintenance phase of latency is an active process that involves the ongoing proliferation or reseeding of latently infected memory B cells.

Gammaherpesviruses such as Epstein-Barr virus (EBV), Kaposi’s sarcoma-associated virus (KSHV, HHV-8), and murine gammaherpesvirus 68 (MHV68, γHV68) are associated with lymphoproliferative diseases and a variety of malignancies of both epithelial and lymphoid origin. The strict species specificity exhibited by gammaherpesviruses has limited research on the human viruses primarily to in vitro studies. MHV68 is genetically colinear to the human gammaherpesviruses and the human viruses primarily to

*Corresponding author. Mailing address: Center for Molecular and Tumor Virology, Department of Microbiology and Immunology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130. Phone: (318) 675-8148. Fax: (318) 675-5764. E-mail: stibble@lsuhsc.edu.

Published ahead of print on 19 May 2010.

© 2010, American Society for Microbiology. All Rights Reserved.
transcriptionally active forms of EBV latency in B cells and in all EBV-associated tumors (56). Similarly, KSHV LANA is expressed in all KSHV-associated malignancies, including primary effusion lymphoma B cells, Kaposi’s sarcoma-derived endothelial cells, and B cells from multicentric Castleman’s disease (MCD) patients (15, 23, 28, 33, 51). During MHV68 infection, transcripts corresponding to orf73 (encoding mLANA) are detectable by quantitative reverse transcription-PCR (qRT-PCR) in sorted splenic germinal-center (GC) and marginal-zone (MZ) B cells at 14 days postinoculation (38), a time point that corresponds with the peak expansion of latently infected cells (10, 38, 64). Consistent with this finding, by limiting-dilution nested RT-PCR, 5 to 10% of viral genome-positive splenocytes express spliced orf73 transcripts at 16 days postinoculation (3).

Taken together, these reports suggest that the expression of episomal maintenance proteins in dividing cells is a critical aspect of the pathogenesis of chronic gammaherpesvirus infection. The MHV68 system provides a means to systematically dissect mechanisms used by a gammaherpesvirus to establish and maintain long-term latency in vivo. Based on the critical role of the episomal maintenance protein in maintaining stable latency, it stands to reason that kinetic studies of the specific cell types expressing episomal maintenance proteins in vivo will provide fundamental insight into gammaherpesvirus pathogenesis. To identify specific cell types expressing mLANA in vivo during the establishment and maintenance of latency, we have generated a recombinant MHV68 expressing a modified β-lactamase enzymatic marker as a C-terminal fusion to mLANA. The use of this virus in conjunction with a β-lactam-based fluorogenic substrate (69) provides a sensitive means to detect and isolate mLANA-expressing cells from in vivo samples using flow cytometry. In work presented here, we demonstrate that mLANA is expressed in naive follicular B cells, germinal-center B cells, and memory B cells during early latency, but that expression in naive follicular B cells wanes over time. The observation that mLANA continues to be expressed in a substantial fraction of memory B cells throughout chronic infection supports the concept that the maintenance of long-term latency is a dynamic process in vivo.

MATERIALS AND METHODS

**Mice and cells.** C57BL/6 (B6) mice obtained from Jackson Laboratory (Bar Harbor, ME) were housed and bred in a pathogen-free facility at Louisiana State University Health Sciences Center–Shreveport in accordance with all federal and university guidelines. Six- to 12-week-old mice were used in all experiments. NIH 3T12 murine fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 100 mg/ml streptomycin, and 2 ml/L-glutamine.

**Generation of MHV68.ORF73plla.** The MHV68.ORF73pla recombinant virus was generated from the parental bacterial artificial chromosome (BAC) (2) using 100 mg/ml streptomycin, and 2 ml/L-glutamine.

**FIG. 1. Generation of the MHV68.ORF73pla marker virus.** (A) Schematic of MHV68.ORF73pla construction. The marker virus was constructed using allelic exchange with a bacteriophage artificial chromosome (BAC) containing the complete wild-type MHV68 genome. orf73 was amplified by PCR with the omission of the stop codon and was fused in-frame upstream of the β-lactamase gene. (B) DNA digestion analysis. DNA was digested with the restriction enzyme AscI, KpnI, or NcoI and subjected to agarose gel electrophoresis to confirm the insertion of the marker molecule and assess the integrity of the viral genome. Shifts in DNA fragment size due to the insertion of the β-lactamase marker are indicated.

The adjacent 1,086-bp region including orf73 coding region upstream of the stop codon (genome coordinates 103927 to 104871; GenBank accession number JX673741) was amplified by PCR with the omission of the stop codon and was ligated into the NotI site of the pGS284 allelic exchange vector. Subsequently, this vector was cut with NotI, yielding a 3,264-bp M11.ORF73pla fragment that was ligated into the NotI site of the pGS284 allelic exchange vector (53).

Allelic exchange was performed using pGS284.M11.ORF73pla + S17pir and MHV68.BAC+ RecA + GSS00 Escherichia coli, as previously described (53). Following positive and negative selection, diagnostic restriction digests were performed on multiple clones to determine the integrity of the viral genome (Fig. 1B), and Southern blots using a β-lactamase probe were performed to determine the correct insertion of the β-lactamase reporter (data not shown). In addition, the region of interest was directly sequenced, and the correct sequence was indicated.

**Viruses and infections.** Wild-type MHV68 clone WUMS originally was obtained from the American Type Culture Collection (ATCC VR1465). BAC-derived MHV68 (MHV68.BAC) has been described previously (1). BAC-deleted stocks of MHV68 and MHV68.ORF73pla were generated from multiple passages on Cre-expressing NIH 3T12 cells, generating MHV68.BAC− and MHV68.ORF73pla.BAC− viruses. For intraperitoneal (i.p.) infections, mice were infected with 10^6 PFU of virus in 500 μl serum-free DMEM. For intranasal (i.n.) infections, mice were anesthetized with isoflurane and inoculated with 10^4 PFU of virus in 30 μl of serum-free DMEM. At indicated time points, spleens
from three to five mice per experimental group were harvested and pooled, unless otherwise stated.

**Plaque assays.** Plaque assays were performed as previously described (59, 60). Briefly, harvested spleens were placed in sterile 2-ml screw-cap tubes containing 1 ml of DMEM and 100 μl of 1-mm-diameter zirconia-silica beads (BioSpec Products, Inc., Bartlesville, OK) and stored at −80°C. Samples were thawed on ice and tissues homogenized using a Mini BeadBeater (BioSpec). Samples were serially diluted 10-fold prior to the infection of NIH 3T12 monolayers. Infected monolayers were overlaid with methylcellulose (Sigma), and plaques were visualized by neutral red staining at day 7. The limit of detection was 50 PFU.

**Limiting-dilution nested PCR analyses.** Single-copy-sensitive nested PCR was used on serial dilutions of harvested cells to determine the frequency of cells harboring viral genome, as previously described (35, 58). In brief, cell samples were washed, resuspended in an isotonic solution, counted, and serially diluted 3-fold in a background of uninfected RAW 264.7 murine macrophages such that a total of 10^5 cells were present in each reaction mixture. Cells were plated in a 96-well PCR plate at 12 wells per dilution. Positive control reaction mixtures contained 10, 1, or 0.1 copies of an MHV68 ORF72 plasmid in a background of RAW 264.7 cells, and negative control reaction mixtures of RAW 264.7 cells only were included on all plates. Cells were lysed using overnight digestion with proteinase K at 56°C followed by enzyme deactivation at 95°C. Nested PCR was performed using primers specific for MHV68 ORF72, and 195-bp amplicons were visualized on a 3% agarose gel with ethidium bromide staining.

**Flow cytometry.** Following spleen harvest, single-cell suspensions were prepared and red blood cells were lysed in red blood cell lysis buffer (144 mM NaCl, 17 mM Tris, pH 7.2) for 7 min at 37°C. For cell surface marker staining, harvested splenocytes were washed twice in cold complete DMEM followed by red blood cell lysis. Cells were adjusted to 2 × 10^6 cells/ml in blocking buffer (phosphate-buffered saline, pH 7.2, 5% bovine serum albumin, and 10% normal rat serum). All samples were blocked with purified anti-mouse CD16/CD32 (Fc block; clone 2.4G2; BD Biosciences, San Jose, CA) followed by antibody staining. Cells then were stained for 30 min on ice in the dark with rat anti-mouse allophycocyanin (APC)/Cy7-conjugated CD19 (clone 1D3; BD Biosciences), rat anti-mouse APC-conjugated CD38 (clone 90; eBioscience, San Diego, CA), and rat anti-mouse biotin-conjugated IgG (clone 11-26c; eBioscience) in blocking buffer. Alexa fluor 700-conjugated streptavidin (Invitrogen, Carlsbad, CA) was used as a secondary reagent to detect bound anti-IgD antibody. For the flow-cytometric analysis of ORF73la expression, cells were washed twice and adjusted to 2 × 10^5 cells/ml in PBS. Freshly prepared 6× coumarin cephalosporin fluorescein acetoxymethyl ester (CCF2/AM) was added to 1 ml of PBS. Cells then were washed twice in PBS and resuspended in PBS containing 10% fetal calf serum. Unstained and isotype-staining controls were included in each experiment. Flow-cytometric analysis was performed on an LSR II flow cytometer, and fluorescent-activated cell sorting (FACS) was performed on a FACS Aria flow cytometer (BD Biosciences). Data were analyzed using FACSDiva software (BD Biosciences).

**Statistical analyses.** All data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). The frequencies of viral genome-positive cells were determined from the nonlinear regression analysis of sigmoidal dose-response best-fit curve data. Based on Poisson distributions, the frequency at which at least one event in a given population is present occurs at the point where the regression analysis line intersects 63.2%.

**RESULTS**

**Generation of the MHV68.ORF73la marker virus.** To generate a recombinant MHV68 that would facilitate the detection of cells expressing mLANA in vivo, we fused a gene encoding a modified β-lactamase molecule to the C terminus of the orf73 coding sequence (Fig. 1A). Although such a fusion could interfere with the functions of mLANA during viral infection, previous work has demonstrated that the fusion of a heterologous protein epitope to the C terminus had no significant effect on latency establishment (7, 52). Although the MHV68 mLANA protein has not been carefully mapped, the orf73 coding region is conserved among the gamma-2-herpesviruses KSHV, herpesvirus saimiri (HVS), and MHV68 (25), and the products of these genes share C-terminal homology with EBNA-1 (20, 25). The episomal tethering function of KSHV LANA is mediated by portions of both the N and C termini, which bind nucleosomes and viral terminal repeats, respectively (24, 36, 43). The central repeat regions of LANA and EBNA-1 are believed to be responsible for cis-acting cytotoxic T-cell evasion by inhibiting translation and proteosomal degradation (7, 34, 48, 68), and fusion to the mLANA C terminus appears to prevent the proteosomal degradation of heterologous fusion proteins (7, 52).

The bacterial enzyme β-lactamase was chosen as a reporter molecule due to its ability to cleave the β-lactam ring of a passively diffusing, membrane-permeant fluorogenic substrate (69), allowing a versatile and sensitive means to detect mLANA expression in living cells. The advantage of this system over other potential markers is that the catalytic activity of β-lactamase allows (i) the detection of cells in which as few as 10 copies of the marker are present, and (ii) detection at frequencies as low as 1 β-lactamase-positive cell in a background of 10^6 negative cells (29). Thus, the use of this marker as an in-frame fusion should allow the detection of mLANA-expressing cells even when mLANA is expressed at a low level in rarely infected cells. To our knowledge, this is the first use of this β-lactamase/fluorogenic substrate reporter system in the context of an intact virus.

The MHV68.ORF73la recombinant virus was generated from the parental MHV68 BAC using allelic exchange, as described in Materials and Methods. Following selection, diagnostic restriction digests were performed on multiple clones to determine the integrity of the viral genome and correct the insertion of the β-lactamase reporter (Fig. 1B). In addition, the region of interest was directly sequenced, and the correct sequence was confirmed (data not shown). BAC DNA from one verified MHV68.ORF73la clone was used to generate high-titer viral stocks on NIH 3T12 murine fibroblasts, and the resulting MHV68.ORF73la.BAC virus was serially passaged in NIH 3T12 cells stably expressing Cre recombinase, resulting in the removal of the loxP-flanked BAC sequence and the generation of the MHV68.ORF73la.BACΔ virus, hereafter termed MHV68.ORF73la.

**MHV68.ORF73la undergoes normal lytic and latent infection.** Because mLANA plays multiple roles in MHV68 infection, we questioned whether the fusion of a marker to the mLANA C terminus would compromise lytic replication or latency. For example, previous studies have demonstrated that the mutation of MHV68 mLANA results in delayed lytic replication and a severe defect in latency establishment (1, 40). To determine whether β-lactamase fusion to mLANA interferes with lytic replication, we first performed single-step (Fig. 2A) and multistep (Fig. 2B) growth curve analyses in vitro, as previously described (59, 60). No significant difference between the MHV68 and MHV68.ORF73la viruses was detected at any time point in single-step growth curves, indicating that the insertion of β-lactamase does not interfere with the ability of the recombinant MHV68 to undergo lytic replication. In multistep growth curves, although the peak viral titers were nearly identical for MHV68 and MHV68.ORF73la, MHV68.ORF73la titers were decreased at 24 and 48 h postinoculation. This finding is consistent with the reported lytic replication defect of a mutant MHV68 lacking the expression of mLANA (19, 40), suggest-
MHV68. ORF73bla establishes and maintains latent infection at levels comparable to those of wild-type MHV68. Taken together, these results demonstrate that the C-terminal fusion of β-lactamase to mLANA does not significantly alter the normal progression of MHV68 acute replication or latency.

mLANA is expressed in a stable fraction of latently infected B cells during chronic infection. To assess whether mLANA/β-lactamase expression could be detected ex vivo from latently infected splenocytes, we initially harvested spleens 16 days postinoculation with MHV68 or MHV68. ORF73bla and loaded cells with the permeable, fluorescent β-lactamase substrate coumarin cephalosporin fluorescein acetoxyethyl ester (CCF2/AM; Invitrogen). CCF2/AM is composed of a 7-hydroxycoumarin (coumarin) molecule linked to a fluorescein is hydrolyzed, disrupting FRET and resulting in fluorescence from the coumarin moiety under violet laser excitation. This detectable shift in fluorescence emission makes it possible to distinguish β-lactamase–expressing cells from non-β-lactamase–expressing cells by fluorescence microscopy or flow cytometry. After CCF2/AM loading, samples were analyzed flow cytometrically to quantify the frequency of cells that displayed β-lactamase activity (Fig. 4A). At 16 days after i.n. inoculation, 0.10% of splenocytes from MHV68. ORF73bla–injected mice displayed β-lactamase activity, corresponding to a frequency of approximately 1 in 1,120. Similarly, following i.p. inoculation with MHV68. ORF73bla, 0.12% of splenocytes were positive for β-lactamase activity, corresponding to an approximate frequency of 1 in 1,510. In contrast to these results, less than 0.0002% of splenocytes from wild-type MHV68–infected mice were detected in the coumarin gate, demonstrating that the spontaneous cleavage of CCF2/AM is negligible. These results demonstrate that the detection of coumarin emission directly correlates with the expression of the mLANA-β-lactamase fusion.

To determine whether cells expressing mLANA were latently infected, we used limiting-dilution assays of sorted cells to quantify the frequencies of mLANA+ cells that harbored viral genome and spontaneously reactivated ex vivo, as previ-
The presence of viral genome-positive cells in this population likely indicates the presence of MHV68-infected cells that either do not express mLANA or express mLANA below the level of detection. Bulk populations of latently infected cells typically exhibit the spontaneous reactivation of MHV68 ex vivo but are free of preformed or infectious virus particles (57, 64). To verify that mLANA⁺ cells were latently infected, we examined the sorted populations of mLANA⁻ or mLANA⁺ cells for reactivation and preformed virus (Fig. 4C). No preformed infectious virus was detected in mLANA⁺ cells, while approximately 1 in 290 mLANA⁺ cells spontaneously reactivated ex vivo. A similarly low proportion of mLANA⁻ cells reactivated ex vivo. Taken together, these data indicate that mLANA⁺ cells are latently infected in vivo.

To determine the frequency of splenocytes that express mLANA throughout chronic infection, C57BL/6J mice were infected i.n. with MHV68.ORF73βla, and the frequency of splenocytes displaying β-lactamase activity was assessed from 7 to 90 days postinoculation (Fig. 4D). The frequency of mLANA-positive cells was highest at 16 days (1 in 1,120), a time point that is coincident with the peak of MHV68 latent cell expansion (Fig. 3 and references 10, 38, and 64). Consistent with the drop in genome-positive cells over time that we observed, the frequency of cells expressing mLANA decreased to approximately 1 in 18,600 by 42 days and 1 in 30,800 by 90 days. Overall, these data indicate that a significant proportion of genome-positive cells express mLANA even during long-term infection, including 18.1% as late as 90 days postinoculation (Fig. 4E). Interestingly, the highest percentage of mLANA-expressing cells (41.6%) occurred at 28 days, indicating that this time point represents a key stage during latency establishment.

mLANA is expressed in germinal-center and memory B cells during chronic infection. MHV68 establishes latent infection in vivo in multiple B-cell subsets, including naïve follicular, germinal-center, and memory B cells (16, 18, 38, 50, 66). To determine in which of these cell populations mLANA is expressed during latency establishment and maintenance, we used flow cytometry to examine the surface phenotype of β-lactamase-expressing cells. Splenocytes were harvested at 16 days postinoculation, and cell samples were stained with antibodies to CD19, IgD, and CD38. While CD19 is a pan-B-cell marker, naïve B cells are the only B subset that express surface IgD and CD38 (aside from a minor antigen-experienced memory population). In mice, CD38 is expressed at high levels on naïve follicular B cells and memory B cells but is downregulated on germinal-center B cells (46). Thus, we defined naïve B cells as CD19⁺ IgD⁺ CD38⁻, germinal-center B cells as CD19⁺ IgD⁻ CD38high, and memory B cells as CD19⁺ IgD⁺ CD38low. Following antibody staining, cells were loaded with CCF2/AM dye, and the distribution of naïve follicular B cells, germinal-center B cells, and memory B cells expressing mLANA was assessed using flow cytometry (Fig. 5). At 16 days, all three B-cell subsets expressed mLANA, a finding that is consistent with previous observations that each of these cell types harbors genome during latency establishment (18, 38, 66).

To gain further insight into the dynamics of mLANA expression in these populations over time, we performed similar experiments throughout a 90-day course of infection (Fig. 6 and Table 1). Seven days postinoculation, a time point at which

![Figure 3](http://jvi.asm.org/) Limiting-dilution nested PCR analysis of splenocytes from MHV68.ORF73βla-infected mice during chronic infection. Single-copy sensitive limiting-dilution nested PCR analysis was performed on splenocytes harvested at 16, 28, 42, and 90 days postinoculation. Harvested cells were serially diluted 3-fold in a background of uninfected RAW264.7 murine macrophages, plated at 12 reactions per cell dilution, and then subjected to lysis followed by nested PCR specific for viral genome. The frequency of cells harboring viral DNA was determined at 63.2% (indicated by the horizontal line) using Poisson distribution (n = 3).
acute replication is apparent (Fig. 1C), the frequencies of mLANA-positive cells in all three subsets were less than 1 in 30,000. Following the clearance of acute replication in the spleen, the number of mLANA-expressing splenocytes increased significantly. At 16 days, a time point that coincides with early latency establishment and correlates with the highest frequency of genome-positive cells during latency (10, 18, 63), the frequencies of germinal-center (1 in 330) and memory (1 in 210) B cells expressing mLANA were significantly higher than that of naïve B cells (1 in 1,190). Although mLANA expression in all three populations diminished by 42 days, expression in the germinal-center and memory B-cell subsets maintained 10-fold higher frequencies than naïve B cells at this time point (1 in 2,500 for germinal-center and 1 in 5,000 for memory versus <1 in 55,000 for naïve cells), and these levels did not decrease through 90 days (1 in 4,300 for germinal-center and 1 in 3,800 for memory cells). In contrast, mLANA expression in naïve B cells waned over time, with less than 1 in 200,000 cells displaying β-lactamase activity 90 days postinoculation. These results therefore demonstrate that mLANA is expressed in all three subsets of mature B cells during early MHV68 latency, and that the expression of mLANA is maintained in a substantial fraction of germinal-center and memory B cells during chronic MHV68 infection.

FIG. 4. Detection of splenocytes expressing mLANA. (A) Detection of mLANA expression using flow cytometry. Spleens were harvested at 16 days after i.n. (top panel) or i.p. (bottom panel) inoculation. Single-cell suspensions were loaded with the CCF2/AM β-lactamase substrate and subjected to flow-cytometric analysis. mLANA/Blα-positive cells (blue) are indicated by the boxed gate. (B) Limiting-dilution nested PCR for viral genome. Sorted mLANA/Blα and mLANA cells populations were subjected to PCR analyses as described for Fig. 3. The frequency of cells harboring viral DNA was determined at 63.2% (indicated by the horizontal line) using Poisson distribution ($n = 6$). (C) Limiting-dilution analyses for ex vivo reactivation and preformed infectious virus. For reactivation assays, sorted mLANA and mLANA cells populations were subjected to serial dilution and then plated on macrophage monolayers to assess cytopathic effect following spontaneous reactivation from latency. The frequency of reactivating cells was determined at 63.2% (indicated by the horizontal line) using Poisson distribution ($n = 3$). (D) Kinetics of mLANA expression in splenocytes. Splenocytes were harvested from MHV68.ORF73Blα-infected mice at the indicated time points. Single-cell suspensions were loaded with the CCF2/AM substrate and subjected to flow-cytometric analysis ($n = 3$ to 5). The $x$ axis is the reciprocal frequency of mLANA-positive cells. The $y$ axis is the number of days postinoculation. (E) Ratio of mLANA-positive to viral genome-positive splenocytes over time. The ratio at each time point was calculated based on the frequency of mLANA-positive cells determined for Fig. 4D and the frequency of genome-positive cells determined for Fig. 3.
DISCUSSION

In work presented here, we describe the use of a novel enzymatic marker to define the types of B cells that express the critical latency protein mLANA during chronic MHV68 infection. The use of this recombinant marker virus presents a unique opportunity to address fundamental questions about the virus-host relationship, in particular about the role played by mLANA in this relationship, during latent infection in vivo. A primary function of MHV68 mLANA and its homologs KSHV LANA and EBV EBNA-1 is the maintenance of the viral episome during mitosis. It has been shown that EBNA-1 expression is induced during cell cycle progression (14), and that EBNA-1 is expressed in latently infected memory B cells upon division (26). Although B-cell subsets that express EBNA-1 have been defined from human samples during chronic infection (4), the kinetics of LANA/EBNA-1 expression during different stages of latency (i.e., establishment versus maintenance) and the subsets of latently infected B cells that express this protein as a function of time have not been determined previously. This information could provide critical insight into the dynamic nature of gammaherpesvirus latency.

Our data demonstrate that mLANA is expressed in a significant proportion of infected B cells throughout latent infection. Interestingly, the highest frequency of infected cells expressing mLANA (42%) occurred at 28 days (Fig. 4C), a time at which germinal-center and effector T-cell responses peak despite the significant loss of cells harboring viral genome (30). In con-
Thus they would not have detected alternatively spliced mLANA primers designed to detect transcripts spliced from exon 2 and E3, E2, and an additional exon downstream of p1 (E1). The (E3) alone, (ii) E3 plus an exon downstream of p2 (E2), or (iii) unique region between **orf75a** mLANA transcript variants include (i) the coding exon to incorporate all potential mLANA transcript fusions just upstream of the **orf73** lactamase gene to the C terminus of the **orf73** lactamase fusion marker. There are multiple mLANA transcript variants that originate from pro-\_\_\_\_lactamase copies per cell are detectable by flow cytometry using the CCF2/AM system (29); however, in all likelihood the expression of the mLANA/\_\_\_\_lactamase fusion marker is not sufficiently sensitive to detect every cell that expresses mLANA in the context of viral infection.

Our data also indicate that the distribution of B-cell subsets expressing mLANA is relatively stable during long-term infection. Taking into account the absolute number of each B-cell subset expressing mLANA at each time point (Table 1 and Fig. 7B), it is apparent that naive B cells represent only a minority of mLANA-expressing cells in all phases of latency. In contrast, memory B cells surprisingly represent the vast majority of mLANA-expressing cells at 16 days, while germinal-center B cells represent the majority of mLANA-expressing cells during chronic infection. Together, germinal-center and memory B cells accounted for more than 80% of mLANA-expressing cells throughout the maintenance phase of latency. These observations are somewhat surprising considering the perception of gammaherpesvirus latency as a dormant state. Although it is well established that B cells are the primary reservoir, it remains unclear whether life-long latency represents a truly quiescent form of infection in which the virus silently resides in resting memory B cells, or whether long-term latency is a dynamic process involving the active turnover of latently infected cells. The prevailing paradigm for EBV is that following the primary infection of naive B cells, the virus provides surrogate signals to drive these cells through germinal-center reactions and into the resting, long-lived memory B-cell compartment. Consistent with such a model, MHV68 genome is detected in a high frequency of naive, germinal-center, and memory B cells during the early stages of latency, but it transitions to the major reservoirs of class-switched B cells during long-term latency (18, 38, 66). While these observations seem to favor a model in which latency is maintained in a dormant state, our findings instead imply that long-term MHV68 latency is maintained through an active process involving the constant generation of new latently infected cells through the division of infected germinal-center and/or memory B cells. In support of this argument, proliferation is critical for long-term latency in B cells (41), and MHV68 genome is detectable in both resting and proliferating germinal-center and memory B cells at 90 days postinfection (18, 41).

The finding that a significant proportion of mLANA-expressing latently infected cells are memory B cells is particularly surprising, because these cells are considered to be primarily resting and therefore presumably would not require the presence of mLANA for the maintenance of the viral genome. There are multiple explanations for our observations. First, it is possible that the memory B cells detected in this assay are indeed undergoing cell division, either as a consequence of virus-driven or antigen-specific activation, or were detected prior to their entering into a resting state after the differentiation of de novo-infected naive or germinal-center B cells. Consistent with this idea, EBV EBNA-1 is expressed in circulating memory B cells only when they are undergoing proliferation (26). Interestingly, memory B cells undergo clonal expansion in response to stimulation by cognate antigen and prior to differentiation to plasma cells (8), and the infection of plasma B cells by MHV68 has been described very recently (12,
49), supporting the possibility that mLANA-expressing cells are transitioning to the plasma B-cell compartment. Second, it is formally possible that memory B-cell proliferation occurs in response to nonspecific inflammatory stimuli, which is a long-standing proposal; however, we feel that this explanation is unlikely in light of recent work indicating that memory B-cell proliferation and differentiation is a tightly regulated process that is induced by cognate antigen but not bystander inflammatory signals (8). Third, it is possible that mLANA performs a critical function separate from episome maintenance that is required in a subset of nondividing memory B cells. Finally, it is conceivable that some memory B cells were detected in this assay due to the slow decay of mLANA following germinal-center B-cell differentiation; however, this is not likely to be the sole explanation, as the half-life of EBNA-1 and LANA are in the range of 20 to 48 h (14, 22, 31, 34, 55).

### TABLE 1. Results of detection of mLANA^+ cells

<table>
<thead>
<tr>
<th>Cell type and inoculation route (n)</th>
<th>% of population in spleen^a</th>
<th>Total no. of cells per spleen^b</th>
<th>% mLANA^+ in population^c</th>
<th>Approx frequency of mLANA^+ in population^d</th>
<th>Approx no. of mLANA^+ cells in spleen^e</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 dpi i.n. (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>52.1 \times 10^6</td>
<td>0.006</td>
<td>18,300</td>
<td>3,100</td>
</tr>
<tr>
<td>CD19^+</td>
<td>55.6</td>
<td>29.2 \times 10^6</td>
<td>0.004</td>
<td>33,700</td>
<td>1,000</td>
</tr>
<tr>
<td>Naïve</td>
<td>30.9</td>
<td>16.3 \times 10^6</td>
<td>0.003</td>
<td>39,000</td>
<td>500</td>
</tr>
<tr>
<td>GC</td>
<td>9.0</td>
<td>4.7 \times 10^6</td>
<td>0.004</td>
<td>35,200</td>
<td>200</td>
</tr>
<tr>
<td>Memory</td>
<td>13.2</td>
<td>6.9 \times 10^6</td>
<td>0.006</td>
<td>28,400</td>
<td>300</td>
</tr>
<tr>
<td>16 dpi i.n. (5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>102.5 \times 10^6</td>
<td>0.10</td>
<td>1110</td>
<td>110,500</td>
</tr>
<tr>
<td>CD19^+</td>
<td>51.3</td>
<td>52.8 \times 10^6</td>
<td>0.18</td>
<td>680</td>
<td>98,300</td>
</tr>
<tr>
<td>Naïve</td>
<td>34.7</td>
<td>35.6 \times 10^6</td>
<td>0.04</td>
<td>2640</td>
<td>14,600</td>
</tr>
<tr>
<td>GC</td>
<td>4.8</td>
<td>5.7 \times 10^6</td>
<td>0.25</td>
<td>530</td>
<td>13,200</td>
</tr>
<tr>
<td>Memory</td>
<td>9.7</td>
<td>9.9 \times 10^6</td>
<td>0.63</td>
<td>210</td>
<td>65,300</td>
</tr>
<tr>
<td>i.p. (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>106.9 \times 10^6</td>
<td>0.11</td>
<td>1500</td>
<td>118,600</td>
</tr>
<tr>
<td>CD19^+</td>
<td>44.1</td>
<td>47.2 \times 10^6</td>
<td>0.26</td>
<td>770</td>
<td>111,800</td>
</tr>
<tr>
<td>Naïve</td>
<td>24.2</td>
<td>25.8 \times 10^6</td>
<td>0.20</td>
<td>950</td>
<td>48,200</td>
</tr>
<tr>
<td>GC</td>
<td>4.8</td>
<td>5.2 \times 10^6</td>
<td>0.63</td>
<td>360</td>
<td>20,600</td>
</tr>
<tr>
<td>Memory</td>
<td>13.9</td>
<td>14.9 \times 10^6</td>
<td>0.14</td>
<td>1830</td>
<td>21,400</td>
</tr>
<tr>
<td>28 dpi i.n. (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>82.3 \times 10^6</td>
<td>0.04</td>
<td>3180</td>
<td>28,600</td>
</tr>
<tr>
<td>CD19^+</td>
<td>47.4</td>
<td>39 \times 10^6</td>
<td>0.07</td>
<td>1750</td>
<td>25,100</td>
</tr>
<tr>
<td>Naïve</td>
<td>36.4</td>
<td>30 \times 10^6</td>
<td>0.01</td>
<td>9710</td>
<td>3400</td>
</tr>
<tr>
<td>GC</td>
<td>4.2</td>
<td>3.5 \times 10^6</td>
<td>0.39</td>
<td>310</td>
<td>13,300</td>
</tr>
<tr>
<td>Memory</td>
<td>13.9</td>
<td>5.6 \times 10^6</td>
<td>0.13</td>
<td>990</td>
<td>7100</td>
</tr>
<tr>
<td>42 dpi i.n. (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>88.1 \times 10^6</td>
<td>0.004</td>
<td>25,700</td>
<td>3600</td>
</tr>
<tr>
<td>CD19^+</td>
<td>49.5</td>
<td>43.6 \times 10^6</td>
<td>0.006</td>
<td>20,600</td>
<td>2400</td>
</tr>
<tr>
<td>Naïve</td>
<td>37.55</td>
<td>33.2 \times 10^6</td>
<td>0.001</td>
<td>116,500</td>
<td>400</td>
</tr>
<tr>
<td>GC</td>
<td>3.5</td>
<td>3.0 \times 10^6</td>
<td>0.040</td>
<td>2800</td>
<td>1200</td>
</tr>
<tr>
<td>Memory</td>
<td>7.5</td>
<td>6.6 \times 10^6</td>
<td>0.014</td>
<td>9700</td>
<td>800</td>
</tr>
<tr>
<td>90 dpi i.n. (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>56.2 \times 10^6</td>
<td>0.003</td>
<td>40,200</td>
<td>1500</td>
</tr>
<tr>
<td>CD19^+</td>
<td>45.7</td>
<td>25.6 \times 10^6</td>
<td>0.004</td>
<td>26,500</td>
<td>1000</td>
</tr>
<tr>
<td>Naïve</td>
<td>34.9</td>
<td>19.6 \times 10^6</td>
<td>0.001</td>
<td>116,600</td>
<td>200</td>
</tr>
<tr>
<td>GC</td>
<td>3.1</td>
<td>1.7 \times 10^5</td>
<td>0.038</td>
<td>4200</td>
<td>500</td>
</tr>
<tr>
<td>Memory</td>
<td>7.0</td>
<td>3.9 \times 10^6</td>
<td>0.008</td>
<td>14100</td>
<td>300</td>
</tr>
</tbody>
</table>

---

^a Percentage of total splenocytes that are CD19^+, naïve follicular (CD19^+ IgD^+ CD38^-), germinal-center (CD19^+ IgD^- CD38^-), and memory (CD19^+ IgD^- CD38^+) B cells.

^b Estimated total number of CD19^+, naïve follicular, germinal-center, and memory B cells per spleen based on the total number of harvested splenocytes and the percentage of analyzed cells in each population.

^c Percentage of mLANA^+ cells in each population based on the number of detected mLANA^+ cells in a population divided by the total number of cells detected in that population multiplied by 100.

^d Approximate frequency of mLANA^+ cells in each population based on the total number of cells detected in a population divided by the number of mLANA^+ cells in that population.

^e Approximate frequency of mLANA^+ cells in each population based on the total number of cells in a population multiplied by the percent of mLANA^+ cells in that population.
nodes, lung, and peritoneal cavity (16, 17, 39, 54, 65). Future experiments will be critical to determine whether mLANA is expressed in other major infected cell populations.

This work demonstrates the utility of a heterologously expressed enzymatic marker to identify gammaherpesvirus-infected cells that express a critical latency protein in vivo. Naive, germinal-center, and memory B cells expressed mLANA during chronic infection, supporting previous reports that these cell types are reservoirs for long-term latency (18, 38, 66). Notably, a stable proportion of memory B cells expressed mLANA during chronic infection, suggesting that the maintenance of latency is an active process, perhaps involving the proliferation of latently infected memory B cells or the de novo infection and generation of new latently infected memory B cells. Future work should uncover other cell types that express mLANA during chronic infection and determine whether mLANA expression in individual cell types correlates with proliferative capacity.

ACKNOWLEDGMENTS

This work was supported by NIH grant CA139984 and NIH COBRE Center for Molecular and Tumor Virology grant P20-RR018724. M.N. was supported by an American Heart Association Predoctoral Fellowship (0815151E). We thank Robert Chevenak, Deborah Chevenak, and Shannon Mumphrey for expert assistance with flow cytometry experiments. We thank Doug White for providing the CRE-NIH 3T12 cell line and Yali Jia for assistance with viral DNA digests.

REFERENCES

31. Knapp, T., E. Hare, L. Feng, G. Zlokarnik, and P. Negulescu. 2003. The Latency-associated nuclear antigen (LANA) cooperatively binds to two sites within the terminal repeat, and both sites contribute to the ability of LANA to suppress transcription and to facilitate DNA replication. J. Virol. 77:2770–2783.


