Blimp-1-Dependent Plasma Cell Differentiation Is Required for Efficient Maintenance of Murine Gammaherpesvirus Latency and Antiviral Antibody Responses[▽]

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Recent evidence from the study of Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus supports a model in which terminal differentiation of B cells to plasma cells leads to virus reactivation. Here we address the role of Blimp-1, the master transcriptional regulator of plasma cell differentiation, in murine gammaherpesvirus 68 (MHV68) latency and reactivation. Blimp-1 expression in infected cells was dispensable for acute virus replication in the lung following intranasal inoculation and in the spleen following intraperitoneal inoculation with MHV68. However, we observed a role for Blimp-1 in both the establishment of latency and reactivation from latency *in vivo*. Additionally, plasma cell-deficient mice also exhibited a significant defect in the establishment of latency in the spleen, as well as reactivation from latency, similar to mice that lacked Blimp-1 only in MHV68-infected cells. In the absence of plasma cells, MHV68 infection failed to elicit a strong germinal center response and fewer B cells in the germinal center were MHV68 infected. Notably, the absence of a functional Blimp-1 gene only in MHV68-infected cells led to a decrease in both B-cell and CD4⁺ T-cell responses during the establishment of latency. Finally, Blimp-1 expression in infected cells played a critical role in the maintenance of both MHV68 latency in the spleen and antibody responses to MHV68. Together, these studies support a model wherein episodic Blimp-1-mediated plasma cell differentiation leads to MHV68 reactivation, which serves to both renew the latency reservoirs and stimulate long-lived plasma cells to secrete virus-specific antibody.

Gammaherpesviruses establish lifelong latent infections in lymphocytes and are associated with a variety of lymphomas and carcinomas. More than 95% of the human population is infected with Epstein-Barr virus (EBV), which is the etiologic agent of infectious mononucleosis and is closely linked to the development of several cancers, including the endemic form of Burkitt's lymphoma and nasopharyngeal carcinoma. Additionally, EBV is well known for its ability to immortalize primary human B cells *in vitro* (20). Kaposi's sarcoma-associated herpesvirus (KHSV or HHV8) infection is found in Kaposi's sarcoma tumors and in primary effusion lymphomas (PELs), as well as an immunoblast variant of multicentric Castlemann's disease (MCD). The murine gammaherpesvirus 68 (MHV68 or γ HV68) is associated with B-cell lymphoma development in β 2-microglobulin-deficient BALB/c mice (52). EBV, KSHV, and MHV68 all establish latency in B cells, and investigation of how B-cell biology shapes gammaherpesvirus pathogenesis is critical to understanding virus-mediated lymphomagenesis (9, 20, 51).

Herpesviruses are characterized by their ability to establish lifelong latent infections with episodic production of progeny virus. During latency, the viral genome is almost completely transcriptionally silent, except for the expression of viral genes necessary for maintenance of the viral genome, allowing the infection to persist without detection and clearance by the host

immune system. However, viral dissemination must occur for viral transmission. Viral genes involved in virus replication need to be transcribed and translated to produce infectious viral particles. This process of change from a dormant infection to active viral shedding is termed reactivation. It is also possible that reactivation plays a critical role in reseeding of latency reservoirs, facilitating maintenance of infection for the lifetime of the host.

EBV establishes latency in the memory B-cell reservoir (3, 24, 46). In the tonsils, the site of viral shedding, latent EBV can be found in both naïve, IgD⁺ and IgD[−] B cells (3). Memory B cells are proposed to traffic latent EBV through the blood into the peripheral tissues, and they harbor latent virus for the lifetime of the host (3, 46). In EBV pathogenesis, reactivation from latency is associated with differentiation from a quiescent memory B cell to a plasma cell (29). Plasma cells isolated from EBV patients have been shown to be positive for the master lytic transcript, BZLF1, and thus are associated with reactivation from latency *in vivo* (13, 29). X-box binding protein 1s (XBP-1s), a transcription factor necessary for plasma cell differentiation, has been shown to bind to the BZLF1 promoter, directly linking plasma cell differentiation and EBV reactivation (38, 49).

Similarly, KSHV reactivation is linked to plasma cell differentiation. Many PELs are of ambiguous origin—lacking cell surface markers clearly indicative of B- or T-cell lineage—yet many have rearranged VDJ genes and express surface CD138 (Syndecan-1, a surface marker of plasma cell differentiation) and Blimp-1 (B-lymphocyte-induced maturation protein 1, discussed below) transcripts (8, 17, 23, 27). Data from microarray

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experiments revealed that PELs display a plasmablastic gene expression profile, a postgerminal center intermediate between plasmablasts and fully differentiated plasma cells (23, 27). Parallel to EBV pathogenesis, XBP-1s is capable of inducing KSHV reactivation by transactivation of the RTA (replication and transcription activator) promoter, the master transcriptional regulator of KSHV reactivation (32, 50, 59, 60). Thus, plasma cell differentiation is associated with both lymphomagenesis and reactivation of KSHV. However, due to the strict species-specific tropism typical of this viral family, study of latency and reactivation *in vivo* is limited.

Upon an encounter with their cognate antigen, T-cell help, and appropriate cytokines, memory B cells can first differentiate into preplasma memory B cells, proliferate, and continue to develop into plasmablasts, finally ceasing proliferation and becoming plasma cells, cellular factories of antibody secretion (42). Plasma cell differentiation is orchestrated by the master transcriptional regulator, Blimp-1, encoded by the gene *prdm1* (54). Ectopic expression of Blimp-1 leads to J-chain synthesis, immunoglobulin secretion, an increase in cell size and granularity, and upregulation of the plasma cell marker *syndecan-1* (54). Blimp-1 directs plasma cell differentiation by repressing a broad range of genes involved in maintaining a mature B-cell phenotype and for driving proliferation (41). Blimp-1 is necessary for differentiation to and maintenance of a plasma cell phenotype, but it is not necessary for the induction of plasma cell differentiation *in vivo* (25, 42, 43). Blimp-1 expression is needed for antibody secretion by all subsets of B cells, including B-1 B cells (40).

MHV68 (γ HV68) is a natural pathogen of wild murid rodents whose pathogenesis parallels that of EBV in many respects. MHV68, too, establishes latency in B cells as well as macrophages and dendritic cells (16, 51). Following intranasal infection, B cells are necessary for trafficking of MHV68-infected cells to the spleen, leading to the establishment of splenic latency (48, 57). Proliferating B cells reactivate at a higher frequency than nonproliferating cells at the onset of splenic latency, and latent MHV68 can be preferentially detected in proliferating B cells as late as 3 months postinfection (35). Recent studies show that viral replication is necessary for the establishment of MHV68 latency in B cells but not macrophages, implicating lytic replication or reactivation in the seeding of the B-cell compartment in the peritoneal cavity following intraperitoneal inoculation (30). In similarity to EBV pathogenesis, memory B cells are the long-term latency reservoir for MHV68 (26, 58).

Due to the many parallels between MHV68, EBV, and KSHV latency, we asked whether plasma cell differentiation plays an important role in chronic MHV68 infection *in vivo*. Here we address the role of plasma cell differentiation in MHV68 pathogenesis using conditional Blimp-1-deficient mice (*prdm1^{c/c}*) and either infection of these mice with a transgenic strain of MHV68 harboring a Cre recombinase expression cassette (MHV68/Cre) (35, 42) or wild-type MHV68 infection of plasma cell null mice (*prdm1^{c/c} CD19^{Cre/+}*). The results of these studies provide important insights into the role of Blimp-1 and plasma cell differentiation in chronic gammaherpesvirus infection in the setting of the infected host and the subsequent impact of the absence of Blimp-1 in infected B cells on the host humoral immune response.

MATERIALS AND METHODS

Mice and infections. Mice were bred, housed, and treated according to the guidelines at Emory University School of Medicine (Atlanta, GA). Mice containing conditional *prdm1* alleles via insertion of loxP sites with the *prdm1* gene (*prdm1^{c/c}* mice) were a generous gift from Kathryn Calame (Columbia University) (42). *CD19^{Cre/+}* mice were a kind gift from Klaus Rajewsky (Harvard University) (39). *prdm1^{c/c} CD19^{Cre/+}* mice were bred at Emory University, and *prdm1^{c/c}* mice were used as controls for infections. Mice were sedated and infected with 1,000 PFU of the respective strain of MHV68 either intraperitoneally in 500 μ l of complete Dulbecco's modified Eagle's medium (cMEM) or intranasally in 20 μ l of cMEM. Mice were allowed to recover from anesthesia before being returned to their cages.

Virus generation. Wild-type MHV68 refers to the WUMS (ATCC VR-1465) strain of MHV68. MHV68/Cre and MHV68/YFP viruses were generated as previously described (12, 35). MHV68/Cre.MR virus was generated by allelic exchange in *Escherichia coli* as developed by Smith and Enquist (1, 2, 45), as follows. The open reading frame 27 (ORF27)-ORF29b intergenic region (WUMS sequence) of MHV68 was cloned into the suicide vector pGS284 to create pGS284-27-29B as previously described (28). pGS284 carries an ampicillin gene and a levansucrose cassette for positive and negative selection, respectively. S17 Δ pir *E. coli* bacteria harboring the pGS284-27-29B plasmid were mated to GS500 *E. coli* (RecA⁺) bacteria containing MHV68/Cre-Kan^r BAC (a virus wherein the Cre gene contains a kanamycin resistance [Kan] cassette), an intermediate virus made during the construction of MHV68/Cre (35). The MHV68 bacterial artificial chromosome (BAC) contains a chloramphenicol (Cam) resistance cassette. Bacteria were mated on Luria-Bertani (LB) agar plates without selection. The following day, cointegrants were selected by culturing the *E. coli* bacteria on LB plates containing both chloramphenicol and ampicillin. Matings were then allowed to resolve by culturing overnight in LB-Cam. The bacteria were then plated on LB plates containing Cam and 7% sucrose to select for loss of the pGS284 backbone sequence. Finally, individual colonies were replica plated on LB-Cam and LB-Kan plates, and Cam^r Kan^s colonies were screened for loss of the Cre-Kan^r cassette by PCR. Positive clones were identified by PCRs for both loss of the Cre cassette and presence of the wild-type locus. The reversion to wild-type sequence and genomic integrity of the ORF27-ORF29b region were confirmed by Southern blotting, resulting in the selection of MHV68/Cre.MR BAC. MHV68/Cre.MR BAC DNA was purified using a Midi Prep kit (Qiagen, Hilden, Germany) with a modified manufacturer's protocol.

BAC DNA was used to transfect Vero-Cre cells using the Superfect reagent (Qiagen, Hilden, Germany) as previously described (34). Following the appearance of a cytopathic effect (CPE), virus was harvested and used to infect fresh Vero-Cre cells to generate high-titer cultures. Viral DNA was then purified from Vero-Cre cells infected with either MHV68/Cre or MHV68/Cre.MR as previously described (28). Viral DNA was then used to transfect Vero-Cre cells, and virus was used to infect 50% confluent monolayers of Vero-Cre to generate high-titer stocks. Once abundant CPE had occurred in the Vero-Cre cultures, supernatants and cells were harvested, the cells were homogenized, and debris was cleared by centrifugation. The resultant viral stocks were tested for mycobacterial contamination, and clean stocks were stored at -80°C . Viral titers were determined by plaque assay as previously described (28).

Virus assays. Plaque assays were used to determine organ titer as previously described (28). Briefly, 1 day prior to the assay, 2×10^5 NIH3T12 cells were plated per well in a 6-well plate. The following day, organs frozen at -80°C in cMEM with 1.0 mM zirconia/silica beads (Biospec Products) were thawed and subjected to four rounds of mechanical disruption for 1 min each in a Mini-Beadbeater 8 cell disrupter (Biospec Products). The resulting homogenates were then serially diluted 10-fold in cMEM. Medium was removed from the 3T12 monolayers and replaced with 200 μ l of each of the dilutions in duplicate. The plates were rocked every 15 min for 1 hour and then overlaid with 2% methylcellulose in cMEM. Approximately 1 week later, plates were stained with neutral red overnight, and following aspiration of the methylcellulose, plaques were scored. The limit of detection of this assay is 50 PFU per organ.

Limiting-dilution assays for viral latency and reactivation were performed as previously described (21, 22). Single-copy-sensitive PCR was performed to determine the frequency of cells harboring latent viral genome. In brief, frozen samples were thawed, washed in isotonic buffer, and counted, and 3-fold serial dilutions were plated in a background of 10^4 NIH 3T12 cells in 96-well plates. Twelve wells were plated per dilution, and cells were lysed by protease K digestion for 6 h at 56°C . Samples were then subjected to two rounds of nested PCR, and the products were resolved on 2% agarose gels. In order to measure the frequency of reactivating cells, cells were counted and plated in serial 2-fold dilutions on mouse embryonic fibroblast monolayers in 96-well tissue culture

plates. Parallel samples of mechanically disrupted cells were plated to detect preformed infectious virus. Wells were scored for cytopathic effect 14 to 21 days postexplant.

Flow cytometry. Splenocytes were incubated in 96-well round-bottom plates with rat anti-mouse CD16/32 (Fc block) prior to staining (BD Biosciences). Cells were stained with conjugated antibodies to the following epitopes: Vβ4-fluorescein isothiocyanate (FITC), GL7-FITC, IgG-FITC, IgE-FITC, IgA-FITC, CD44-phycoerythrin (PE), CD95-PE, IgD-PE, CD19-PE, CD138-PE, CD4-peridinin chlorophyll protein (PerCP), CD67-PE-Cy7, CD95-PE-Cy7, CD62L-allophycocyanin (APC), CD19-APC, B220-APC, CD3-Pacific Blue, and CD8-Pacific Blue (BD Biosciences). Additionally, some experiments used anti-GL7-biotin antibodies detected with streptavidin-APC (eBiosciences). Most samples were fixed with 1% formalin, and all were analyzed on an LSR II flow cytometer (BD Biosciences). Data were analyzed using the FlowJo software program (TreeStar, Inc., San Carlos, CA).

ELISA for MHV68-specific antibody. Enzyme-linked immunosorbent assay (ELISAs) to measure the MHV68-specific antibody response were performed as previously described (19). Briefly, plates were coated with viral antigen fixed in 1% paraformaldehyde to detect MHV68-specific antibody. To create a standard curve, each plate contained wells coated with 2 μg/ml of donkey anti-goat IgG (Jackson ImmunoResearch) in coating buffer (0.1 M Na₂CO₃, 0.2% NaH₂PO₄ [pH 9.6]) for 2 h at 37°C. Wells were blocked in phosphate-buffered saline (PBS)–3% bovine serum albumin for 2 h at 37°C. Wells were washed with wash buffer (PBS–0.1% Tween) three times before addition of samples or standards. Three dilutions of 1 μg/ml mouse IgG in ELISA diluent (BD Biosciences) were plated in duplicate to create a standard curve. Appropriate dilutions of serum samples from infected mice were made in ELISA diluent and plated on wells coated with viral antigen. Plates were incubated for 3 h at 37°C or overnight at 4°C. Wells were washed three times, and bound antibody was detected with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) diluted 1:5,000. Plates were incubated for 2 h at 37°C, washed three times, and incubated with 100 μl of 1:1 solution of developer (BD Biosciences). Reactions were stopped after 10 to 30 min with 50 μl of 2 N H₂SO₄ per well. Plates were analyzed on a Synergy HT microplate reader (BioTek) using KC4 software (BioTek).

Statistical analyses. All data analysis was conducted using the GraphPad Prism software program. Error bars in graphs depict the standard errors of the means. Limiting-dilution assays were analyzed by subjecting the data to nonlinear regression analysis with a sigmoidal dose-response algorithm for best fit. Poisson distribution predicts that the frequency at which 63.2% of the wells are positive for an event (PCR product or cytopathic effect) is the frequency at which there is at least one positive event in the population. Statistical significances of flow cytometry and ELISA data were determined by a two-tailed, unpaired Student's *t* test with a confidence level of 95%.

RESULTS

Blimp-1 expression is dispensable for lytic replication in the lung and spleen. In the *prdm1*^{cl/c} mice, exons 6 to 8, encoding the zinc finger motifs of Blimp-1, are flanked by loxP sites (42). Expression of Cre recombinase in cells leads to deletion of the sequence between the loxP sites and loss of Blimp-1 protein expression (42). Previously we generated and characterized a recombinant MHV68, MHV68/Cre, which harbors a Cre recombinase expression cassette inserted into a phenotypically neutral locus in the MHV68 genome (35). To assess the role of plasma cell differentiation in acute replication, *prdm1*^{cl/c} mice were infected with 1,000 PFU of MHV68/Cre or MHV68/Cre.MR either intranasally to examine acute replication in the lungs or intraperitoneally to examine acute replication in the spleen. Viral titers in the lungs (Fig. 1A) and spleens (Fig. 1B) at days 4 and 9 postinfection were quantified by plaque assay. We observed no significant differences in virus replication in the lungs at either day 4 or day 9 postinfection (Fig. 1A), although there was significant mouse-to-mouse variation in viral titers observed at day 9, presumably due to rapid clearance of the virus at this relatively late acute time point (i.e., asynchronous clearance of acute virus replication). Similarly,

FIG. 1. Blimp-1 is dispensable for acute virus replication in the lung and spleen. *Prdm1*^{cl/c} mice were infected either intranasally (A) or intraperitoneally (B) with 1,000 PFU of either MHV68/Cre or MHV68/Cre.MR. Lungs and spleens were harvested at days 4 and 9 postinfection, and viral titers were determined by plaque assay (see Materials and Methods). A statistically significant difference in titers between the groups of mice at day 9 postinfection in the spleen was observed (*, *P* = 0.0292). Data represent results compiled from two independent experiments with five mice per group.

we observed no significant difference in viral titers in the spleens of MHV68/Cre-infected mice (457.5 ± 720.4 PFU) compared to those for MHV68/Cre.MR mice ($735.0 \pm 1,003.0$ PFU) at day 4 postinfection (Fig. 1). There was a statistically significant difference in splenic viral titers at day 9 postinfection, with the Cre-recombinase-expressing virus exhibiting slightly higher levels of viral replication (MHV68/Cre, 642 ± 579.0 PFU; MHV68/Cre.MR, 169.4 ± 140.7 PFU), but we think this <4-fold difference is unlikely to be significant to viral pathology (Fig. 1). We conclude from this that plasma cell differentiation plays little or no role in acute virus replication. Alternatively, we cannot rule out the possibility that Cre recombinase expressed from the viral genome failed to efficiently delete exons 6 to 8 of *prdm1* at the early stages of infection, thereby leaving Blimp-1 expression intact and masking a role for Blimp-1 in MHV68 lytic replication. Regardless, under these experimental conditions there was no detectable defect in acute virus replication that might confound subsequent analyses of viral latency.

Blimp-1 expression is necessary for the efficient establishment of latency in the spleen. We next investigated the role of plasma cell differentiation in the establishment of, and reactivation from, latency. *Prdm1*^{cl/c} mice were infected intranasally

FIG. 2. A functional Blimp-1 gene is required in infected splenocytes for both efficient establishment of latency and reactivation from latency at day 18 postinfection. *Prdm1*^{cl/c} were infected via either intranasal inoculation (A and B) or intraperitoneal inoculation (C and D) with 1,000 PFU of either MHV68/Cre or MHV68/Cre.MR. At 18 days postinfection, the frequency of cells establishing latency and capable of reactivating from latency was determined by limiting-dilution assay. (A and C) Limiting-dilution determination of the frequency of splenocytes harboring latent MHV68 in *prdm1*^{cl/c} mice infected with either MHV68/Cre or MHV68/Cre.MR. (B and D) Limiting-dilution determination of the frequency of splenocytes spontaneously reactivating MHV68 upon explant from *prdm1*^{cl/c} mice infected with either MHV68/Cre or MHV68/Cre.MR. Data represent results compiled from three independent experiments with three to four mice per group.

with 1,000 PFU of either MHV68/Cre or MHV68/Cre.MR, and MHV68 latency and reactivation in the spleen were measured by limiting-dilution analyses at day 18 postinfection. Somewhat surprisingly, splenic latency was reduced almost 11-fold in the *prdm1*^{cl/c} mice infected with MHV68/Cre (1/5,687 splenocytes harbored latent viral genomes) compared to results for those animals infected with MHV68/Cre.MR (1/523) (Fig. 2A). In line with the decreased efficiency in the establishment of latency, *prdm1*^{cl/c} mice infected with MHV68/Cre exhibited decreased virus reactivation compared to *prdm1*^{cl/c} mice infected with MHV68/Cre.MR (1/109,065 splenocytes in MHV68/Cre-infected mice versus 1/15,931 splenocytes in MHV68/Cre.MR-infected mice) (Fig. 2B). Notably, the contribution of virus reactivation from the latently infected non-B-cell reservoirs (e.g., macrophages and dendritic cells) in the spleen may obscure the magnitude of the reactivation defect in B cells upon loss of a functional Blimp-1 gene. Regardless, these results demonstrate an important role for plasma cell differentiation during the establishment of MHV68 latency in the spleen, perhaps reflecting a role for virus reactivation from latently infected B cells trafficking from distal sites in seeding acute replication in the spleen at early times postinfection.

Because we have previously noted for some MHV68 mutants differences in the establishment of splenic latency that are dependent on the route of inoculation (21, 22), we infected *prdm1*^{cl/c} mice via intraperitoneal inoculation with 1,000 PFU

of either MHV68/Cre or MHV68/Cre.MR. As before, we again observed a defect in the establishment of latency (7-fold in the *prdm1*^{cl/c} mice infected with MHV68/Cre [1/906 splenocytes harbored latent viral genomes] compared to those animals infected with MHV68/Cre.MR [1/124]) (Fig. 2C). Furthermore, infection of *prdm1*^{cl/c} mice with MHV68/Cre led to a 12.7-fold defect in the frequency of splenocytes reactivating from latency (1/89,493 splenocytes in MHV68/Cre-infected mice versus 1/7,006 splenocytes in MHV68/Cre.MR mice) (Fig. 2D). Thus, taken together with the decreased establishment of latency, we observed a modest impact on the efficiency of splenocyte reactivation. Notably, previous characterizations of the MHV68/Cre virus in C57BL/6 mice did not reveal any defect in establishment or reactivation from latency (34), indicating that the observed latency phenotypes in the *prdm1*^{cl/c} mice infected with the MHV68/Cre virus are due to the loss of a functional Blimp-1 gene.

Mice deficient in plasma cells exhibit decreased establishment of, and reactivation from, splenic latency *in vivo*. The experiments described above depended upon efficient excision of exons 6 to 8 in the *prdm1* gene by Cre recombinase expressed from the MHV68 genome. We were concerned that limited expression of Cre recombinase from the viral genome might lead to a failure to efficiently excise exons 6 to 8 from both floxed *prdm1* alleles and thus could mask viral latency and reactivation phenotypes that are dependent upon plasma cell

FIG. 3. *Prdm1^{c/c}* *CD19^{Cre/+}* mice lack plasma cells in the spleen. Mice were infected via intranasal inoculation with 1,000 PFU of recombinant wild-type MHV68/YFP. At day 16 postinfection, splenocytes were stained with CD3-Pacific Blue, B220-APC, and CD138-PE and analyzed by flow cytometry. Cells were gated on live lymphocytes as determined by forward and side scatter followed by exclusion of CD3⁺ lymphocytes. Representative median flow cytometry of naïve and infected mice demonstrates a significant loss of B220^{low} CD138^{high} plasma cells.

differentiation. To address this concern, we bred *prdm1^{c/c}* mice to *CD19^{Cre/+}* mice and then backcrossed *prdm1^{c/+}* *CD19^{Cre/+}* mice to *prdm1^{c/c}* mice to generate Cre-expressing mice that were homozygous for the conditional *prdm1* allele. The resulting *prdm1^{c/c}* *CD19^{Cre/+}* mice express Cre recombinase under the control of the CD19 promoter, and thus, these mice lack Blimp-1 expression in all peripheral B cells. Previous characterization of *prdm1^{c/c}* *CD19^{Cre/+}* mice has demonstrated that they lack preplasma memory and plasma B cells (42). We confirmed that the *prdm1^{c/c}* *CD19^{Cre/+}* mice bred in our laboratory were plasma cell deficient by flow cytometry, although due to background fluorescence, we cannot rule out the presence of a small population of plasma cells in these mice (Fig. 3 and 4B).

To examine MHV68 pathogenesis in the absence of plasma cells, mice were infected and B-cell populations were monitored by flow cytometry. Recently our laboratory generated and described a transgenic strain of MHV68 that expresses the yellow fluorescent protein (MHV68/YFP) from a neutral locus within the viral genome (12). This recombinant MHV68 has been shown to behave like wild-type MHV68 *in vivo* and is capable of marking latently infected cells following intranasal inoculation early after the establishment of latency (12). *Prdm1^{c/c}* *CD19^{Cre/+}* mice and *prdm1^{c/c}* mice were infected via intranasal inoculation with 1,000 PFU of MHV68/YFP. Splenocytes were harvested at 16 days postinfection, stained with appropriate antibodies, and analyzed by flow cytometry. In parallel, *prdm1^{c/c}* mice were infected with wild-type MHV68 and used as staining controls to ensure that the observed YFP fluorescence was not due to autofluorescence (data not shown). At day 16 postinfection, we observed a 5-fold decrease in the frequency of YFP-positive B cells in spleens of the plasma cell-null mice compared to levels for control mice (0.75% in *prdm1^{c/c}* mice versus 0.15% in *prdm1^{c/c}* *CD19^{Cre/+}* mice) (Fig. 4A). Additionally, we noted a 2-fold decrease in the frequency of germinal center B cells (CD95^{high} GL7^{high}) in the plasma cell-null mice (Fig. 4C). Earlier studies have shown that more than 85% of MHV68-infected B cells (YFP⁺ B cells) exhibit a germinal center surface phenotype, and the majority

of the other infected B cells are detected in the plasma cell compartment (12). In line with the observed lower frequency of MHV68-infected splenocytes in the plasma cell null mice (Fig. 4A), when we examined the frequency of the germinal center B cells that were harboring MHV68, we observed a 3.5-fold decrease in the frequency of YFP⁺ cells in the CD95^{high} GL7^{high} B-cell population (Fig. 4D). These data suggest a link between plasma cell differentiation and seeding of latency in the germinal center compartment; perhaps in the absence of efficient virus reactivation from MHV68-infected plasma cells, there is less-efficient infection of germinal center B cells. Furthermore, these data demonstrate a perturbation in the entry of YFP⁺ cells into the germinal center reservoir in plasma cell-null mice, supporting the idea of a dynamic phase of MHV68 splenic latency that is dependent on plasma cell differentiation in the spleen.

We examined the effect of a lack of plasma cells in the entire mouse on latency and reactivation by comparing MHV68 infection of *prdm1^{c/c}* *CD19^{Cre/+}* mice and that of *prdm1^{c/c}* mice. Following intranasal inoculation with 1,000 PFU of MHV68/YFP, at day 16 postinfection there was a 6-fold decrease in the establishment of latency in the *prdm1^{c/c}* *CD19^{Cre/+}* mice (Fig. 5A). In the absence of plasma cells, 1/1,062 cells harbored latent MHV68 compared to 1/168 cells in the *prdm1^{c/c}* mice infected with MHV68/YFP (Fig. 5A). We observed a 9.5-fold reduction in the frequency of reactivation for the plasma cell-null mice (1/202,065) compared to that for the wild-type animals (1/21,161) (Fig. 5B). These data demonstrate a requirement for Blimp-1 for MHV68 establishment of latency and reactivation in B cells.

Following intraperitoneal infection with 1,000 PFU of MHV68, we observed a 12-fold decrease in the establishment of latency in *prdm1^{c/c}* *CD19^{Cre/+}* mice (1/1,073 splenocytes) compared to results for the control *prdm1^{c/c}* mice (1/90 splenocytes) (Fig. 5C). In similarity to infection of *prdm1^{c/c}* with the transgenic MHV68/Cre virus, we observed a further 5-fold reduction in the frequency of splenocytes capable of reactivating from latency in the *prdm1^{c/c}* *CD19^{Cre/+}* mice (1/129,320) versus results for the control animals (1/7,664) for a combined

FIG. 4. Fewer B cells are marked by MHV68/YFP in plasma cell-deficient mice. *Prdm1^{c/c}* and *prdm1^{c/c} CD19^{Cre/+}* mice were infected intranasally with 1,000 PFU of MHV68/YFP. At day 16 postinfection, spleens were harvested and cells were stained with CD19-PE, CD95-PE-Cy7, GL7-Biotin, and CD3-Pacific Blue, followed by incubation with streptavidin-APC. *Prdm1^{c/c}* mice infected with MHV68 were used as gating controls to exclude autofluorescent cells (data not shown). Cells were gated on live lymphocytes as determined by forward and side scatter, followed by exclusion of CD3⁺ lymphocytes. CD19 was used to discriminate B cells. (A) The frequency of YFP⁺ B cells was significantly lower in the *prdm1^{c/c} CD19^{Cre/+}* plasma cell-null mice. (B) Absence of plasma cells for MHV68-infected *prdm1^{c/c} CD19^{Cre/+}* mice compared to levels for *prdm1^{c/c}* mice. (C) Lower frequency of total germinal center (GL7^{high} CD95^{high}) B cells for the plasma cell-null mice compared than for control mice. (D) Plasma cell-null mice exhibited a significant decrease in the frequency of YFP⁺ B cells in the germinal center population. Data represents results from two independent infections with one naïve mouse and three infected mice per group. *, $P = 0.0023$; **, $P < 0.0001$; ***, $P = 0.0349$; ****, $P = 0.0014$.

17-fold reduction in reactivation frequency (Fig. 5D). Overall, there was only a modest decrease in reactivation efficiency (0.8% versus 1.2%) in the *prdm1^{c/c} CD19^{Cre/+}* mice, although, as discussed above, reactivation from the latently infected non-B-cell reservoirs may mask a more substantial defect in B-cell reactivation. Finally, following intraperitoneal inoculation, we did not observe any significant difference in the frequency of peritoneal exudate cells (PECs) capable of establishing or reactivating from latency for the *prdm1^{c/c} CD19^{Cre/+}* mice (Fig. 5E and F).

Taken together, these studies provide evidence that Blimp-1 expression in B cells (presumably linked to plasma cell differentiation) plays an important role in both the establishment of latency and reactivation from latency *in vivo*. Importantly, the data demonstrate that B-cell differentiation plays a critical role in the seeding of latency and reactivation in the spleen but not in the peritoneum. Furthermore, the results obtained with MHV68 infection of plasma cell null mice were entirely consistent with the data obtained following infection of *prdm1^{c/c}* mice with the MHV68/Cre virus, in which Blimp-1 expression is lost only in virus-infected cells (compare results in Fig. 2 and 5).

B-cell responses to MHV68 are diminished in the absence of Blimp-1. To follow up on the analysis of MHV68 latency in specific reservoirs, we next assessed whether there were alterations in the host immune response to MHV68 infection in the absence of Blimp-1 expression in infected cells. To this end, we examined the B- and T-cell responses following intraperitoneal MHV68 infection at days 18, 42, and 90 postinfection by multicolor flow cytometry. At day 18 postinfection, at the peak of MHV68 latency, we observed a significant decrease in the frequency of activated (CD69^{high}) B cells for the *prdm1^{c/c}* mice infected with MHV68/Cre compared to results for those infected with the marker rescue virus, MHV68/Cre.MR (Fig. 6A and B). Notably, under these experimental conditions, Blimp-1 is absent only in B cells that are MHV68 infected (<1% of B cells). Thus, global B-cell activation is influenced by the inability of MHV68-infected B cells to differentiate into plasma cells.

In the absence of B-cell activation, we hypothesized that the frequency of B cells capable of entering a germinal center reaction and undergoing class switch recombination would be reduced. Indeed, we observed a low frequency of CD95^{high} GL7^{high} B cells in the *prdm1^{c/c}* mice infected with MHV68/Cre versus results with MHV68/Cre.MR infection (Fig. 6A and C). Furthermore, a lower frequency of B cells underwent class switch recombination, becoming IgD^{low} IgG/A/E⁺, in the *prdm1^{c/c}* mice infected with MHV68/Cre (Fig. 6A and C). However, by day 42 postinfection, there was no significant difference in the B-cell responses in the *prdm1^{c/c}* mice infected with MHV68/Cre and those infected with MHV68/Cre.MR, and the B-cell responses were largely quiescent, as previously observed (data not shown) (19, 28). Interestingly, we have observed similar decreases in B-cell responses in other instances where there are defects in the establishment of latency and reactivation from latency, such as a loss of MyD88 signaling, inhibition of NF- κ B signaling, or infection with an M2-null virus (19, 28) (A. M. Siegel and S. H. Speck, unpublished data).

V β 4⁺ CD8⁺ T cells expand to wild-type levels in absence of Blimp-1. Infection of C57BL/6 mice with MHV68 leads to an expansion of a T-cell receptor (TCR)-specific population of

FIG. 5. Global loss of plasma cells in MHV68-infected mice leads to a defect in both the establishment of latency and reactivation from latency in the spleen but has no impact on latency in the peritoneal cavity. *Prdm1*^{c/c} *CD19*^{Cre/+} mice, deficient in plasma cells, and *prdm1*^{c/c} mice were inoculated intranasally with 1,000 PFU of MHV68/YFP (A and B) or intraperitoneally (C to E) with wild-type MHV68 at a dose of 1,000 PFU. At day 18 postinfection, splenocytes and PECs were subjected to limiting-dilution analyses for establishment of latency and reactivation from latency as described in Materials and Methods. (A) The frequency of splenocytes harboring latent MHV68 was significantly reduced in the *prdm1*^{c/c} *CD19*^{Cre/+} mice infected via intranasal inoculation. (B) A lower frequency of splenocytes capable of reactivation from latency was also observed in the *prdm1*^{c/c} *CD19*^{Cre/+} mice following intranasal infection. (C) The frequency of latently infected splenocytes was reduced in *prdm1*^{c/c} *CD19*^{Cre/+} mice infected via intraperitoneal inoculation. (D) There was a further reduction in the frequency of splenocytes reactivating from latency in *prdm1*^{c/c} *CD19*^{Cre/+} mice infected intraperitoneally. (E and F) No significant difference in the establishment of latency or reactivation from latency was observed in PECs isolated from MHV68-infected *prdm1*^{c/c} or *prdm1*^{c/c} *CD19*^{Cre/+} mice. Data represent results from two to three independent experiments with three to five mice per experimental group.

CD8⁺ T cells that share the V β 4 chain of the TCR (15, 53). Notably, the V β 4⁺ CD8⁺ T-cell population fails to contract over time, and we have previously shown that the M1 ORF is required for the expansion of this population of T cells *in vivo* (14). We have shown that M1 limits the degree of reactivation *in vivo* through induction of gamma interferon, and B cells are required for the V β 4⁺ CD8⁺ T-cell expansion (6, 11). Thus, we hypothesized that during an infection where reactivation is decreased from B cells, we might observe a defect in the

expansion of this T-cell subset if M1 expression is tied to MHV68 reactivation. However, over the course of the infection, we observed no significant decrease in the frequency of V β 4⁺ CD8⁺ T cells in the *prdm1*^{c/c} mice infected via intraperitoneal inoculation with MHV68/Cre (Fig. 7A). These data argue that Blimp-1-mediated virus reactivation is not necessary for the expansion of the V β 4⁺ CD8⁺ T-cell population in the spleen.

We next assessed the CD4⁺ T-cell response to MHV68

FIG. 6. The B-cell response to MHV68 infection is lower in the absence of a functional Blimp-1 gene in infected cells. *Prdm1^{c/c}* mice were infected with 1,000 PFU of MHV68/Cre or MHV68/Cre.MR. At day 18 postinfection, spleens were harvested and single-cell suspensions were stained with CD19-APC, CD69-PE-Cy7, CD95-PE, and GL7-FITC or CD19-APC, IgD-PE, and IgG/A/E-FITC and analyzed by flow cytometry. (A) Representative flow cytometry data from the median mice per group. Cells were gated on live lymphocytes as determined by forward and side scatter followed by discrimination of B cells by gating on CD19⁺ lymphocytes. (B) Fewer B cells from *prdm1^{c/c}* mice infected with MHV68/Cre were activated (CD69^{high}). (C) A lower frequency of B cells from *prdm1^{c/c}* mice infected with MHV68/Cre entered the germinal center reaction (CD95^{high} GL7^{high}). (D) There was a significant decrease in the frequency of isotype-switched (IgD^{low} IgG/A/E⁺) B cells in *prdm1^{c/c}* mice infected with MHV68/Cre. Data represent results from two independent infections with three to four animals per group and two naïve control mice per experiment. *, $P = 0.0027$; **, $P = 0.0015$; ***, $P = 0.0043$.

infection by measuring the frequency of effector CD4⁺ T cells in the spleen (CD62L^{low} CD44^{high}). We observed a 20% decrease in the frequency of effector CD4⁺ T cells in the *prdm1^{c/c}* mice infected with MHV68/Cre intraperitoneally at day 18 postinfection (51% for MHV68/Cre-infected mice versus 63% for MHV68/Cre.MR-infected mice) (Fig. 7B). However, during a long-term MHV68 infection, there was no significant difference in the effector CD4⁺ T-cell response in the two groups of animals, and the CD4 response was largely quiescent in both groups by day 90 postinfection (Fig. 7B). We observed no difference in the CD8⁺ effector T-cell populations in the

two groups of mice throughout the time course of infection (data not shown).

Blimp-1 is necessary for the prolonged antibody response to MHV68 *in vivo*. MHV68 infection leads to a delayed increase in MHV68-specific serum IgG beginning at day 20 postinfection that is maintained for more than 100 days postinfection (47). We asked if the loss of Blimp-1 expression was associated with a decrease in the antibody response to MHV68. Beginning at day 42 postinfection, we observed a significant 4-fold decrease in the levels of MHV68-specific IgG in the *prdm1^{c/c}* mice infected with MHV68/Cre intraperitoneally versus those

FIG. 7. T-cell responses in *prdm1^{c/c}* mice infected with MHV68/Cre. *Prdm1^{c/c}* mice were infected intraperitoneally with 1,000 PFU of MHV68/Cre or MHV68/Cre.MR, and splenocytes were harvested at days 18, 42, and 90 postinfection. Cells were stained with Vβ4-FITC, CD44-PE, CD4-PerCP, CD62L-APC, and CD8-Pacific Blue and analyzed by flow cytometry. (A) The Vβ4⁺ CD8⁺ T-cell population expands to wild-type frequencies in *prdm1^{c/c}* mice infected with MHV68/Cre. (B) Fewer effector CD4⁺ (CD62L^{low} CD44^{high}) T cells were observed in *prdm1^{c/c}* mice infected with MHV68/Cre at day 18 postinfection. At days 42 and 90, there was no significant difference in the CD4⁺ T-cell response in the two groups of mice. Data represent results of two independent experiments with three to four mice per infection with two naïve controls per experiment.

infected with MHV68/Cre.MR (Fig. 8). Importantly, the lower antibody titers in the *prdm1^{c/c}* MHV68/Cre-infected animals persisted until the end of the time course at day 90 postinfection. Importantly, as noted above, this experimental approach

FIG. 8. MHV68-specific antibody responses are decreased in *prdm1^{c/c}* mice infected with MHV68/Cre. *Prdm1^{c/c}* mice were infected with MHV68/Cre and MHV68/Cre.MR, and serum MHV68-specific IgG levels were measured by quantitative ELISA. Data represent results of three independent experiments with three to four infected mice per group with two naïve controls per experiment (*, $P < 0.0001$; **, $P = 0.0010$).

FIG. 9. A functional Blimp-1 gene is necessary for long-term maintenance of MHV68 latency. *Prdm1^{c/c}* mice were infected via intraperitoneal inoculation with 1,000 PFU of MHV68/Cre or MHV68/Cre.MR, and the frequency of latently infected splenocytes was determined by limiting-dilution PCR at days 42 (A) and 90 (B) postinfection. Data represent results of three independent experiments with three to four mice per group.

leads to a loss of Blimp-1 only in infected B cells, which represent <1% of B cells in the infected mouse. Previously a transient decrease in MHV68-specific IgG was observed in *MyD88^{-/-}* animals, but as the infection progressed, antibody levels became equivalent to wild-type levels (19). We observed no such return of antibody titers, suggesting that perhaps differentiation to a plasma cell phenotype and the associated reactivation from latency are needed to maintain the high levels of MHV68-specific IgG observed in wild-type mice.

Loss of Blimp-1 expression in infected splenocytes leads to a decrease in latency by day 90 postinfection. We next examined the requirement of Blimp-1 expression for the maintenance of MHV68 latency at late time points (day 42 and day 90) postinfection. Mice were inoculated intraperitoneally with 1,000 PFU of MHV68/Cre and MHV68/Cre.MR, splenocytes were harvested at day 42 and day 90, and latency was measured by limiting-dilution PCR. At day 42 postinfection, there was no significant difference in the frequency of latently infected splenocytes between the two groups of mice (1/8,953 splenocytes harbored latent MHV68/Cre compared to 1/3,295 splenocytes in MHV68/Cre.MR animals, a roughly 3-fold difference in the frequency of latently infected splenocytes) (Fig. 9A). Interestingly, we observed a significant decrease in the frequency of latently infected splenocytes at day 90 postinfection in the mice infected intraperitoneally with MHV68/Cre (Fig. 9B). Only 1/19,210 splenocytes from MHV68/Cre mice carried latent MHV68, compared to 1/2,350 splenocytes from MHV68/Cre.MR animals, an 8-fold decrease in latency (Fig. 9B). These results are significant because previously characterized mutations affecting latency and reactivation have resulted

in an eventual return to wild-type levels of latency at late times postinfection (19, 21). Although longer infection time points will be required to adequately address the role of Blimp-1 in maintaining chronic MHV68 infection, these data suggest that Blimp-1 plays a role, presumably through episodic reactivation and reseeding of the latency reservoirs.

DISCUSSION

In this report, we demonstrate that Blimp-1 expression plays a role in both the establishment of latency and reactivation from splenic latency. Importantly, while the observed difference in the initial establishment of latency in the spleen at day 18 between Blimp-1-sufficient and Blimp-1-deficient latently infected B cells was largely abrogated by day 42 postinfection, the loss of Blimp-1 expression in infected splenocytes ultimately led to a significant decrease in long-term latency discernible by day 90 postinfection in the spleen. Notably, this decrease in viral latency was coupled with significantly lower levels of MHV68-specific antibodies. Together, these results support a model in which periodic reactivation of MHV68 from the plasma cell compartment is necessary for long-term latency maintenance as well as the protracted, high serum levels of MHV68-specific antibodies observed in infected mice.

Notably, murine gammaherpesvirus 68 carries genes capable of facilitating virus reactivation (21, 55). One such gene, designated M2, encodes a latency-associated protein that plays a role in both the establishment of latency and reactivation from latency (21, 22, 33). M2 expression in primary murine B cells leads to B-cell proliferation, survival, and differentiation of primary murine B cells in culture to a preplasma memory B-cell phenotype (44). M2-mediated proliferation is dependent on interleukin 10 (IL-10), and M2 expression in B cells leads to copious secretion of IL-10 *in vitro*, as well as upregulating secretion of IL-2, IL-6, and MIP-1 α (44). Additionally, infection of mice with an M2-null strain of MHV68 leads to a significant reduction in serum IL-10 levels compared to those for wild-type virus (44). Notably, IL-10 interrupts human B cells in the germinal center and triggers them to differentiate into plasma cells (10). Consistent with the latter observation, we have recently shown that the M2 gene is necessary for the appearance of MHV68-infected plasma cells in the spleen at the peak of viral latency (31). Furthermore, the majority of the spontaneous MHV68 reactivation observed upon explant of splenocytes, harvested at day 18 postinfection, arises from the plasma cells (31). These studies directly link M2, a specific MHV68 viral protein that plays a critical role in virus reactivation from B cells, to the manipulation of plasma cell differentiation *in vivo*. Finally, M2 expression in the murine B lymphoma cell line BCL-1 leads to upregulation of Blimp-1, XBP-1s, and IRF4 transcripts, directly linking M2 and plasma cell differentiation (31). Thus, we hypothesize that MHV68 is able to directly regulate plasma cell differentiation and subsequent virus reactivation, removing any obligatory requirement for antigenic stimulation to initiate plasma cell differentiation-associated virus reactivation.

Although highly compromised, reactivation was not completely eliminated in the plasma cell-deficient animals. This is consistent with our recent observation that plasma cells recovered from infected mice accounted for much, but not all, of the

spontaneous reactivation observed upon explant of splenocytes into tissue culture (31). As discussed above, it is likely that reactivation from the latently infected non-B-cell reservoir (e.g., macrophages and dendritic cells) contributes to the reactivation observed in the absence of plasma cells. In addition, it is likely that there are other pathways to MHV68 reactivation from B cells that are independent of Blimp-1 and plasma cell differentiation (e.g., stress response pathways). These alternative pathways of virus reactivation are echoed in the humoral response to MHV68, which, although significantly lower in the absence of Blimp-1, is still maintained. The antibody response likely reflects the low levels of persistent viral reactivation that cannot be measured by *ex vivo* limiting-dilution reactivation assays but nonetheless continues to occur.

Plasma cell differentiation triggers reactivation in both EBV and KSHV. The transcription factor X-box binding protein 1s (XBP-1s) is required for plasma cell differentiation, and XBP-1s expression in latently EBV- and KSHV-infected cell lines leads to reactivation (38). Expression of XBP-1s in latently KSHV-infected B cells leads to transactivation of the RTA promoter and expression of the lytic protein K8 (60). XBP-1s binds directly to the RTA promoter, leading to induction of KSHV reactivation (59). In latently infected epithelial cells and lymphoblastoid cells, XBP-1s, in combination with protein kinase D, induces transcription of the immediate-early BZLF1 and BRLF1 genes encoding the two viral proteins capable of activating the entire EBV lytic cascade (5). Furthermore, as previously mentioned, XBP-1s binds the BZLF1 promoter, directly linking plasma cell differentiation and EBV reactivation (49). It is unknown whether there is a similar mechanism of induction of RTA expression in MHV68 that is activated by transcription factors associated with plasma cell differentiation. Due to the parallel requirement for plasma cell differentiation in EBV, KSHV, and MHV68 reactivation, it is likely that a similar mechanism exists to drive MHV68 immediate-early gene expression leading to virus reactivation.

How is periodic MHV68 reactivation triggered *in vivo*? Stimulation of B cells through the B-cell receptor (BCR), Toll-like receptors (TLRs), and CD40, as well as exposure to IL-21, IL-2, IL-6, IL-5, and IL-10, have been shown to lead to *prdm1* expression (reviewed in reference 7). Stimulation of latently infected splenocytes with anti-IgM/IgG and anti-CD40 leads to an increase in reactivation *ex vivo*, implying that direct BCR stimulation of latently infected memory B cells with their cognate antigen could lead to plasma cell differentiation and reactivation (36). However, the likelihood of a memory B cell encountering the specific antigen that its BCR recognizes is very low. Based on data presented here, we proposed that reseeding of latency reservoirs plays an important role in maintaining chronic infection; thus, it seems likely that mechanisms other than latently infected B cells encountering cognate antigen are involved in promoting virus reactivation *in vivo*. As discussed above, one such mechanism is the ability of the MHV68 M2 antigen to drive plasma cell differentiation and subsequent virus reactivation (31). We also hypothesize that stimulation of memory B cells with a heterologous pathogen through TLRs may also play an important role. Indeed, humoral memory is known to be maintained through TLR signaling (4). *In vivo* stimulation with TLR ligands leads to an increase in MHV68 reactivation, seeding more latently in-

fects splenocytes, "refilling" the latency reservoir (18). Additionally, MyD88-deficient mice have a significant defect in reactivation from latency (19). Mice deficient in TLR signaling have a decreased ability to form germinal centers, express Blimp-1, and secrete antibody in a T-dependent fashion (37). EBV LMP2a has been shown to enhance B-cell sensitivity to TLR9 stimulation (56). Thus, signaling through TLRs may be a shared mechanism involved in driving gammaherpesvirus reactivation *in vivo*.

In conclusion, Blimp-1 expression in MHV68-infected splenocytes plays a role in both the establishment of latency and reactivation from latency. We have demonstrated that mice lacking the entire plasma cell compartment have a phenotype similar to that of those mice in which only infected B cells have lost the ability to express a functional Blimp-1 gene product. It seems likely that the role of Blimp-1 in the establishment of splenic latency is associated with virus reactivation and subsequent replication in the spleen, which serves to seed latency. Notably, we provide evidence to support a model in which periodic reactivation from latency is needed for long-term maintenance of MHV68 genomes and the sustained MHV68-specific humoral response. Moreover, these studies extend the tissue culture models of EBV and KSHV reactivation, demonstrating another parallel aspect of gammaherpesvirus pathogenesis that is conserved across species.

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