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B cell expression of E3 ubiquitin ligase Cul4b promotes chronic gammaherpesvirus infection *in vivo*

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ABSTRACT The gammaherpesviruses, including human Epstein-Barr virus, human Kaposi's sarcoma-associated herpesvirus, and murine gammaherpesvirus 68 (MHV68, γHV68, MuHV-4), are ubiquitous pathogens that directly contribute to the genesis of a wide variety of malignancies, including B cell lymphomas. *In vivo*, these viruses infect naïve B cells and then usurp B cell signaling pathways to drive infected cells, independent of antigen stimulation, through germinal center (GC) reactions and thereby establish lifelong latency in the memory B cell compartment. However, the specific molecular determinants by which these viruses establish chronic latent infection in B cells *in vivo* remain largely unknown. The E3 ubiquitin ligase Cullin 4b (Cul4b) is well recognized as a central player in regulating cell proliferation, DNA damage repair, and gene transcription by catalyzing the ubiquitination and degradation of numerous cellular proteins. Here, we determined whether B cell-intrinsic expression of Cul4b is required for chronic gammaherpesvirus infection *in vivo*. Through MHV68 infection of mice with B cell-specific Cul4b deficiency, we found that loss of Cul4b expression in B cells severely impeded the establishment of latency at peripheral sites. In particular, a lack of Cul4b expression in B cells significantly attenuated the expansion of virus-infected GC B cells, suggesting that gammaherpesviruses may manipulate a previously unknown function of Cul4b in GC B cell biology. Cumulatively, these findings demonstrate that Cul4b promotes chronic gammaherpesvirus infection *in vivo*.

IMPORTANCE The human gammaherpesviruses Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus are etiologic agents of numerous B cell lymphomas. A hallmark of gammaherpesvirus infection is their ability to establish lifelong latency in B cells. However, the specific mechanisms that mediate chronic infection in B cells *in vivo* remain elusive. Cellular E3 ubiquitin ligases regulate numerous biological processes by catalyzing ubiquitylation and modifying protein location, function, or half-life. Many viruses hijack host ubiquitin ligases to evade antiviral host defense and promote viral fitness. Here, we used the murine gammaherpesvirus 68 *in vivo* system to demonstrate that the E3 ligase Cul4b is essential for this virus to establish latency in germinal center B cells. These findings highlight an essential role for this E3 ligase in promoting chronic gammaherpesvirus infection *in vivo* and suggest that targeted inhibition of E3 ligases may provide a novel and effective intervention strategy against gammaherpesvirus-associated diseases.

KEYWORDS B cell, E3 ligase, Cullin 4b (Cul4b), germinal center, herpesvirus, gammaherpesvirus, murine gammaherpesvirus 68 (MHV68), latency

The human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) are etiologic agents of numerous types of malignancies, including Burkitt B cell lymphoma, Hodgkin's B cell lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and Kaposi's sarcoma (1, 2). One hallmark of

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gammaherpesvirus infection is the establishment of lifelong latency in B cells, which is vital for chronic infection and virus-associated tumorigenesis. A central aspect of this process is the manipulation of germinal center (GC) reactions. GCs are specialized niches in secondary lymphoid tissue in which antigen-activated follicular B cells undergo extensive proliferation and selection, providing a critical gateway for the differentiation of naïve follicular B cells into antigen-experienced, long-lived plasma cells and memory cells. Gammaherpesviruses are thought to mimic this natural process by driving infected naïve follicular B cells, independent of antigen stimulation, into GCs, where they proliferate and expand before differentiating into resting memory B cells, which serve as the primary long-term latency reservoir (3–5). However, the specific underlying mechanisms by which these human gammaherpesviruses establish chronic latent infection in B cells *in vivo* remain poorly understood due to their strict species restriction. Murine gammaherpesvirus 68 (MHV68, MuHV-4, γHV68) is a natural pathogen of murid rodents that is genetically and pathogenically related to EBV and KSHV (6, 7). Like the human gammaherpesviruses, MHV68 establishes chronic latent infection in B cells (6, 7) and directly contributes to the development of lymphoproliferative diseases and B cell lymphomas (8, 9). Thus, MHV68 infection in mice represents a highly tractable model system to define key virus and host determinants of *in vivo* gammaherpesvirus infections.

Protein ubiquitylation is a form of posttranslational modification that plays a critical role in almost every biological process (10, 11). Ubiquitylation occurs through sequential enzymatic reactions that are carried out by a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligating enzyme (E3) that catalyzes the final ligation of ubiquitin to a lysine residue on the target protein (10, 11). In contrast to the limited number of E1 and E2 enzymes, mammalian genomes encode more than 600 E3 ligases, providing this group the ability to bind specifically to a wide range of substrates and thereby more precisely regulate diverse biological processes (12). The Cullin-RING ligases (CRL) are the largest family of E3 ligases, catalyzing the ubiquitylation of approximately 20% of cellular proteins that undergo proteasome-mediated degradation (13). As such, the CRLs regulate a wide range of biological processes, including cell development, differentiation, proliferation, apoptosis, gene transcription and translation, signal transduction, and cell metabolism (14).

The CRLs are multi-subunit E3 ligases in which a Cullin family protein functions as a scaffold to bring together the target substrate (bound to a substrate receptor and adaptor proteins) and an E2 enzyme (bound to a RING-domain-containing protein) (14). Either of two highly homologous Cullin proteins, Cul4a and Cul4b, can form the backbone for Cullin RING ligase 4 (CRL4) E3 ligases, complexes that are critical for promoting DNA integrity in rapidly proliferating cells (15). Cul4a and Cul4b exert overlapping but not fully redundant functions. While both Cul4a and Cul4b promote DNA damage response and proliferation in several forms of cancer, the absence of Cul4b in T cells severely attenuates T cell proliferation despite the presence of intact Cul4a (16). In T cells, Cul4b associates with chromatin, which may facilitate its role in maintaining DNA integrity during rapid clonal expansion (16). Although activated B cells proliferate at a rapid rate similar to activated T cells and cancer cells, the importance of Cul4b in B cells is unknown.

Gammaherpesviruses such as EBV and MHV68 are well known for their ability to manipulate normal B cell signaling pathways in order to establish stable, lifelong infection in the B cell compartment. In the work described here, we examine the requirement of Cul4b expression in B cells for *in vivo* MHV68 infection. We show that B cell-specific Cul4b deficiency severely impaired the establishment of *in vivo* latency. In particular, loss of Cul4b in B cells significantly attenuated latent infection of GC B cells. Taken together, these findings demonstrate that B cell-intrinsic expression of Cul4b is a critical determinant for the establishment of chronic gammaherpesvirus infections.

RESULTS

B cell-specific expression of Cul4b promotes the establishment of gammaherpesvirus latency *in vivo*

MHV68 primarily infects and establishes latent infection in B cells (17, 18). To determine whether Cul4b was required for MHV68 infection in B cells *in vivo*, we generated mice with B cell-specific deletion of Cul4b. Mice homozygous for LoxP-flanked (floxed) *Cul4b* exon 4 ($Cul4b^{fl/fl}Mb1^{+/+}$; designated Cul4b WT mice) (16) were crossed with Mb1-Cre mice (19) to generate conditional knockout (CKO) mice in which Cul4b expression is specifically abrogated in B cells ($Cul4b^{fl/fl}Mb1^{Cre/+}$; designated Cul4b CKO mice). PCR analysis of genomic DNA demonstrated the presence of the *Cul4b* floxed region (Fig. 1A) and the *Cre* transgene in Cul4b CKO mice (Fig. 1B). Further, western blot for Cul4b protein expression in purified B cells validated that excision of *Cul4b* exon 4 resulted in a complete loss of Cul4b in B cells in both unstimulated and CpG-stimulated conditions (Fig. 1C). Cul4b CKO mice are healthy, with normal body weights and lifespans, and offspring are generated with expected Mendelian ratios.

To determine whether B cell-specific deletion of Cul4b altered normal B cell development, we first examined basal levels of B cell subsets in Cul4b WT vs CKO mice using flow cytometric analyses. In the spleen, Cul4b CKO mice demonstrated reproducible decreases in the absolute numbers of total B cells (~34% reduction). The distribution and numbers of mature naïve cells in the spleen were also skewed, with decreased follicular B cells (~42% reduction) and increased marginal zone B cells (~56% increase). In contrast, Cul4b KO mice demonstrated no appreciable difference in activated B cell subsets at baseline, and the numbers of GC B cells and switched memory B cells in the spleen were comparable to WT mice (Fig. 2A). The differences in follicular and marginal zone B cells were not the result of a global defect in B cell development, as immature B cell populations in the bone marrow were not affected by Cul4b deficiency (Fig. 2B). Consistent with this conclusion, Cul4b CKO mice demonstrated a reduction in basal IgM levels (~25% decrease), with no significant change in basal IgG1 or IgG2c levels (Fig. 2C). In summary, B cell Cul4b modulated the numbers of marginal zone and follicular B cells in the spleen but had no impact on basal levels of developing, immature, or spontaneously activated B cells. Thus, Cul4b shapes the establishment or maintenance of mature B cell pools but does not impact the baseline levels of spontaneously activated B cells or circulating IgG.

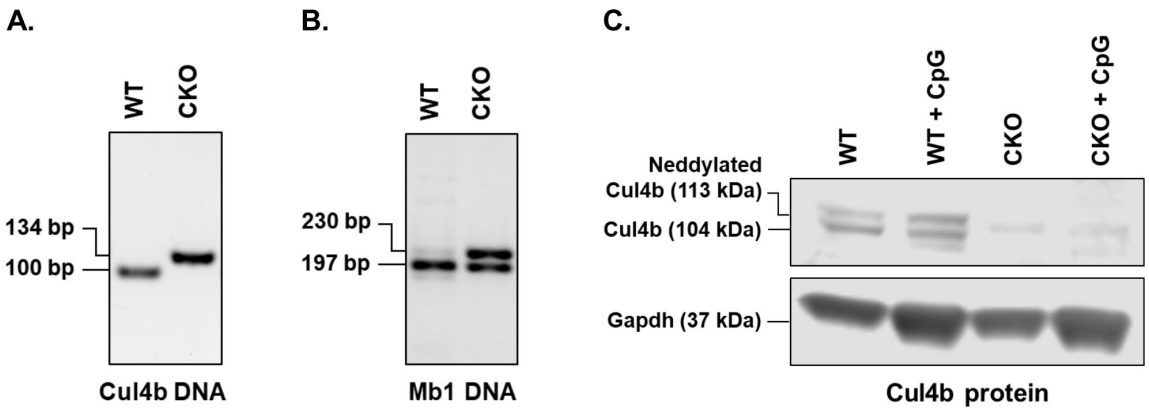

FIG 1 Generation of conditional knockout mice with B cell-specific deletion of Cul4b. (A and B) Genotyping Cul4b CKO mice by genomic PCR. DNA was extracted from mouse tail samples, and PCR was then performed to confirm the presence of the floxed region (A) and the Cre transgene (B) in the Cul4b CKO mice. In (A), the 100-bp amplicon indicates the WT alleles, and the 134-bp amplicon represents the region containing the 5′ LoxP site. In (B), the 197-bp amplicon indicates the WT alleles, and the 230-bp amplicon represents a portion of the Cre transgene. (C) Validation of the Cul4b CKO mouse genotype by western blots. B cells from Cul4b WT and CKO mice were isolated by immunomagnetic negative selection and then unstimulated or stimulated with CpG for 24 hours. The protein lysates were then harvested, and the Cul4b protein expression was determined by western blots. Gapdh was included as a loading control.

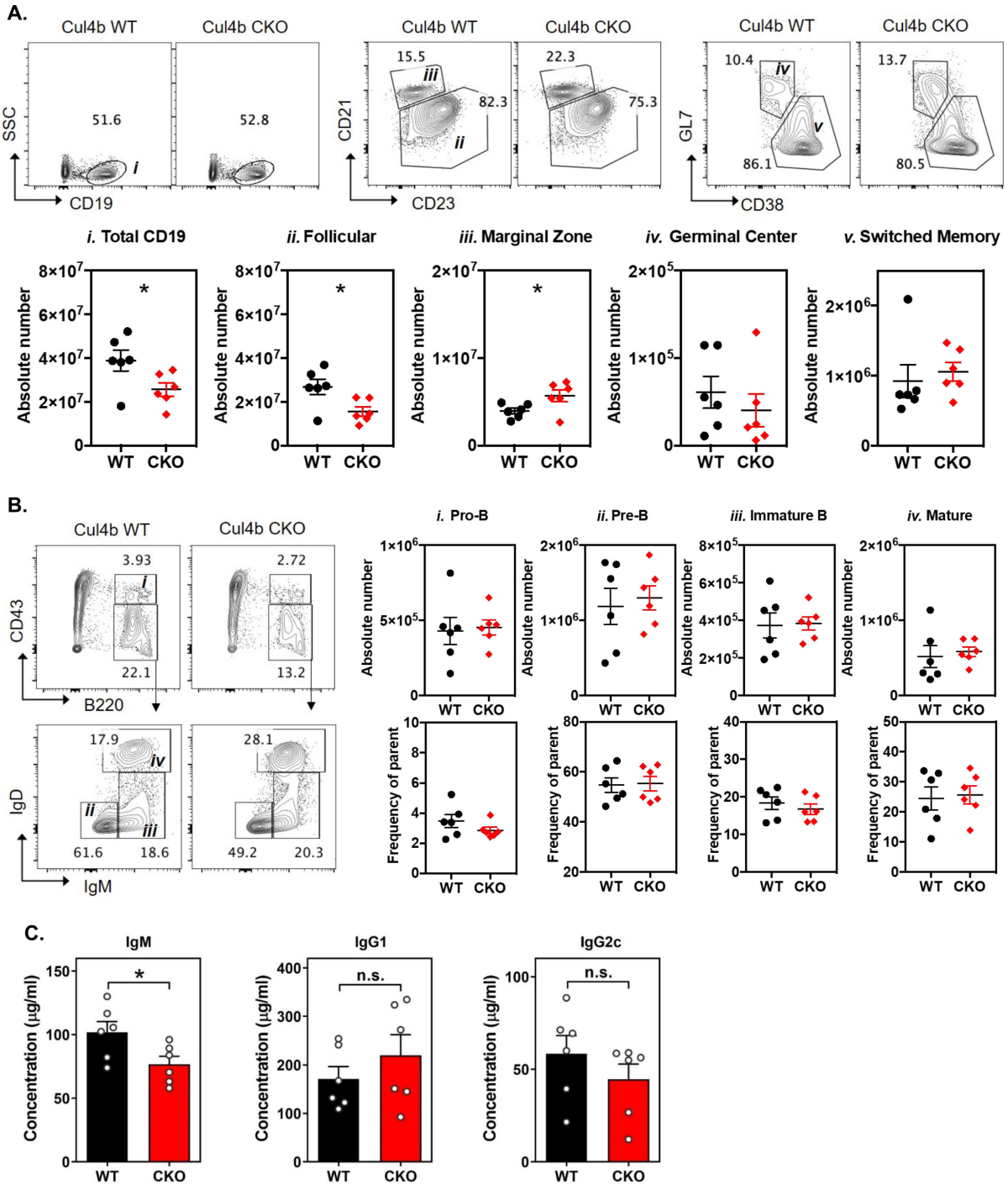

FIG 2 B cell subsets in uninfected mice. (A–C) Spleen, bone marrow, and serum were harvested from 8- to 10-week-old Cul4b WT and CKO mice. (A) Representative flow plots and quantification of spleen B cell subsets. Gating of flow plots: left, live singlets; center, live > singlet > $CD19^+$; and right live > singlet > IgM^-IgD^- > $CD19^+$. (B) Representative flow plots and quantification of bone marrow B cell subsets. Flow plots were gated on live > singlets. (C) Serum antibodies in naïve mice were quantified by enzyme-linked immunosorbent assay. The values represent the means ± SEM of all mice over three independent experiments. Each symbol represents an individual mouse. Significance was determined by a two-tailed, unpaired *t*-test (*, $P < 0.05$). n.s.,not significant.

Following intranasal inoculation, MHV68 undergoes lytic replication in the lungs prior to the establishment of latent infection in B cells at peripheral sites. To first determine whether Cul4b deficiency in B cells impacts lytic virus infection, we quantified MHV68 replication in the lungs of CKO mice. WT and Cul4b CKO mice were intranasally (i.n.)

inoculated with 10^4 PFU MHV68.H2bYFP virus, a phenotypically wild-type (WT) virus that expresses eYFP under the control of the H2b promoter (20). Lung viral titers were quantified at 7 days post-infection (dpi) (Fig. 3A). Although the mean virus titer in the lungs of Cul4b CKO mice was slightly decreased compared to that of WT control mice, this minor difference was not statistically significant. Therefore, these results demonstrated that loss of Cul4b expression in B cells did not significantly impact lytic virus replication *in vivo*.

To determine whether B cell-intrinsic expression of Cul4b was required for latent infection, we quantified standard parameters of chronic virus infection. WT and Cul4b CKO mice were inoculated i.n. with 10^4 PFU virus. At 16 days, spleens were harvested and weighed, and splenocytes were subjected to limiting dilution nested PCR analysis (21–23) to determine the frequency of cells carrying the virus genome. In mock-infected control experiments, WT and Cul4b CKO mice exhibited no apparent difference in spleen weights (WT, mean 71 mg; CKO, mean 82 mg) (Fig. 3B). In contrast, following infection, the virus-induced splenomegaly observed in Cul4b CKO mice (mean 142 mg) was substantially diminished compared to that of WT control mice (mean 291 mg)

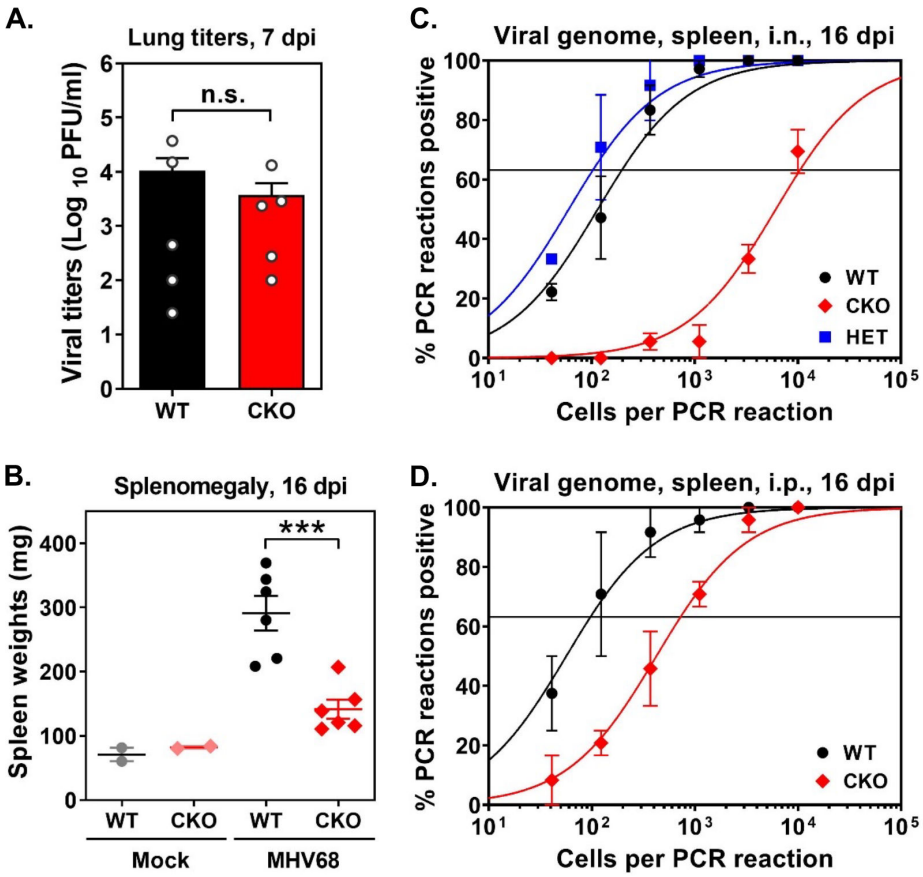

FIG 3 B cell-specific deletion of Cul4b attenuates the establishment of gammaherpesvirus latency *in vivo*. (A) Viral titers during acute lytic replication in the lungs of Cul4b WT and CKO mice at 7 dpi. The values represent means ± SEM of a single experiment. Each symbol represents an individual mouse. Significance was determined by a two-tailed, unpaired *t*-test (n.s., not significant). (B) Splenomegaly. The Cul4b WT and CKO mice were mock-inoculated or inoculated with MHV68.H2bYFP virus, the spleens were harvested and weighed at 16 dpi. The values represent means ± SEM of two independent experiments. Each symbol represents an individual mouse. Significance was determined by a two-tailed, unpaired *t*-test (***, $P < 0.001$). (C and D) Frequencies of latently infected splenocytes during the establishment phase of latency. At 16 dpi, the splenocytes from Cul4b WT, CKO, and HET (heterozygous) mice were prepared and subjected to LDPCR for the detection of the viral genome. The frequencies of splenocytes carrying the viral genome were determined by a Poisson distribution from non-linear regression, as indicated by the line at 63.2%. The values represent means ± SEM of two independent experiments.

(Fig. 3B). Further, while infection of WT mice resulted in virus establishment latency in approximately 1 in 200 splenocytes (Fig. 3C), infection of Cul4b CKO mice resulted in a striking 53-fold reduction in latently infected splenocytes, to a frequency of 1 in 10,500. This attenuation was not simply the result of a defect in hematogenous dissemination, as latency establishment remained highly attenuated in the spleen even when dissemination was bypassed by delivering the virus via intraperitoneal inoculation (Fig. 3D). Together, these results demonstrated that B cell-intrinsic expression of Cul4b was required for the establishment of MHV68 latency *in vivo*.

To determine whether the profound latency attenuation in Cul4b CKO mice was accompanied by compromised reactivation, we performed limiting dilution *ex vivo* reactivation analyses (21, 22). In accordance with our previous findings (22, 24), spontaneous reactivation from latency occurred in the splenocytes of WT mice at a frequency of 1 in 15,800 (Fig. 4A). In contrast, the frequency of reactivation in splenocytes of Cul4b CKO mice was reduced to a level below the detection limit (regression estimate 1 in 3,600,000), consistent with the striking attenuation in latency establishment observed in virus genome assays. As expected, parallel splenocyte samples from both mouse strains demonstrated very little or no detectable preformed infectious virus at the time of harvest (Fig. 4B), indicating no ongoing lytic replication. Cumulatively, these findings demonstrated that B cell-specific expression of Cul4b promotes the establishment of the MHV68 latency reservoir during *in vivo* infection.

B cell-specific expression of cul4b promotes the expansion of gammaherpesvirus-infected germinal center B cells

Gammaherpesviruses, including EBV and MHV68, are thought to mimic antigen-specific BCR and co-stimulatory signals to intrinsically activate infected naive follicular B cells. Mimicking antigen-specific signaling events, intrinsic activation of these pathways drives infected cells through the GC reaction, resulting in proliferation and expansion of the latently infected B cell reservoir (3–5). Terminal differentiation of these cells results in a circulating reservoir of latently infected resting memory B cells (3–5). Therefore, GC B cells play a central role in gammaherpesvirus-driven B cell differentiation, and infected GC B cells represent a critical nexus for mutational events that can result in gammaherpesvirus-induced transformation. In the context of natural infection, latent

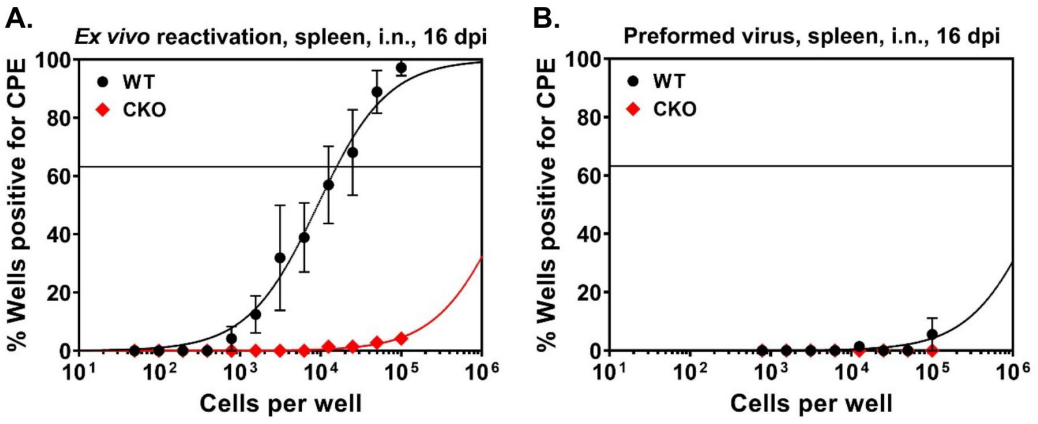

FIG 4 B cell-specific deletion of Cul4b reduces gammaherpesvirus reactivation *ex vivo*. (A) Frequencies of reactivation from latency. The Cul4b WT and CKO mice were infected i.n. with the MHV68.H2bYFP virus for 16 days. The splenocytes were then harvested and subjected to a limiting dilution *ex vivo* reactivation assay for the presence of the reactivating virus. The frequencies of splenocytes carrying reactivating viruses were determined by a Poisson distribution from non-linear regression, as indicated by the line at 63.2%. (B) Frequencies of splenocytes harboring preformed viruses. Parallel splenocyte samples from (A) were mechanically disrupted prior to plating on mouse embryonic fibroblast monolayers. The frequencies of splenocytes carrying preformed viruses were determined by a Poisson distribution from non-linear regression, as indicated by the line at 63.2%.

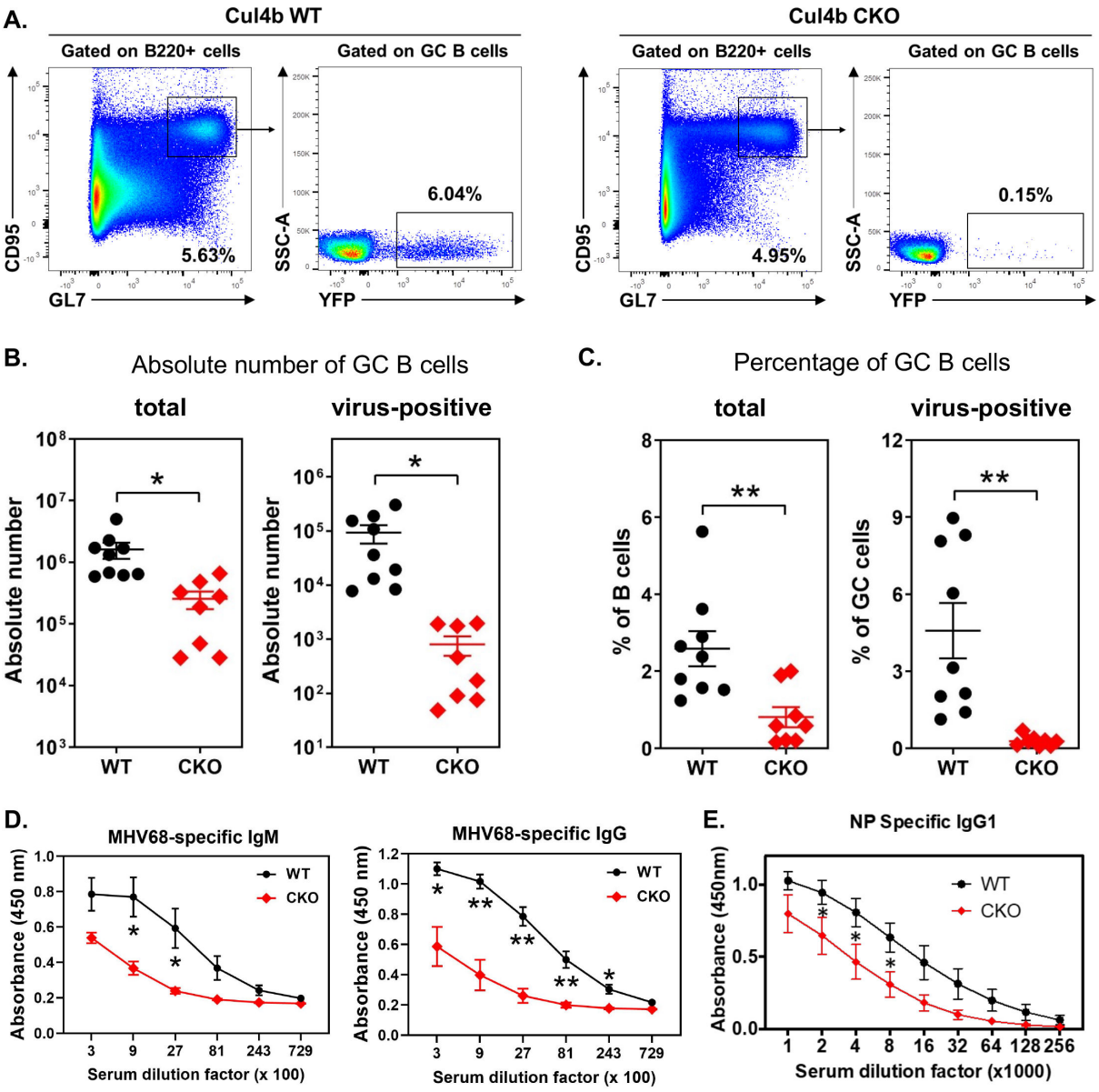

FIG 5 B cell-specific deletion of Cul4b decreases gammaherpesvirus latency in germinal center B cells. The Cul4b WT and CKO mice were infected i.n. with MHV68.H2bYFP virus for 16 days, the splenocytes were then harvested and subjected to flow cytometric quantification for total and virus-positive (YFP^+) GC B cells ($B220^+$ $GL7^+$ $CD95^+$). (A) The representative flow plots show the gating of YFP^+ GC B cells. (B and C) The absolute number and percentage of total GC B cells and virus-positive GC B cells. The values represent the means ± SEM of all mice over two independent experiments. Each symbol represents an individual mouse. (D) MHV68-specific IgM and IgG antibody levels at 16 days post-infection. (E) NP-specific IgG1 antibody levels at 14 days post-immunization. Significance was determined by a two-tailed, unpaired *t*-test (*, $P < 0.05$; *, $P < 0.01$).

virus is preferentially maintained in GC B cells during the expansion phase of latency (20, 25, 26). Thus, we sought to determine whether Cul4b was specifically required for efficient infection of the GC B cell compartment. To test this possibility, Cul4b WT and CKO mice were inoculated i.n. with the MHV68.H2bYFP virus. After 16 days, splenocytes were harvested for flow cytometric quantification (Fig. 5A). The absolute number of total GC B cells ($B220^+$ $GL7^+$ $CD95^+$) was sixfold reduced (WT 1,618,650 vs CKO 255,053) in infected Cul4b CKO mice compared to WT mice (Fig. 5B), supporting the idea that Cul4b is important in antigen-driven GC B cell responses. Intriguingly, this disparity was significantly amplified in virus-infected cells (Fig. 5C), with the number of infected GC B cells ($B220^+$ $GL7^+$ $CD95^+$ YFP^+) reduced 116-fold in Cul4b CKO mice as compared to

WT mice (WT 93,158 vs CKO 805). These findings were also reflected in the percentages of GC B cells, with the overall percentage of GC B cells decreasing in CKO mice by 3-fold and the percentage of infected GC B cells decreasing in CKO mice by 15-fold. To determine if Cul4b regulated antigen-driven GC responses, we examined the levels of MHV68-specific IgM and IgG in the serum on day 16 post-infection, and found that CKO mice had significantly reduced levels of antigen-specific antibodies (Fig. 5D). To exclude the possibility that the reduction of virus-specific antibodies was due to differences in antigen loads between the two strains, we examined humoral responses after immunization with the non-replicating T-dependent antigen NP-ova. Mice were immunized with NP-ova in alum, and levels of NP-specific serum IgG1 were measured after 14 days (Fig. 5E). Consistent with the results in virus-infected animals, CKO mice demonstrated a fourfold decrease in NP-specific IgG1 following immunization. Together, these data demonstrate that B cell-intrinsic expression of Cul4b (a) promotes antigen-driven GC B cell responses and (b) plays a profound role in the accumulation of large numbers of the MHV68 latently infected GC B cells.

Finally, to determine whether the attenuation of latency was sustained throughout the maintenance phase of chronic infection, we infected WT and Cul4b CKO mice with virus for 45 days, then harvested spleens for latency analyses. Cul4b CKO mice demonstrated an apparent reduction in the frequency of splenocytes harboring viral genomes as compared to WT control mice (Fig. S1), although this difference was not statistically testable due to the low frequency of infection at this time point. These data strongly suggest that B cell-intrinsic Cul4b expression promotes the maintenance of MHV68 long-term latency *in vivo*; however, future experiments will be required to define the full impact of Cul4b on long-term infection.

DISCUSSION

In the work presented here, we identified Cul4b, a scaffolding component of the multi-subunit CRL family E3 ubiquitin ligases, as a critically important host factor that promotes gammaherpesvirus latency *in vivo*. Cul4b is a scaffold protein that assembles the Cul4b-RING E3 ligase complex (CRL4) by binding its N-terminus to adaptor protein DDB1 (damaged DNA binding protein 1), which interacts with a substrate receptor, and binding its C-terminus to RING domain-containing protein RBX1 (RING box protein 1), which interacts with E2 enzymes (27). CRL4 regulates a myriad of biological processes, including cell proliferation, survival, and apoptosis; cell cycle regulation; DNA replication; DNA damage repair; and gene transcription, by catalyzing monoubiquitylation for histone modification and polyubiquitylation for proteasomal degradation of substrate proteins (28). Through MHV68 infection of mice with B cell-specific Cul4b deficiency, we demonstrated that Cul4b is required for the efficient establishment of *in vivo* latency. Notably, Cul4b deficiency resulted in a particularly marked reduction in infection in the GC B cell compartment. Cumulatively, these findings demonstrate that B cell-intrinsic expression of Cul4b is required for gammaherpesvirus latency *in vivo* and point to the possibility that Cul4b may play a crucial role in normal GC B cell responses.

The GC is a microanatomical structure that forms in secondary lymphoid organs when B cells are activated by antigen and cognate T cell help and is critical for the development of robust humoral immune responses. *In vivo*, gammaherpesviruses usurp this critical structure by infecting naïve B cells and intrinsically manipulating B cell signaling pathways to drive infected cells, independent of antigen stimulation, to proliferate and expand in the GC before differentiating into the memory B cell compartment (3–5). Not surprisingly, then, GC B cells represent a primary reservoir for latent viruses during the establishment phase of latency (20, 25, 26). Although the specific molecular mechanism by which Cul4b promotes GC B cell infection and/or expansion remains unknown, it is plausible that Cul4b may promote MHV68 latency by supporting normal GC B cell functions and ensuring a large population of target cells for the establishment of latency. This is consistent with the results in Fig. 5, showing that CKO mice had reduced numbers of uninfected, antigen-driven GC B cells and reduced titers of antigen-driven antibodies

after both virus infection and NP-ova immunization. In this manner, Cul4b might be critical for the proliferative expansion or survival of infected GC B cells or the differentiation of infected naïve B cells into the GC B cell stage. In support of this concept, Cul4b has recently been reported to promote the repair of DNA damage to support the proliferation, expansion, and differentiation of activated T cells (16, 29). Newly activated B cells proliferate for several days before differentiating into GC B cells. Within the GC, B cells proliferate every 4–6 hours and undergo somatic mutations in immunoglobulin genes at a rate 1 million times higher than mutations accrued during normal replication (30). Thus, similar to its role in T cells, Cul4b may facilitate DNA damage repair in B cells to support B cell proliferation in pre-GCs or in GCs undergoing somatic hypermutation.

Cul4b deficiency in B cells led to a significant skewing of the baseline levels of marginal zone and follicular B cells, as well as the levels of serum IgM, although these phenotypes were less dramatic than the changes in GC B cells and viral latency after MHV68 infection. The decrease in serum IgM was particularly surprising, given the increased numbers of marginal zone B cells, which are significant sources of serum IgM in unchallenged mice (31–33). Cul4b could play a role in plasmablast differentiation of marginal zone B cells (34, 35), such that CKO mice have more marginal zone B cells but less marginal zone B cell-derived IgM. Natural IgM can also be produced by B1-B cells (36), and future studies should determine if Cul4b plays a role in the development or function of these cells.

One alternate possibility is that Cul4b may directly interact with viral proteins that hijack its enzymatic function to help the virus establish latency. As the largest family of E3 ligases, the CRLs catalyze the ubiquitylation and degradation of numerous cellular proteins and are thus capable of regulating a variety of biological processes, from cell division to tumor development (11, 12, 14). Not surprisingly, many viruses, including gammaherpesviruses, have evolved to encode viral proteins that assemble the CRL E3 ligase complexes to recruit host target proteins for ubiquitylation and degradation, thus promoting viral fitness (27, 37–41). The most well-known examples use viral episome maintenance proteins such as KSHV latency-associated nuclear antigen (kLANA) and MHV68 LANA (mLANA) to re-purpose the E3 ligase complex CRL5, containing ElonginC, Cullin5, and Rbx2 (37, 38, 42). For example, MHV68 mLANA, like KSHV kLANA (38), assembles an E3 ligase complex $CRL5^{mLANA}$ (mLANA-ElonginC-Cullin5-Rbx2) to recruit host nuclear factor-kappa B (NF-κB) family member p65/RelA for polyubiquitination and subsequent proteasomal degradation, which promotes viral latent infection of GC B cells (42). Consistent with this, deletion of the mLANA E3 ligase domain severely impairs MHV68 latency establishment in GC B cells, suggesting that the mLANA E3 ligase activity is required for the proliferative expansion of GC B cell latency *in vivo* (43). Similarly, the MHV68 latency protein M2, which is essential for B cell latency *in vivo* (44, 45), interacts with DDB1 to hijack CRL4 (46). Thus, it would be interesting to test whether M2 serves as a substrate receptor for the assemble E3 ligase complex $CRL4^{M2}$ (M2-DDB1-Cul4b-Rbx1) to recruit host proteins for proteasomal degradation, thereby promoting latent infection of GC B cells.

Consistent with the possibility that Cul4b may play a conserved role in gammaherpesvirus biology, Cul4b has recently been shown to regulate KSHV latency in primary effusion lymphoma B cells *in vitro* through the restriction of lytic gene expression (47). However, we did not observe increased lytic infection, reactivation, or persistent replication in Cul4b-deficient mice, indicating that Cul4b is unlikely to restrict lytic gene expression *in vivo*. Nevertheless, these studies strongly suggest that gammaherpesviruses employ the E3 ubiquitin ligases to manipulate the fate of host proteins in order to create a favorable cellular environment for establishing viral latency. Taken together, these observations clearly indicate that defining the functions of the CRL family of E3 ligases on cellular and viral protein degradation during gammaherpesvirus infection warrants significant further investigation.

In summary, the findings described here demonstrate a critical role for the E3 ligase Cul4b in facilitating gammaherpesvirus latency in GC B cells. This work highlights the

essential roles of E3 ligases in promoting chronic gammaherpesvirus infection *in vivo* and implies that targeted inhibition of E3 ligases may provide a novel and effective intervention strategy against gammaherpesvirus-associated diseases.

MATERIALS AND METHODS

Cell culture

NIH 3T12 murine fibroblasts (ATCC, CCL-164) and mouse embryonic fibroblasts (MEFs; ATCC, SCRC-1008) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Corning, 10–013-CM) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, S12450) and 1× penicillin-streptomycin solution (Corning, 30–002-CI) at 37°C with 5% CO_2.

Genotyping of conditional knockout mice with B cell-specific cul4b deficiency

Tail biopsies were digested in DirectPCR Lysis Reagent (Viagen) plus 200 µg/mL proteinase K (Sigma). Crude lysate was amplified with primers (IDT) to detect the 5′ LoxP within the Cul4b locus as well as the WT and Cre alleles in the MB1 locus. Primer sequences were Cul4b 5′ LoxP, Forward: 5′-TGT TGT GAT TCT GTG ACT GCT-3′, and Reverse: 5′-GGT GAA GAA AGT GAG CCT GG-3′; MB1 WT, Forward: 5′-CTC TTT ACC TTC CAA GCA CTG A-3′, and Reverse: 5′-ACT GAG GCA GGA GGA TTG G-3′; and MB1 Cre, Forward: 5′-CAT TTT CGA GGG AGC TTC A-3′, and Reverse: 5′-ACT GAG GCA GGA GGA TTG G-3′. PCR products were run on a 2% agarose gel, and DNA was detected with ethidium bromide using a BioRad ChemiDoc system.

Western blots

B cells were isolated from the spleens of $Cul4b^{fl/fl}MB1^{+/+}$ (Cul4b WT) and $Cul4b^{fl/fl}MB1^{Cre/+}$ (Cul4b CKO) littermate controls using negative magnetic selection (StemCell). Cells were cultured for 24 hours in the presence or absence of 0.1 µM CpG (5′-TCC ATG ACG TTC CTG ACG TT-3′, IDT). Cells were washed 2× in phosphate-buffered saline (PBS) and lysed with SDS-containing buffer (EasyPep Lysis Buffer; Thermo) supplemented with 25 U/mL universal nuclease (Pierce). Lysate was clarified by centrifugation, and the supernatant was mixed with Laemmli SDS sample buffer (Trident) with 0.2 M dithiothreitol. Protein ladder (BioRad) and lysates were run on a 4%–12% Bis-Tris polyacrylamide gel (Nupage) and transferred onto a 0.45-µM PVDF membrane (Thermo). Membrane was blocked with Intercept TBS blocking buffer (LI-COR). Primary antibodies were incubated overnight and washed 4× with phosphate buffered saline with 0.1% Tween-20 (PBST), then secondary antibodies were incubated for 1 hour at room temperature and washed 4× with PBST. Primary antibodies were polyclonal rabbit anti-Cul4b (Cat# 12916–1-AP; Proteintech) and mouse anti-Gapdh (clone D4C6R; Cell Signaling). Secondary antibodies were donkey anti-rabbit IRDye 680 and goat anti-mouse IgG IRDye 800 (LI-COR). Signal was detected with an Odyssey Imager (LI-COR), and images were analyzed with Image Studio Lite (LI-COR).

Mouse infections

Eight- to twelve-week-old Cul4b WT and CKO mice were housed at the University of Florida (Gainesville, FL) in accordance with all federal and university guidelines. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida. Mice were inoculated i.n. with 10^4 PFU of MHV68.H2bYFP recombinant marker virus, a phenotypically WT virus that expresses eYFP under the control of the H2b promoter (20), in 30 µL serum-free DMEM under isoflurane anesthesia.

Viral lytic replication assays

Mice were i.n. infected with the MHV68.H2bYFP virus; the lungs were then harvested and stored at −80°C at 7 dpi. Subsequently, the lung samples were thawed and homogenized, and viral titers were determined by plaque assay as previously described (22, 23, 48). Briefly, NIH 3T12 murine fibroblasts were seeded at 2×10^5 cells/well in six-well plates. Twenty-four hours later, the lung samples were serially 10-fold diluted in serum-free DMEM and added to the plates in duplicate. One hour later, the wells were overlaid with a 1:1 mixture of methyl cellulose (Acros Organics, 258115000) and 2× modified Eagle medium (Gibco, 11935-046) supplemented with 10% FBS and 1× penicillin-streptomycin solution. Seven days later, a neutral red solution (MilliporeSigma, N6264-50ML) was diluted at 1:25 in PBS and added. The plaques were then counted.

Latency assays

Mice were i.n. infected with the MHV68.H2bYFP virus for 16 days; the spleens were then harvested and homogenized. Subsequently, the cells were treated with red blood cell lysis buffer (0.144 M ammonium chloride, 0.017 M Tris, pH 7.2) to remove red blood cells, and then filtered through a 100-µm nylon cell strainer (Corning, 352360). The resulting single splenocyte suspensions were used to determine the frequency of cells harboring the viral genome by limiting dilution-nested PCR (LDPCR), as previously described (21–23). Briefly, the cells were serially threefold diluted and added to 96-well PCR plates (Eppendorf, 951020460) at 12 wells per dilution. RAW 264.7 murine macrophages served as background cells to maintain a total of 10^4 cells per well. The plasmid containing the MHV68 ORF72 gene was added at 10, 1, or 0.1 copies per well as positive controls on a background of 10^4 RAW 264.7 cells. RAW 264.7 cells without plasmid were plated at 10^4 per well as a negative control. The cells were then lysed with proteinase K at 56°C for 8 hours, followed by inactivation at 95°C for 20 min. Two rounds of PCR were performed using primers specific for MHV68 ORF72 (primers for round 1 PCR: 5′-GAGATCTGTACTCAGGCACCTGT-3′ and 5′-GGATTTCTTGACAGCTCCCTG-3′, and primers for round 2 PCR: 5′-TGTCAGCTGTTGTTGCTCCT-3′ and 5′-CTCCGTCAGGATAACAACGTC-3′), as previously described (21, 22). The resultant 195-bp PCR products were visualized on a 3% agarose gel. Data are expressed as the mean and standard errors of the percentages of PCR reactions that are positive for the MHV68 genome per dilution.

Reactivation assays

The single splenocyte suspensions were prepared as described above at 16 dpi. Limiting-dilution *ex vivo* reactivation assays were then carried out to measure the frequency of cells that reactivate from latency, as previously described (21, 22). Briefly, one million splenocytes were serially twofold diluted and then plated onto permissive MEF monolayers in 96-well tissue culture plates (Corning, 3585) at 24 wells per dilution, starting at 10^5 cells/well. MEF monolayers were observed for the presence of CPE induced by the reactivating virus after 14–21 days. Data are expressed as the mean and standard errors of the percentages of wells that are positive for CPE per dilution.

Flow cytometry

The latent infection of GC B cells was quantified by flow cytometry based on the eYFP expression at 16 dpi, as previously described (23). The single splenocyte suspensions were prepared as above and blocked in PBS containing 2% FBS, 0.1% sodium azide, and purified rat anti-mouse CD16/CD32 at 1:50 (BD Biosciences, 553141). The cells were stained with specific GC B cell surface markers: V450 rat anti-mouse CD45R (B220) at 1:200 (BD Biosciences, 560472), Alexa Fluor 647 rat anti-mouse T- and B-cell activation antigen GL7 at 1:200 (BD Biosciences, 561529), PE hamster anti-mouse CD95 (BD Biosciences, 554258), Zombie aqua (BioLegend, #423102), IgM (BD, 550881), IgD (Bioleged, 405712), and CD38 (BioLegend, 102742). Fluorescence-activated cell sorting acquisition was performed on a BD FACSCanto II flow cytometer (BD Biosciences) or

a three-laser Aurora Spectral Cytometer (Cytek) at the University of Florida Center for Immunology and Transplantation flow cytometry core. Latently infected GC B cells were identified as $B220^+$, $GL7^+$, $CD95^+$, and YFP^+. The data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay plates were coated overnight with anti-mouse Ig (Southern Biotech), then plates were blocked with 10% FBS, and standards (Southern Biotech) and serum sample dilutions between 1:5,000 and 1:125,000 were added and incubated for 2 hours at room temperature. After washing, isotype-specific detection antibodies conjugated to horseradish peroxidase (Southern Biotech) were added for 1-hour at room temperature. 3,39,5,59-tetramethylbenzidine substrate was added, and the reaction was stopped by adding 1 M phosphoric acid. Absorbance at 450 nM was determined.

Statistical analyses

All data were analyzed using GraphPad Prism 9 software (GraphPad, San Diego, CA). The frequencies of splenocytes that are positive for the viral genome or positive for reactivating viruses were determined by Poisson distribution from non-linear regression, as indicated by the line at 63.2%. Statistical significance was determined using a two-tailed, unpaired Student *t*-test, and *P* values less than 0.05 were considered to be statistically significant. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig. S1 (JVI101008-23-S0001.tif). Frequency of latently infected cells at 45 dpi.
Supplemental legend (JVI01008-23 S0002.docx). Legend for Fig. S1.

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