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The Antagonism between the Murine Gammaherpesvirus Protein Kinase and Global Interferon Regulatory Factor 1 Expression Shapes the Establishment of Chronic Infection

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ABSTRACT Gammaherpesviruses infect most vertebrate species and are associated with B cell lymphomas. Manipulation of B cell differentiation is critical for natural infection and lymphomagenesis driven by gammaherpesviruses. Specifically, human Epstein-Barr virus (EBV) and murine gammaherpesvirus 68 (MHV68) drive differentiation of infected naive B cells into the germinal center to achieve exponential increase in the latent viral reservoir during the establishment of chronic infection. Infected germinal center B cells are also the target of viral lymphomagenesis, as most EBV-positive B cell lymphomas bear the signature of the germinal center response. All gammaherpesviruses encode a protein kinase, which, in the case of Kaposi's sarcoma-associated herpesvirus (KSHV) and MHV68, is sufficient and necessary, respectively, to drive B cell differentiation *in vivo*. In this study, we used the highly tractable MHV68 model of chronic gammaherpesvirus infection to unveil an antagonistic relationship between MHV68 protein kinase and interferon regulatory factor 1 (IRF-1). IRF-1 deficiency had minimal effect on the attenuated lytic replication of the kinase-null MHV68 *in vivo*. In contrast, the attenuated latent reservoir of the kinase-null MHV68 was partially to fully rescued in IRF-$1^{-/-}$ mice, along with complete rescue of the MHV68-driven germinal center response. Thus, the novel viral protein kinase–IRF-1 antagonism was largely limited to chronic infection dominated by viral latency and was less relevant for lytic replication during acute infection and *in vitro*. Given the conserved nature of the viral and host protein, the antagonism between the two, as defined in this study, may regulate gammaherpesvirus infection across species.

IMPORTANCE Gammaherpesviruses are prevalent pathogens that manipulate physiological B cell differentiation to establish lifelong infection. This manipulation is also involved in gammaherpesvirus-driven B cell lymphomas, as differentiation of latently infected B cells through the germinal center response targets these for transformation. In this study, we define a novel antagonistic interaction between a conserved gammaherpesvirus protein kinase and a host antiviral and tumor suppressor transcription factor. The virus-host antagonism unveiled in this study was critically important to shape the magnitude of gammaherpesvirus-driven germinal center response. In contrast, the virus-host antagonism was far less relevant for lytic viral replication *in vitro* and during acute infection *in vivo*, highlighting the emerging concept that nonoverlapping mechanisms shape the parameters of acute and chronic gammaherpesvirus infection.

KEYWORDS gammaherpesvirus, IRF-1, chronic infection, germinal center response, protein kinase

Gammaherpesviruses establish lifelong infection in multiple vertebrate species and are associated with several cancers, including B cell lymphomas (1). At least 95% of humans are infected with Epstein-Barr virus (EBV), with the prevalence of Kaposi's sarcoma-associated herpesvirus (KSHV), the second known human gammaherpesvirus, ranging

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from 5% to 15% of the general U.S. population (2, 3). Unfortunately, the molecular and immune mechanisms that control human gammaherpesvirus infection and pathogenesis remain poorly understood, in part due to the species specificity of these viruses. To overcome the species restriction, this study utilizes murine gammaherpesvirus 68 (MHV68), a rodent pathogen genetically related to EBV and KSHV (4–6) that represents a tractable model of natural gammaherpesvirus infection and lymphomagenesis.

All gammaherpesviruses encode a conserved protein kinase (EBV, BGLF4; KSHV, orf36; MHV68, orf36). *In vitro*, EBV and KSHV protein kinases facilitate reactivation and lytic replication in transformed cell lines (7, 8). Similarly, MHV68 orf36 supports *de novo* lytic replication in primary macrophages but not fibroblasts (9). While the role of EBV/KSHV protein kinases in chronic infection is not known, we and others demonstrated that expression and enzymatic activity of MHV68 orf36 facilitate acute lytic replication, the establishment of chronic infection, and viral reactivation *in vivo* (9–12). In the elegant study from the Damania group, expression of KSHV orf36 in transgenic mice was sufficient to drive aggressive B cell lymphomas that expressed orf36 (13), highlighting the lymphomagenic potential of this conserved gammaherpesvirus protein.

Despite the significance of viral protein kinase for chronic infection and, likely, lymphomagenesis, its mechanisms and targets *in vivo* remain poorly understood. A plethora of cellular and viral proteins can be phosphorylated by gammaherpesvirus protein kinases *in vitro* (9, 12, 14–17). However, only a few of these substrates and mechanisms have been confirmed *in vivo*. Importantly, EBV, MHV68, and KSHV protein kinases attenuate type I interferon (IFN) expression and signaling *in vitro*, in part via direct antagonism of interferon regulatory factor 3 (IRF-3) (12, 18). This viral kinase-IFN antagonism also manifests *in vivo*, as global type I IFN signaling deficiency (IFNAR1$^{-/-}$ mice) partially rescues acute and chronic infection of the orf36-null MHV68 mutant (12). In contrast, we found that global IRF-3 deficiency fails to rescue the attenuated latency establishment and acute replication of the orf36-null MHV68 mutant (19).

IRF-1 is the founding member of the IRF family of transcription factors that share a homologous DNA binding domain. Unlike IRF-3, IRF-1 is dispensable for induction of type I IFN by most viruses, including MHV68 (20–23). Despite little effect on IFN expression, IRF-1 attenuates lytic replication of diverse viruses (24) via poorly understood mechanisms. We previously showed that IRF-1 suppresses replication of wild-type (WT) MHV68 in primary macrophages (23, 25), a physiologically relevant cell type that supports gammaherpesvirus infection (26–32). Interestingly, the increased replication of WT MHV68 in IRF-1$^{-/-}$ macrophages occurred despite similar induction of type I IFN signaling (23).

Unlike lytic replication, the establishment of gammaherpesvirus latency *in vivo* is intimately tied to the B cell differentiation process. Specifically, EBV and MHV68 infect naive B cells and drive differentiation of latently infected and uninfected B cells through the germinal center response to achieve long-term latency in memory B cells or reactivation from plasma cells (33–35). Due to the rapid proliferation of germinal center B cells, gammaherpesviruses, in a clever evolutionary feat, use germinal center reaction to drive an exponential increase in the cellular reservoir of latent infection. Germinal center B cells are susceptible to transformation due to intense proliferation, downregulation of tumor suppressors (36), and expression of a mutagenic enzyme (37, 38). Not surprisingly, most EBV-positive lymphomas are of germinal center or post-germinal center origin (39), highlighting the importance of the gammaherpesvirus-driven germinal center response in chronic infection and lymphomagenesis.

We showed previously (10) that expression and enzymatic activity of MHV68 orf36 are required for optimal expansion of germinal center B cells and increase in class-switched antibodies during the establishment of chronic infection. In addition to decreased abundance of germinal center B cells in orf36 mutant infections, fewer of these germinal center B cells were latently infected. Our findings are not unique to the MHV68 system, as KSHV orf36 transgenic mice demonstrated an increase in germinal center class-switched B cells prior to lymphomagenesis (13), indicating that the ability of gammaherpesvirus protein kinases to drive B cell differentiation is conserved across species.

In contrast, we identified IRF-1 as the first host factor that selectively suppresses

MHV68-driven germinal center response (40). Specifically, the abundance of germinal center B cells was significantly elevated in MHV68-infected but not sheep red blood cell-immunized or lymphocytic choriomeningitis virus (LCMV)-infected IRF-1$^{-/-}$ mice. Further, given the increased germinal center B cell population, the overall latent reservoir was significantly increased in the spleens of MHV68-infected IRF-1$^{-/-}$ mice (40). Having observed opposite viral and host phenotypes during host IRF-1 and MHV68 orf36 deficiencies, we took advantage of the combination of host and viral genetics offered by the MHV68 system to probe for the potential biological interplay between the viral and host genes. We found that IRF-1 deficiency supported an increase in lytic replication of the orf36-null MHV68 mutant and WT MHV68 *in vitro*. Interestingly, acute titers of the MHV68 orf36-null mutant were minimally increased *in vivo*, compared to a 20-fold increase in wild-type MHV68 acute replication in IRF-1$^{-/-}$ lungs. In contrast, global IRF-1 deficiency provided significant or full rescue of the attenuated latent reservoir of the orf36-null MHV68 mutant during chronic infection. Further, MHV68 orf36 expression was no longer necessary for the exaggerated MHV68-driven germinal center response in the IRF-1$^{-/-}$ mice. Thus, our study uncovers a novel interaction between host IRF-1 and orf36 that selectively manifests during the chronic phase of natural infection and is responsible for the tug of war that defines the parameters of gammaherpesvirus-driven germinal center response, an immunological process critical for both the natural course of infection and viral lymphomagenesis.

RESULTS

IRF-1 deficiency increases the replication of orf36-deficient MHV68 mutant and WT MHV68 in primary macrophages. Conserved gammaherpesvirus protein kinases interact with IRF-3 (12, 18), an IRF family member that is critical for induction of type I IFN expression, including during MHV68 replication in primary macrophages (41). In contrast, the interplay between viral protein kinases and other IRF family members, including IRF-1, has not been examined. We previously showed that IRF-1 attenuates replication of wild-type MHV68 in primary macrophages (23). Further, IRF-1 mRNA and protein levels were increased in BL6 primary macrophages infected with the MHV68 mutant unable to express orf36 (N36S) (23). Having observed increased IRF-1 levels in N36S-infected macrophages and given the established antiviral role of IRF-1, we examined replication of the N36S mutant in macrophages derived from BL6 or IRF-1$^{-/-}$ bone marrow. Consistent with previous studies, lytic titers of WT MHV68 were increased ~20-fold in IRF-1$^{-/-}$ macrophages at the completion of a single replication cycle (Fig. 1A). Attenuated titers of the N36S mutant observed in BL6 macrophages were also increased (~7-fold) in IRF-1$^{-/-}$ cultures, with the N36S mutant titers remaining below those observed for WT MHV68 (Fig. 1A). Thus, IRF-1 deficiency resulted in increased lytic replication of the N36S mutant and WT MHV68 in primary macrophage cultures.

Despite being dispensable for induction of type I IFN expression during several virus infections, including MHV68 (20–23), IRF-1 is known to mediate expression of select interferon-stimulated genes (ISGs); however, such IRF-1-dependent ISGs appear to be cell type and context dependent. Specifically, IRF-1 facilitated induction of a subset of ISGs in human monocytic leukemia cells stimulated with Toll-like receptor 4 (TLR4) but not TLR7/8 ligands (42). We also showed that IRF-1-driven expression of cholesterol-25-hydroxylase (CH25H) in primary macrophages contributed to but did not fully account for the antiviral effects of IRF-1 during WT MHV68 infection (23). It is without a doubt that additional, IRF-1-dependent genes attenuate MHV68 infection; however, the identity of such genes is currently unknown. The identity of IRF-1-driven antiviral genes may be further obscured by putative viral antagonists, such as MHV68 orf36, that oppose IRF-1-dependent changes in gene expression during infection. Thus, having observed increased N36S lytic replication in IRF-1$^{-/-}$ macrophages, we hypothesized that the viral protein kinase might antagonize IRF-1-dependent expression of ISGs that were previously shown to attenuate MHV68 replication in human cell lines (43). As expected (23), induction of overall type I IFN signaling, as measured by the increase in MX-1 mRNA, was not IRF-1 dependent (Fig. 1B and C). In contrast to our hypothesis,

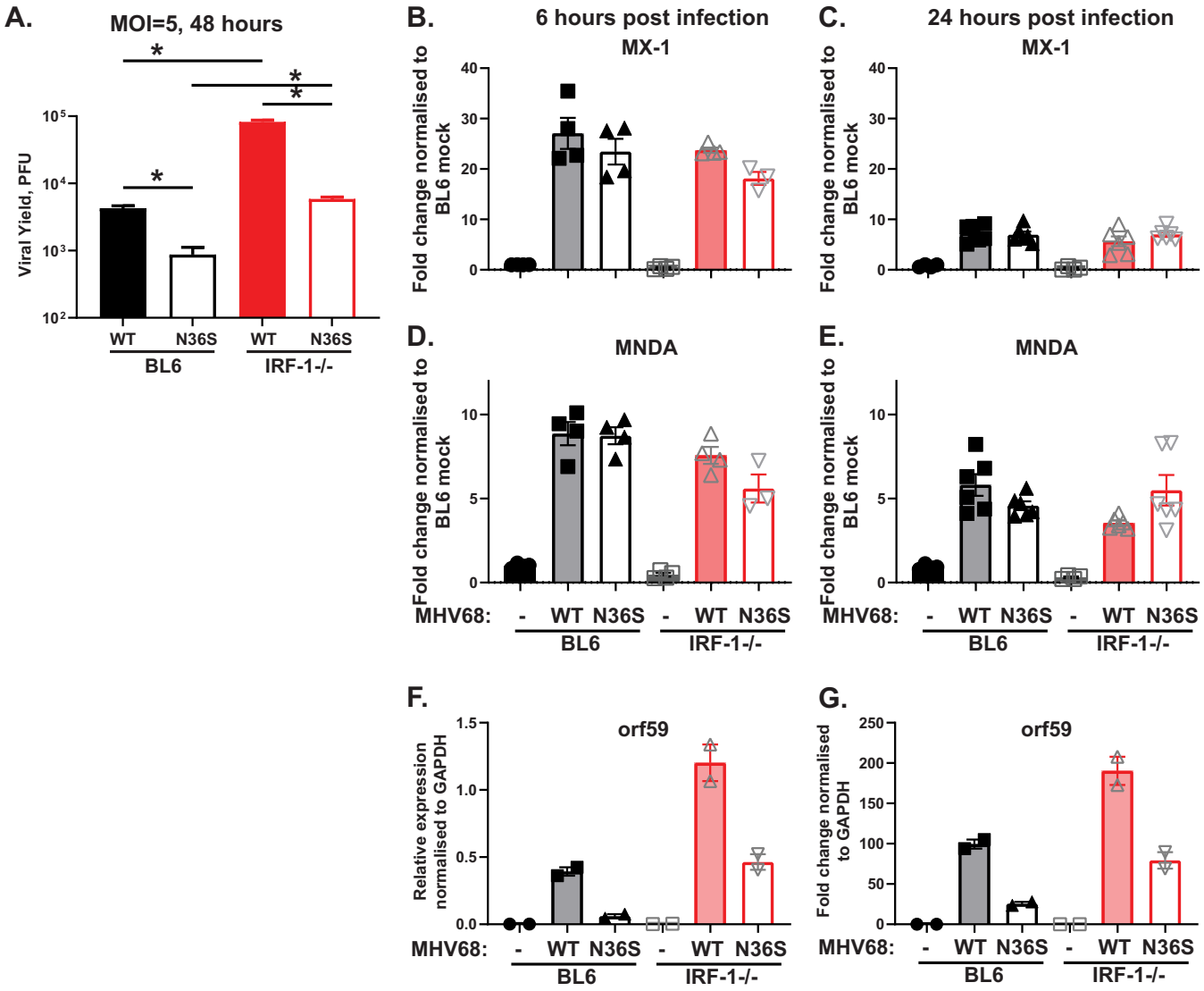

FIG 1 IRF-1 deficiency increases the replication of orf36-deficient MHV68 mutant and WT MHV68 in primary macrophages. BL6 and IRF-1$^{-/-}$ bone marrow-derived macrophages were infected with either WT or N36S mutant virus at an MOI of 5 PFU/cell. (A) Total MHV68 titers were measured at 48 h postinfection. (B to G) RNA was isolated from BL6 and IRF-1$^{-/-}$ macrophages infected at an MOI of 5 PFU/cell for 6 or 24 h, and the mRNA levels of ISGs (MX-1 and MNDA) and the viral gene orf59 were measured by qRT-PCR and normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Data shown here are representative results of 2 to 5 experiments. Each experiment was performed using independently derived batches of macrophages, with at least two replicates within each experiment. Mean and standard error of the mean are shown. *, $P < 0.05$.

mRNA levels of MNDA, ADAR, IFIH, MT1, MITD, and MX2 were also not regulated by the postulated antagonism between the viral protein kinase and IRF-1 (Fig. 1D and E and data not shown). Similar to our previous studies (23), there was an increase in the expression of MHV68 orf59, an early viral gene, in IRF-1$^{-/-}$ macrophages infected with WT MHV68 (Fig. 1F and G). While orf59 mRNA levels were also increased in N36S-infected IRF-1$^{-/-}$ macrophages, the expression of orf59 remained below that observed in WT MHV68 infection. In summary, IRF-1 deficiency resulted in increased replication and viral gene expression of both the N36S MHV68 mutant and WT MHV68 in primary macrophages.

Global IRF-1 deficiency leads to a minimal increase in N36S, compared to WT MHV68 lytic replication in the lungs. Having observed increased replication of the N36S mutant in IRF-1$^{-/-}$ primary macrophages, we next examined parameters of acute replication. Inoculation of a naive host leads to a once-in-a-lifetime acute gammaherpesvirus infection, with peak MHV68 lytic titers observed ~7 days postinfection. We previously showed that

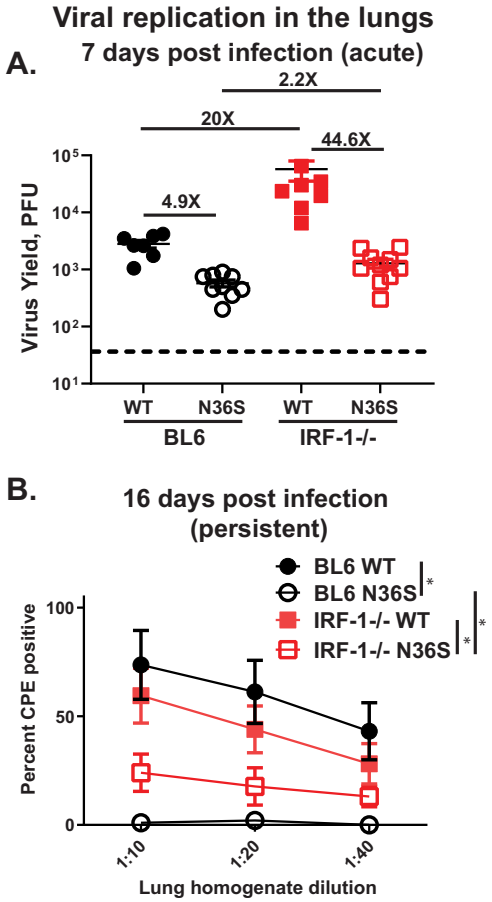

FIG 2 Global IRF-1 deficiency leads to a minimal increase in N36S, compared to WT MHV68 lytic replication in the lungs. BL6 and IRF-1$^{-/-}$ mice were intranasally infected with 10,000 PFU of WT or N36S mutant MHV68. (A) Acute-phase lung titers at 7 days postinfection. Each symbol represents an individual lung; the dashed line represents the limit of detection. There is a statistically significant difference between any two experimental groups ($P < 0.05$). (B) Persistent viral replication was measured by a highly sensitive semiquantitative assay (see Materials and Methods) using homogenized lungs harvested at 16 days postinfection. Data were pooled from 6 to 8 animals/group. CPE, cytopathic effect; *, $P < 0.05$.

IRF-1$^{-/-}$ mice infected with a low dose of WT MHV68 (500 PFU) showed a small (~5-fold) increase in the peak acute lung titers compared to BL6 mice (25). A low (500-PFU) dose of inoculation severely attenuates the ability of the N36S mutant to establish acute and chronic infection (10) (data not shown), making it challenging to perform a valid comparison of host responses induced by WT versus those induced by N36S MHV68. Thus, we used a higher inoculation dose of 10,000 PFU that enables the N36S mutant to execute both lytic replication and latent infection *in vivo*, albeit at attenuated levels. Following intranasal inoculation with a higher viral dose, IRF-1$^{-/-}$ mice exhibited a significant, 20-fold increase in acute lung titers of WT MHV68 compared to titers observed in BL6 mice (Fig. 2A). As expected, while the N36S MHV68 mutant was able to replicate in BL6 lungs following 10,000-PFU inoculation, the N36S titers were attenuated 4.9-fold, compared to WT MHV68. Interestingly, peak N36S lung titers were increased only 2.2-fold in IRF-1$^{-/-}$ compared to BL6 lungs. Thus, global IRF-1 deficiency led to a preferential increase in WT MHV68 but not N36S acute replication in the lungs.

Following clearance of acute lytic viral replication, MHV68 continues to replicate in the lungs of chronically infected BL6 mice, albeit at minimal levels. These very low levels of persistent replication that are no longer detectable by the conventional plaque assay can be detected via a semiquantitative assay that utilizes mouse embryonic fibroblasts (MEF) to amplify low levels of preformed virions in lung homogenates. Using this

assay, we previously showed increased persistent WT MHV68 replication in IRF-1$^{-/-}$ lungs examined at 16 days after infection with a 500-PFU dose (40). In contrast to our previous observation, inoculation with 10,000 PFU resulted in similar levels of persistent WT MHV68 replication in BL6 and IRF-1$^{-/-}$ lungs (Fig. 2B). Persistent lung replication of the N36S mutant was increased in the absence of IRF-1; however, the levels of N36S persistent replication did not reach those observed for WT MHV68. Thus, global IRF-1 deficiency resulted in a minimal increase of acute and persistent N36S lytic replication in the lungs.

Global IRF-1 deficiency rescues the latent reservoir but not reactivation of the N36S MHV68 mutant during chronic infection. Acute MHV68 replication is typically controlled by 10 to 12 days postinfection, with a concomitant rise in the latent viral reservoir that peaks at 16 to 18 days postinfection. In the spleen, the expansion and establishment of the MHV68 latent reservoir are intimately tied to B cell differentiation. Specifically, MHV68 infects naive B cells, with the virus-infected and bystander naive B cells subsequently driven to differentiate through the germinal center reaction, where the cellular latent reservoir is exponentially increased via proliferation of latently infected germinal center B cells (44–46). Further differentiation of an infected germinal center B cell into a memory cell ensures long-term infection, whereas differentiation into an antibody-secreting plasma cell triggers reactivation, a switch from latent to lytic life cycle (45). Because germinal center-based differentiation is limited to lymphoid organs, peritoneal macrophages and a distinct lineage of B cells, B-1 B cells, host the latent MHV68 reservoir in the peritoneal cavity (28).

We previously reported that MHV68 orf36 expression and enzymatic activity are required for optimal MHV68-driven B cell differentiation, the establishment of the latent reservoir in the spleen, and spread to the peritoneal cavity of BL6 mice (10, 11). In contrast, IRF-1 attenuates these viral and host parameters, as global IRF-1 deficiency leads to a significant and selective increase in WT MHV68-driven expansion of germinal center response along with an increase in the viral latent reservoir (40). Having observed little effect of IRF-1 deficiency on the attenuated acute lung replication of the N36S mutant, we next examined parameters of chronic N36S infection in IRF-1$^{-/-}$ mice, as the mechanisms that regulate acute MHV68 replication only partially overlap those that shape parameters of chronic infection.

As expected (10, 11), the frequency of latently infected splenocytes was significantly lower (9-fold, $P < 0.05$) in N36S-infected compared to WT MHV68-infected BL6 mice at 16 days postinfection (Fig. 3A). Infection of IRF-1$^{-/-}$ mice with WT MHV68 produced a 3-fold ($P < 0.05$) increase in the frequency of latently infected splenocytes. Unexpectedly, there was a 9-fold increase in the frequency of N36S-infected IRF-1$^{-/-}$ compared to BL6 splenocytes ($P < 0.05$, Fig. 3A), making the N36S latent reservoir in IRF-1$^{-/-}$ spleens numerically indistinguishable from that of WT MHV68 in BL6 splenocytes. Thus, global IRF-1 deficiency resulted in the rescue of the N36S splenic latent reservoir despite minimal alteration of the attenuated acute replication.

Whereas germinal center B cells host the majority of latently infected MHV68, viral reactivation is almost exclusively supported by the splenic plasma cells in BL6 mice (45). The frequency of *ex vivo* reactivation of WT MHV68 was significantly increased (10-fold, $P < 0.05$) in IRF-1$^{-/-}$ compared to BL6 splenocytes (Fig. 3B). Interestingly, unlike the significant rescue observed for the N36S splenic latent reservoir, the frequency of *ex vivo* reactivation of the N36S mutant remained 19-fold lower ($P < 0.05$) than that of WT MHV68 in IRF-1$^{-/-}$ splenocytes (Fig. 3B). Persistent replication in the spleen was equally low in all experimental groups (Fig. 3C). Thus, global IRF-1 deficiency largely rescued the establishment of the splenic latent reservoir but not the viral reactivation of the N36S mutant.

Next, the viral latent reservoir was examined in the peritoneal cavity. As expected (47), the frequency of WT MHV68-infected peritoneal cells was significantly increased (13-fold, $P < 0.05$) in IRF-1$^{-/-}$ compared to BL6 mice at 16 days postinfection (Fig. 3D). The increase in the latent reservoir was even more dramatic for the N36S mutant, such

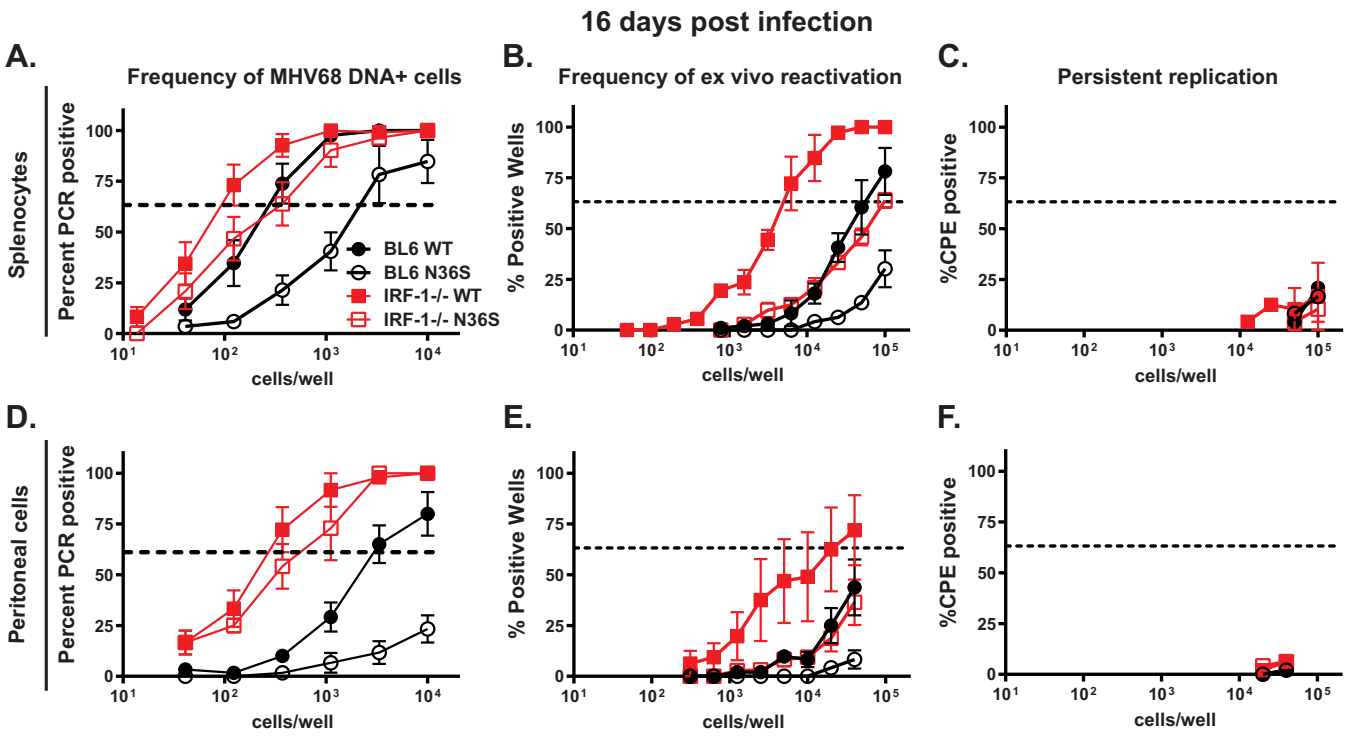

FIG 3 Global IRF-1 deficiency rescues the latent reservoir but not reactivation of the N36S MHV68 mutant during chronic infection. BL6 and IRF-1$^{-/-}$ mice were infected with WT or N36S mutant MHV68 as described for Fig. 2. Splenocytes (A to C) and peritoneal cells (D to F) were harvested at 16 days postinfection and subjected to limiting dilution assays to determine the frequency of MHV68-positive cells (A and D) and the frequency of *ex vivo* viral reactivation (B and E). Persistent viral replication in splenocytes (C) and peritoneal cells (F) was determined by physically disrupting the cells and subjecting them to limiting dilution assays to determine the presence of preformed virus. Each experimental group consists of 3 to 4 animals; data are pooled from 2 to 6 independent experiments.

that the N36S latent reservoir in IRF-1$^{-/-}$ peritoneal cells exceeded that observed for WT MHV68 in BL6 mice (6-fold increase, $P < 0.05$) and became statistically indistinguishable from that of WT MHV68 in IRF-1$^{-/-}$ peritoneal cells. However, similar to that observed in the spleen (Fig. 3B), the frequency of the N36S reactivation from IRF-1$^{-/-}$ peritoneal cells remained below that observed for WT MHV68 (Fig. 3E). No persistent MHV68 replication was observed in peritoneal cells of all experimental groups (Fig. 3F). In summary, global IRF-1 deficiency led to a significant rescue of the N36S latent reservoir, but not reactivation, during the establishment of chronic infection.

MHV68 orf36 expression is no longer required for the exaggerated MHV68-driven germinal center response under conditions of global IRF-1 deficiency. Germinal center B cells host the majority of MHV68 latent reservoir in the spleen. We showed that MHV68 orf36 is required for efficient germinal center B cell infection and the optimal magnitude of the MHV68-driven germinal center response in BL6 mice (10, 48). Given the significant rescue of the N36S splenic latent reservoir in IRF-1$^{-/-}$ mice (Fig. 3A), parameters of the germinal center response were examined next. As previously shown (10, 40), the expansion of germinal center B cells was attenuated in N36S-infected BL6 mice (Fig. 4A and B). The germinal center B cell population was increased in WT MHV68-infected IRF-1$^{-/-}$ mice compared to that observed in BL6 mice. Excitingly, the magnitude of the germinal center B cell population in N36S-infected IRF-1$^{-/-}$ mice was indistinguishable from that observed in WT MHV68-infected IRF-1$^{-/-}$ mice.

CD4^{+} T follicular helper cells are critical for the expansion of germinal center B cells, including during chronic MHV68 infection (49). As observed for germinal center B cells, the magnitudes of the T follicular helper cell population were similar in WT MHV68- and N36S-infected IRF-1$^{-/-}$ mice (Fig. 4C and D). Thus, global IRF-1 deficiency eliminated the requirement for MHV68 orf36 expression in the MHV68-driven germinal center response.

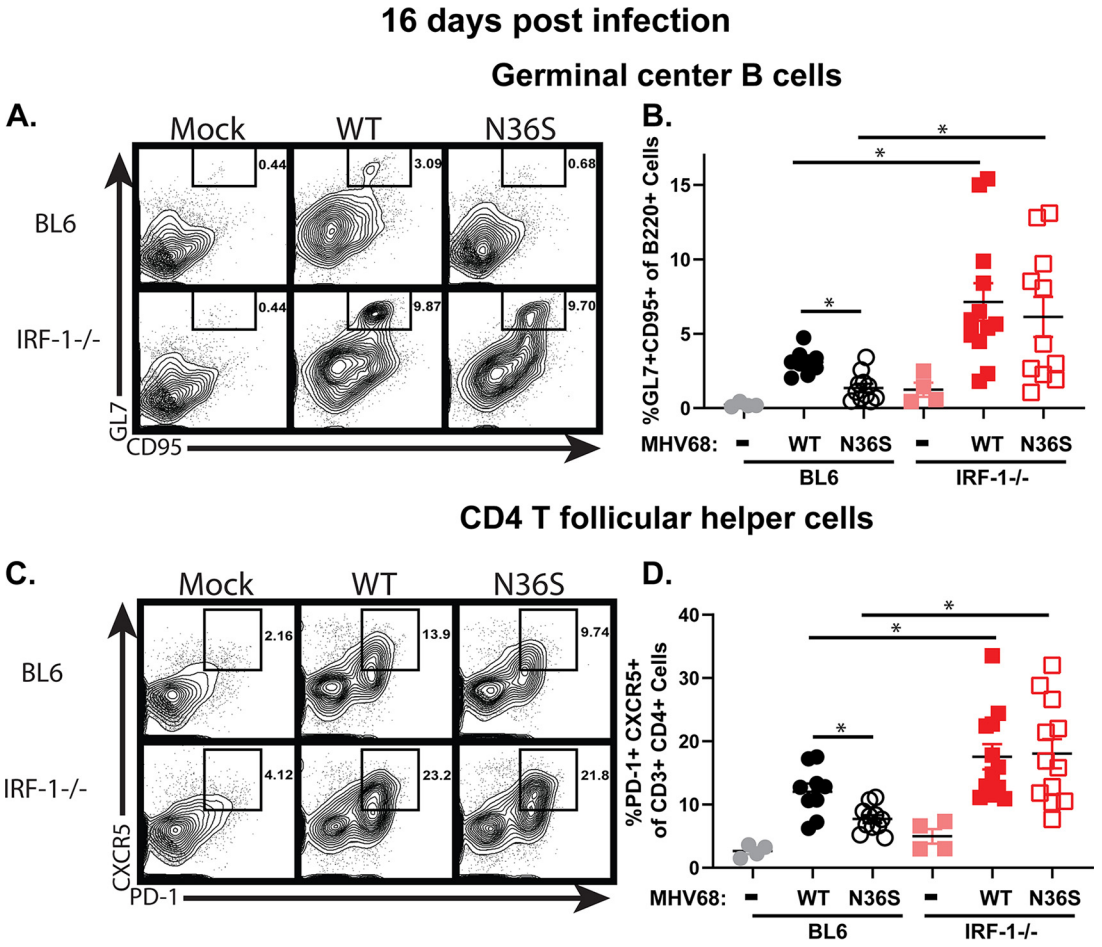

FIG 4 MHV68 orf36 expression is no longer required for the exaggerated MHV68-driven germinal center response under conditions of global IRF-1 deficiency. BL6 and IRF-1$^{-/-}$ mice were infected as described for Fig. 2. The germinal center response was measured at 16 days postinfection, with germinal center B cells (A and B) defined as B220^{+} GL7^{+} CD95^{+} cells and T follicular helper cells (C and D) defined as CD3^{+} CD4^{+} CXCR5^{+} PD-1^{+} cells. Each symbol represents an animal; data are pooled from 2 to 3 independent experiments. Mean and standard error of the mean are shown. *, $P < 0.05$.

Global IRF-1 deficiency selectively leads to a decrease of self-reactive but not MHV68-specific class-switched antibodies. Having observed minimal rescue of the N36S mutant reactivation in the IRF-1$^{-/-}$ splenocytes, we next assessed the abundance of plasma cells, as MHV68 preferentially reactivates from plasma cells in the spleens of BL6 mice (45). The abundance of class-switched plasma cells was decreased in N36S- compared to WT MHV68-infected BL6 and IRF-1$^{-/-}$ mice (Fig. 5A and B). Further, the low abundance of plasma cells remained independent of the IRF-1 genotype for N36S-infected mice. Thus, global IRF-1 deficiency did not alter attenuated levels of class-switched plasma cells induced in the absence of orf36 expression.

Terminal differentiation into class-switched plasma cells results in production of antibodies. The gammaherpesvirus-driven B cell differentiation differs from the typical response to viral infection, as it encompasses both virus-specific and an extensive self- and foreign species-specific B cell differentiation. Because of the latter, there is a robust, albeit transient, increase in the titers of antibodies reactive against self and foreign species antigens (50, 51). In fact, the presence of high-titer antibodies against horse antigens is diagnostic of a recent EBV infection (52). This self- and foreign species-directed B cell response is proviral, as the lifelong latent reservoir is established in germinal center experienced memory B cells that do not encode a gammaherpesvirus-specific antibody that could interfere with viral reactivation (53, 54). Importantly, we have previously shown that the expression of MHV68 orf36 is required for the optimal induction of self-reactive but not MHV68-specific antibodies (10). To define the extent to which IRF-1 deficiency interacted with the MHV68 orf36 to affect the

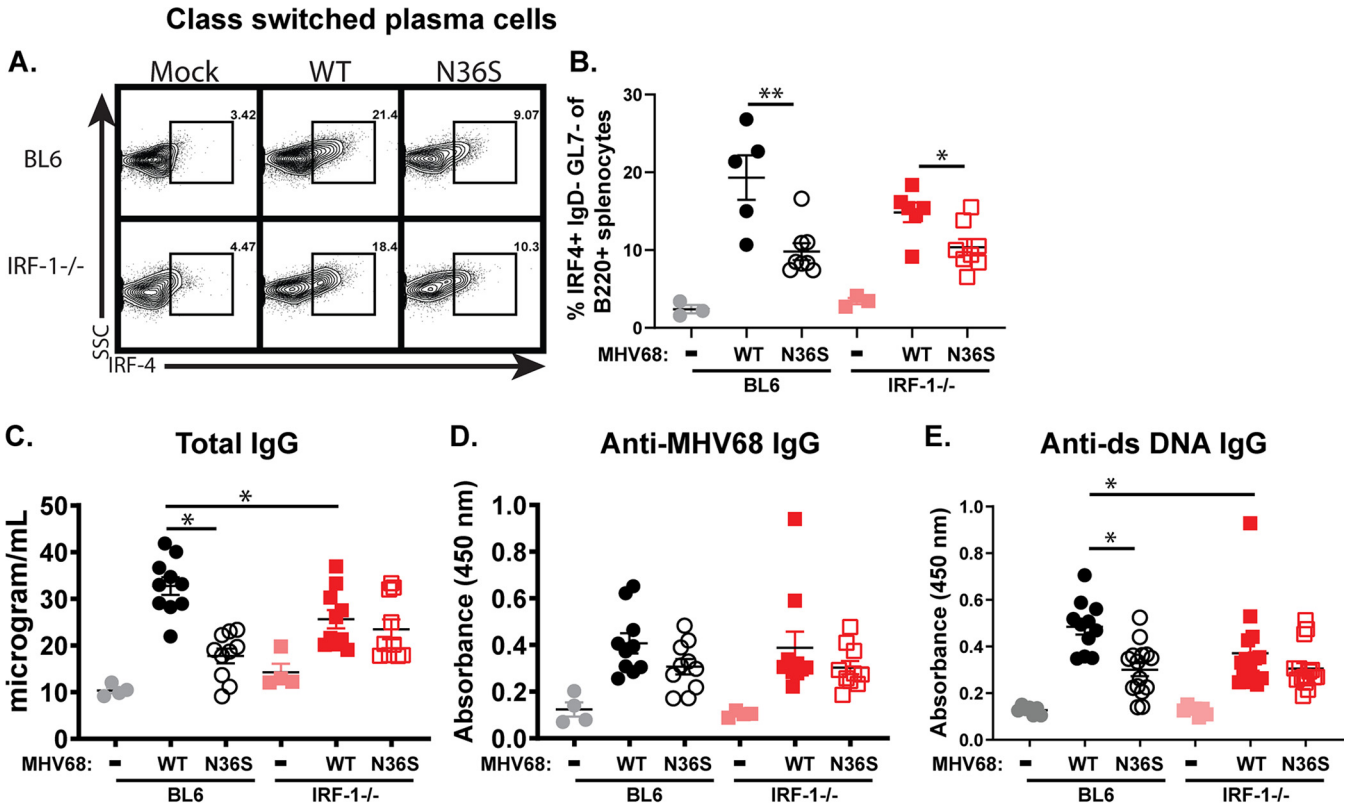

FIG 5 Global IRF-1 deficiency selectively leads to a decrease of self-reactive but not MHV68-specific class-switched antibodies. BL6 and IRF-1$^{-/-}$ mice were infected as described for Fig. 2. (A and B) Splenocytes were harvested at 16 days postinfection, and the frequency of class-switched plasma cells was determined. Plasma cells were defined as B220^{+} IgD^{-} GL7^{-} IRF-4^{+} cells. SSC, side scatter. (C to E) Sera were used to determine total IgG (C), MHV68-specific IgG (D), and dsDNA IgG (E). Data are pooled from 2 to 3 independent experiments, with each symbol representing an individual mouse. Mean and standard error of the mean are shown. *, $P < 0.05$; **, $P < 0.01$.

humoral responses, titers of class-switched antibodies were examined next. As expected, total IgG titers were significantly decreased in BL6 mice infected with the N36S mutant, compared to those infected with WT MHV68 (Fig. 5C). Interestingly, total IgG titers driven by WT MHV68 infection were also decreased in IRF-1$^{-/-}$ compared to BL6 mice. Assessment of class-switched anti-MHV68 antibody levels demonstrated that the generation of antiviral antibodies was independent of either MHV68 orf36 or global IRF-1 expression (Fig. 5D). In contrast, the induction of self-reactive, anti-double-stranded DNA (anti-dsDNA) antibodies was facilitated by MHV68 orf36 and IRF-1 (Fig. 5E). Interestingly, the attenuated induction of anti-dsDNA by the N36S mutant was similar in BL6 and IRF-1$^{-/-}$ mice (Fig. 5E). Thus, global IRF-1 expression selectively promoted orf36-driven induction of self-reactive antibody response.

Global IRF-1 deficiency leads to rescued N36S latent reservoir in splenic non-B cells but only partially rescues latent infection of B cells. We have previously shown that lack of MHV68 orf36 expression led to decreased efficiency of latent infection across all relevant splenic cell types, including germinal center B cells (10, 11). Having observed a significant rescue of the N36S splenic latent reservoir and a complete rescue of the germinal center response under the conditions of global IRF-1 deficiency (Fig. 3A and Fig. 4A and B), we next examined the infection of splenic populations relevant for the MHV68 latent reservoir. In an experimental design shown in Fig. 6A, total splenocytes were first separated into B cell and non-B cell populations using anti-CD19 antibody-conjugated magnetic beads. The B cell population was subsequently subjected to fluorescence-assisted cell sorting (FACS) to obtain purified germinal center B cells. The frequency of latently infected cells was measured in the highlighted splenic populations.

As expected (10, 11), there was a 10-fold decrease ($P < 0.05$) in the frequency of latently

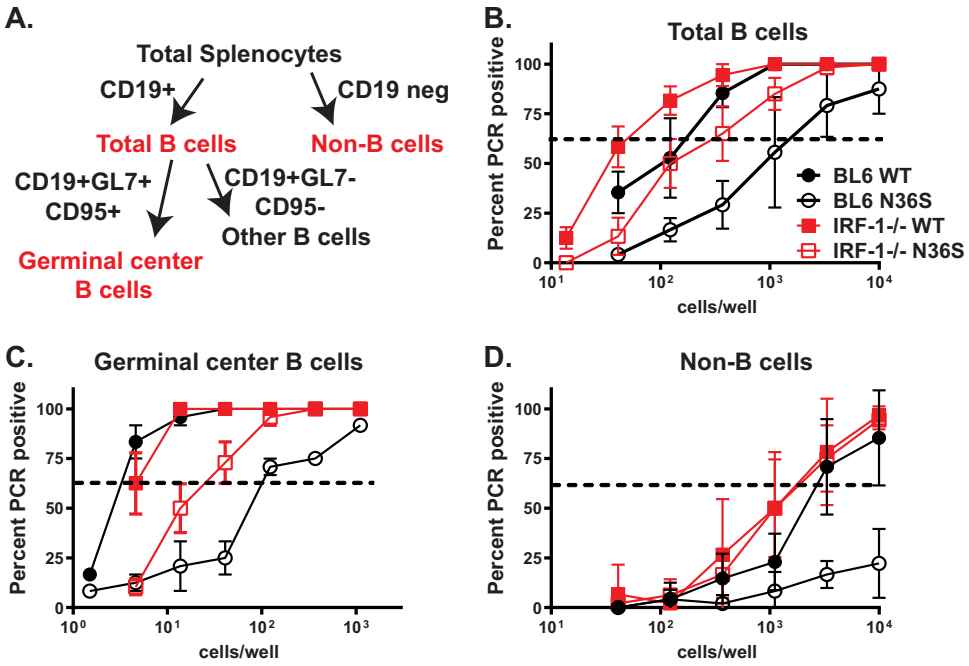

FIG 6 Global IRF-1 deficiency leads to rescued N36S latent reservoir in splenic non-B cells but only partially rescues latent infection of B cells. BL6 and IRF-1$^{-/-}$ mice were infected as described for Fig. 2. (A) At 16 days postinfection, splenocytes were isolated and sorted into B cells and non-B cells using anti-CD19-conjugated magnetic beads. CD19^{+} B cells were then stained for germinal center B cell markers, and CD95^{+} GL7^{+} germinal center B cells were isolated via fluorescence-assisted cell sorting (FACs). (B to D) Total B cells (B), FACs-sorted germinal center B cells (C), and non-B cells (D) were subjected to limiting dilution PCR assay to determine the frequency of MHV68^{+} cells. Each experimental group consists of 3 to 4 animals; data are pooled from 2 to 6 independent experiments.

infected total B cells in N36S-infected compared to WT MHV68-infected BL6 mice (Fig. 6B). The frequency of latently infected total B cells was increased 3-fold in WT MHV68-infected IRF-1$^{-/-}$ spleens compared to BL6 spleens (Fig. 6B). Surprisingly, the frequency of N36S-infected IRF-1$^{-/-}$ B cells remained decreased compared to WT MHV68 latent infection in the same population, albeit the magnitude of decrease was now only 5-fold (Fig. 6B). Given that germinal center B cells support most of the splenic latent reservoir, latent infection was next measured in this population. As previously shown (40), the frequencies of latently infected germinal center B cells were similar in BL6 and IRF-1$^{-/-}$ mice infected with WT MHV68 (Fig. 6C). Surprisingly, only a partial increase in the frequency of infection was observed in N36S-infected IRF-1$^{-/-}$ compared to BL6 germinal center B cells. Thus, global IRF-1 deficiency led to a partial rescue of the N36S mutant latent infection of total and germinal center B cells.

While B cells, particularly germinal center B cells, host most of the WT MHV68 reservoir in BL6 spleens, a small proportion of splenic myeloid cells, such as dendritic cells, is also latently infected (55). The frequency of latent infection of non-B cells in the spleen is further reduced when orf36 cannot be expressed (10). Interestingly, while IRF-1 expression had very little effect on the frequency of WT MHV68 latent infection of non-B cells in the spleen, the latent infection of non-B cells by the N36S mutant was completely rescued in IRF-1$^{-/-}$ mice (Fig. 6D). Thus, while global IRF-1 deficiency led to a complete rescue of N36S latent infection in splenic non-B cell population, the efficiency of total and germinal center B cell latent infection was only partially rescued.

DISCUSSION

In this study, we uncover a novel interplay between host IRF-1 and a conserved gammaherpesvirus protein kinase in the context of natural infection of an intact host. Importantly, given the conserved roles of gammaherpesvirus protein kinases, the interaction between the viral protein kinase and host IRF-1 is likely to transcend species.

Interestingly, the antagonistic interaction between IRF-1 and viral protein kinase was most evident during the chronic and not the acute phase of infection, highlighting the emerging concept that distinct mechanisms regulate acute and chronic gammaherpesvirus infection *in vivo*.

IRF-1 versus orf36 during lytic replication. An extensive body of literature defined the steps in gammaherpesvirus replication affected by gammaherpesvirus protein kinase. We showed that MHV68 orf36 facilitates immediate early viral gene expression prior to the initiation of viral DNA synthesis (56). Importantly, expression of the MHV68 orf36 was also required for optimal viral DNA replication regardless of immediate early viral gene expression, a phenotype that is shared by EBV BGLF4 (7). Induction of late gene expression downstream of viral DNA synthesis was also facilitated by transcriptional complexes that incorporated EBV BGLF4 (57). Finally, EBV BGLF4 enzymatic activity is required for disassembly of the nuclear lamins to promote virion egress (58), a function shared with protein kinases encoded across the beta- and gammaherpesvirus families (59). Given the extensive involvement of gammaherpesvirus protein kinases in every stage of viral lytic replication, it is, perhaps, not surprising that IRF-1 deficiency had little effect on the N36S lytic replication *in vitro* and *in vivo*.

Although IRF-1 is not involved in the induction of global type I IFN response during MHV68 replication (Fig. 1) (23, 25), we hypothesized that orf36 may antagonize IRF-1-driven expression of a select ISG(s) that interferes with MHV68. Of hundreds of ISGs induced by type I IFN, fewer than a dozen affect lytic replication for each viral family and individual virus (24, 60). Thus, we tested expression of ISGs that were reported to attenuate MHV68 replication in human cell lines (43). However, none of the tested ISGs (Fig. 1 and data not shown) had demonstrated antagonistic regulation by IRF-1 and MHV68 orf36, at least in primary macrophage cultures. Given the minimal effect of global IRF-1 deficiency on the attenuated N36S lytic replication *in vivo*, it is likely that, in the context of lytic replication, orf36- and IRF-1-dependent pathways act in parallel to facilitate and repress, respectively, lytic MHV68 replication. The mechanisms underlying IRF-1-dependent antiviral effects are likely to involve differential regulation of yet-unidentified cellular genes that attenuate viral replication, with some of these genes IFN inducible (25).

IRF-1–viral protein kinase antagonism of B cell differentiation driven by gammaherpesvirus infection. During physiological B cell differentiation, pathogen-specific naive B cells receive CD4 T cell help and enter germinal centers, where, in a series of carefully orchestrated and spatially restricted events, the germinal center B cells undergo many cycles of proliferation and mutagenesis followed by interactions with T cells and antigen-presenting cells. When mutagenesis results in the B cell receptor with a high affinity for the pathogen, the germinal center B cell differentiates into a long-lived memory B cell or a short-lived plasma cell that secretes antibodies. Gammaherpesviruses uniquely usurp B cell differentiation to establish long-term latency in memory B cells (33–35, 44, 46, 49). EBV and MHV68 infect naive B cells and drive entry of infected and bystander B cells, independent of the specificity of the B cell receptor, into the germinal center response (33–35). Latent infection of germinal center B cells coupled with rapid proliferation leads to exponential increase in the viral reservoir over a very short period. In addition to their importance in natural infection, germinal center B cells are also the target of viral transformation, as a majority of EBV-positive lymphomas bear the genetic signature of mutagenesis that occurs in germinal center B cells.

We have previously shown that global IRF-1 expression selectively suppresses MHV68-driven germinal center response throughout the course of chronic infection (40). Further, we showed that EBV-positive but not EBV-negative B cell lymphomas in transplant patients are more likely to display decreased IRF-1 protein levels in malignant cells (40), suggesting that IRF-1 is suppressing not only natural gammaherpesvirus infection but also viral lymphomagenesis. In contrast, expression of KSHV protein kinase orf36 was sufficient to drive B cell differentiation and, eventually, B cell lymphomas in transgenic mice (13). Further, we demonstrated that expression and enzymatic activity of MHV68 orf36 were necessary for optimal MHV68-driven germinal center response during chronic infection (10). In the current study, we found that the exaggerated, MHV68-driven germinal center response no

longer requires orf36 expression in IRF-1$^{-/-}$ mice (Fig. 4). This finding indicates that the antagonism between IRF-1 and conserved gammaherpesvirus protein kinase is the primary mechanism that regulates the magnitude (and, perhaps, the transformation risk) of the abnormal germinal center response driven by gammaherpesviruses. Interestingly, the efficiency of N36S germinal center B cell infection was only partially rescued in IRF-1$^{-/-}$ mice (Fig. 6C), indicating that there are additional, IRF-1-independent functions of the viral protein kinase that are important for the optimal establishment and/or maintenance of latent MHV68 infection in germinal center B cells.

Curiously, the attenuated splenic latent reservoir of the N36S mutant was rescued to a much greater degree than was viral reactivation in IRF-1$^{-/-}$ mice (Fig. 3A and B). As discussed above, the sustained attenuation of reactivation could reflect the extensive involvement of gammaherpesvirus protein kinases in almost every step of the viral lytic replication cycle. However, as plasma cells support the majority of MHV68 reactivation in the spleen (45), decreased numbers of plasma cells could offer an additional plausible explanation for the attenuated N36S reactivation. Indeed, despite an increase in germinal center response, the abundance of class-switched plasma cells remained decreased in the N36S-infected IRF-1$^{-/-}$ mice (Fig. 5A and B). Interestingly, there was also no increase in class-switched plasma cell population in IRF-1$^{-/-}$ mice compared to BL6 mice infected with WT MHV68, despite clear difference in the germinal center response that normally drives the generation of class-switched plasma cells, at least in a wild-type host. Thus, the progression of B cell differentiation from germinal center to the plasma cell stage may be affected by global IRF-1 deficiency, a hypothesis to be tested in the future.

Uniquely, gammaherpesvirus-driven B cell differentiation is largely represented by B cells that are not specific for gammaherpesvirus proteins (50). Instead, a majority of antibodies generated during the establishment of chronic EBV and MHV68 infection are directed against self and foreign species antigens. The mechanisms responsible for the gammaherpesvirus-driven differentiation of self-reactive B cells are still poorly understood; however, we showed that MHV68 orf36 selectively facilitates this process during chronic infection (10). Interestingly, in the current study we found that levels of self-reactive, anti-double-stranded DNA (anti-dsDNA) antibodies were decreased in WT MHV68-infected IRF-1$^{-/-}$ mice (Fig. 5E). In contrast, IRF-1 deficiency had no effect on the low levels of anti-dsDNA induction by the N36S infection, indicating that IRF-1 may function downstream of MHV68 orf36 to drive self-reactive B cell differentiation. Interestingly, higher IRF-1 expression was linked to increased risk of autoimmune manifestations in patients with myelodysplasia (61), suggesting that IRF-1 may be a positive regulator of autoimmune diseases in a susceptible host and that gammaherpesvirus protein kinases may usurp this poorly explored IRF-1 function to enhance self-reactive B cell differentiation.

IRF-1 versus IRF-3 in MHV68 infection. IRF-1 is the founding member of the IRF family and was cloned based on the premise that IRF-1 is the factor that regulates (i.e., induces) expression of type I IFN (62). However, it soon became clear that IRF-1 is dispensable for induction of type I IFN in many virus infections, despite its given name (20–23). Instead, it was the related IRF-3 that was found to be critical for induction of IFN-β expression during viral replication (63). Interestingly, conserved protein kinases encoded by EBV, KSHV, and MHV68 directly interact with IRF-3 *in vitro* to attenuate IRF-3-mediated transcription of IFN-β (12, 18). Correspondingly, we showed that the replication of the N36S viral mutant is increased in IRF-3$^{-/-}$ primary macrophage cultures, concurrent with a significant reduction in type I IFN induction and pan-ISG expression (41).

Intriguingly, when the interplay between IRF-3 and MHV68 orf36 was explored *in vivo*, the attenuated acute titers of the N36S mutant were not increased in IRF-3$^{-/-}$ lungs, despite a measurable increase in the titers of WT MHV68 in the absence of IRF-3 expression (19). Similarly, the attenuated establishment of chronic infection observed for the N36S mutant in BL6 mice was not altered in mice with global IRF-3 deficiency, at least under the experimental conditions that were examined (19).

Similar to that observed for IRF-3 deficiency, we found increased replication of the N36S mutant in IRF-1$^{-/-}$ macrophages (Fig. 1). However, this increased replication occurred under

conditions when the global type I IFN signaling was equally induced in BL6 and IRF-1$^{-/-}$ infected macrophages, indicating that, unlike IRF-3, IRF-1 has a more precise, surgical approach to modify host gene expression to establish an antiviral environment. Similar to that observed in IRF-3$^{-/-}$ mice, acute N36S replication in the lungs was minimally affected by the IRF-1 deficiency, indicating that the antiviral functions of IRF-1 may be further modified in a cell-type-specific manner, as multiple cell types, including epithelial cells and macrophages, support MHV68 acute replication in the lungs.

The most unexpected observation, however, was made during the establishment of chronic infection, where the overall latent reservoir of the N36S mutant was partially or completely (depending on the anatomic location) rescued in the absence of IRF-1 expression (Fig. 2A and D). This observation suggests that the antagonism between MHV68 orf36 and IRF-1 is selectively manifest during chronic and not acute phases of infection, with precise cell types and molecular mechanisms responsible for this antagonism to be defined in future studies.

MATERIALS AND METHODS

Animals used. C57BL/6J mice and IRF-1$^{-/-}$ (B6/129S2-Irf1^{tm1Mak}/J stock 002762) (64) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). All mice were housed and bred in a specific-pathogen-free facility. All experimental manipulations of mice were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (protocol AUA971). Both male and female mice were used in all studies, and no sex-specific effects were observed.

Viruses and infections. Virus stocks were prepared and their titers were determined on NIH 3T12 cells. Infections with the N36S MHV68 mutant were controlled by the parental virus retaining a single LoxP site (referred to as wild type in the figures and text) (12). Infections were performed by intranasal inoculation of 3 to 5 mice per group at 6 to 7 weeks of age under light anesthesia. Mice were inoculated with 10^4 PFU of virus or sterile carrier (mock) in an inoculum volume of 15 μL per mouse. Virus was diluted in sterile serum-free Dulbecco's modified Eagle's medium (Corning, Tewksbury, MA). The lungs, spleen, and peritoneal cells were harvested from euthanized mock-treated, WT, and N36S mutant MHV68-infected animals at indicated times postinfection. Mice were bled prior to euthanasia via the submandibular route, and serum was isolated using BD Microtainer blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). For acute studies, lungs were harvested at 7 days postinfection and viral titers were measured as previously described (25).

Primary cell isolation, viral infection, and cell treatment. Bone marrow was harvested from mice between 3 and 10 weeks of age. Primary bone marrow-derived macrophages (BMDMs) were generated as previously described (9). Bone marrow-derived macrophages were infected with WT or N36S MHV68 at a multiplicity of infection (MOI) of 5 for 1 h to allow for adsorption and washed 2 to 3 times with phosphate-buffered saline (PBS) prior to medium replenishment. Total virus yield in each replicate was determined by plaque assay using NIH 3T12 cells. For gene analysis from infected BMDMs, cells were solubilized in TRIzol (Thermo Fisher), at the indicated time point postinfection, and total RNA was isolated and subjected to quantitative reverse transcription-PCR (qRT-PCR) analysis.

qRT-PCR analysis. Total RNA was harvested, DNase treated, reverse transcribed, and analyzed by quantitative reverse transcription-PCR (qRT-PCR) as previously described (23). cDNA was assessed in triplicate, along with corresponding negative RT reactions, by real-time PCR using the CFX Connect system (Bio-Rad, Hercules, CA). Primers for MX-1 (65), MNDA (23), and orf59 (66) were as previously published.

Limiting dilution assays. The frequency of virally infected cells (cells harboring viral DNA) was determined by limiting dilution PCR analysis, while the frequency of *ex vivo* reactivation to identify cells capable of producing infectious virus was determined by limiting dilution assay as previously described (11). Briefly, to determine the frequency of cells reactivating virus *ex vivo*, serial 2-fold dilutions of splenocytes or peritoneal cell suspensions harvested from infected mice were plated onto monolayers of mouse embryonic fibroblasts (MEF) immediately following harvest, at 24 replicates per dilution. To control for any preformed infectious virus, 2-fold serial dilutions of mechanically disrupted lungs, splenocytes, or peritoneal cells were plated as described above. MHV68 was allowed to reactivate from primary cells, and virus was further amplified within the same well via subsequent replication in MEF. At 21 days postplating all replicates and dilutions were scored in a binary fashion for the presence of live fibroblasts (no viral reactivation/replication) or absence of such (dead fibroblasts as a result of cytopathic effect). Because primary MEF were used to amplify the virus, the sensitivity of the limiting dilution reactivation assay was below a single PFU of MHV68 defined using a 3T12-based plaque assay. Because the endpoint of viral amplification in MEF was measured, the limiting dilution reactivation assay was not susceptible to variability of titers released from primary cells upon viral reactivation *ex vivo* (40).

To determine the frequencies of MHV68 DNA-positive splenic B cells, germinal center B cells, and non-B cells, splenocytes pooled from mice within an experimental group were subjected to B cell magnetic sorting using the EasySep Mouse CD19 Positive Selection kit II (Stemcell Technologies, Cambridge MA), to get B and non-B cell populations. The B cells were then stained with germinal center B cell markers CD95 and GL7, prior to fluorescence-assisted cell sorting (FACS) (Aria III; BD Biosciences, San Jose, CA) to obtain germinal center B cells. The sorted populations were then subjected to limiting dilution PCR analyses as described above.

Flow cytometry. Single cell suspensions of splenocytes and peritoneal cells from mock and infected mice were prepared in FACS buffer (phosphate-buffered saline [PBS] plus 2% fetal calf serum plus 0.05% sodium azide) at 1×10^7 nucleated cells/mL. A total of 1×10^6 cells were treated with Fc block (24G2) prior to extracellular staining with an optimal antibody concentration for 30 min on ice. Data acquisition was performed on an LSR II flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (Tree Star, Ashland, OR). The following antibodies were purchased from BioLegend (San Diego, CA) for use in this study: CD3 (17A2), CD4 (RM4-5), CD95 (Jo2), PD-1 (29f.1A12), B220 (RA3-6B2), and CXCR5 (2G8). Compensation controls were done using OneComp eBeads (Thermo Fisher Scientific, Waltham, MA). Briefly, a negative control (beads alone) was used to establish a baseline photomultiplier tube (PMT) voltage and fluorescent background. Positive controls for each fluorochrome (beads with a single fluorochrome) were used to establish spillover of the individual fluorochrome into the other channels being used. PMT values were adjusted for each fluorochrome to minimize spillover. Gating strategies followed what has been previously described (67).

Enzyme-linked immunosorbent assay (ELISA). Total, MHV68-specific, and dsDNA immunoglobulin levels were determined as previously described (10). Briefly, Nunc MaxiSorp plates (Fisher Scientific, Pittsburgh, PA) were coated with anti-IgG (heavy plus light) (Jackson ImmunoResearch, West Grove, PA), UV-irradiated MHV68 virus stock in PBS (740,000 microjoules/cm^2 $\times$ 2) (Stratalinker UV Crosslinker 1800; Agilent Technologies, Santa Clara, CA), or dsDNA from *Escherichia coli* (12.5 μg/mL; Sigma-Aldrich, St. Louis, MO) overnight at 4°C. Plates were washed with PBS-Tween (0.05%), blocked for 1 h with PBS-Tween (0.05%)–bovine serum albumin (BSA) (3%), incubated with 5-fold serial dilutions of serum in PBS-Tween (0.05%)–BSA (1.5%) for 2 h, and washed with PBS-Tween (0.05%). Bound antibody was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse total IgG (heavy plus light chain) (Jackson ImmunoResearch, West Grove, PA) using a 3,3′,5,5′-tetramethylbenzidine substrate (Life Technologies, Gaithersburg, MD). HRP enzymatic activity was stopped by the addition of 1 N HCl (Sigma-Aldrich, St. Louis, MO), and the absorbance was read at 450 nm on a model 1420 Victor^{3}V Multilabel plate reader (PerkinElmer, Waltham, MA).

Statistical analyses. Statistical analyses were performed using Student's *t* test (Prism; GraphPad Software, Inc.).

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C.N.J. and V.L.T. led the conceptual design of the study and wrote the manuscript. C.N.J., P.A.S., D.L.S., K.N., and V.L.T. designed, performed, and analyzed experiments.

We declare no competing interests.

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