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A Replication-Deficient Murine γ-Herpesvirus Blocked in Late Viral Gene Expression Can Establish Latency and Elicit Protective Cellular Immunity[1]

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The human γ-herpesviruses, EBV and Kaposi's sarcoma-associated herpesvirus, are widely disseminated and are associated with the onset of a variety of malignancies. Thus, the development of prophylactic and therapeutic vaccination strategies is an important goal. The experimental mouse γ-herpesvirus, γHV68 (or MHV-68), has provided an in vivo model for studying immune control of these persistent viruses. In the current studies, we have examined infectivity, immunogenicity, and protective efficacy following infection with a replication-deficient γHV68 blocked in late viral gene expression, ORF31STOP. The data show that ORF31STOP was able to latently infect B cells. However, the anatomical site and persistence of the infection depended on the route of inoculation, implicating a role for viral replication in viral spread but not the infectivity per se. Furthermore, i.p. infection with ORF31STOP elicited strong cellular immunity but a non-neutralizing Ab response. In contrast, intranasal infection was poorly immunogenic. Consistent with this, mice infected i.p. had enhanced control of both the lytic and latent viral loads following challenge with wild-type γHV68, whereas intranasal infected mice were not protected. These data provide important insight into mechanisms of infection and protective immunity for the γ-herpesviruses and demonstrate the utility of replication-deficient mutant viruses in direct testing of "proof of principal" vaccination strategies. *The Journal of Immunology,* **2007, 179: 8392–8402.**

The human γ-herpesviruses, EBV and Kaposi's Sarcoma-associated herpesvirus, establish persistent infection in the immunocompetent host via multiple immune evasion strategies (1). Although the acute stage of infection is rapidly cleared by a strong immune response, latency is established and maintained for life. Because the latent stage of infection is associated with immunosuppression-induced lymphoproliferative disorders and a variety of malignancies, the development of prophylactic and therapeutic vaccines targeting the latent stage of infection is a high priority. Analysis of infection of mice with the rodent γ-herpesvirus, γHV68, or MHV-68, has provided insight into mechanisms for the establishment and immune control of latent γ-herpesvirus infection and provides an important in vivo model for testing vaccination strategies (2–6).

Intranasal infection of mice with γHV68 results in a lytic infection in the lung, which is effectively cleared, but the virus "sneaks" through and establishes life-long latency. Early γHV68 vaccination studies focused on generating protection against the initial lytic infection using subunit vaccines targeting defined T cell or Ab neutralizing epitopes (7–10). Although these approaches were successful in lowering the lytic viral load and the early latent load, they were unable to prevent the establishment of long-term latency. An explanation for why these approaches failed was revealed by accumulating evidence that latency can be established independently of the lytic infection. First, it was shown by Tibbetts et al. (11) that γHV68 latency was independent of the infectious dose of virus. Second, Coleman et al. (12) used replication-attenuated viruses deficient in thymidine kinase to show that latency was independent of lytic load. Third, we showed that latency is established in lung B cells as early as 3 days following infection, concurrent with ongoing lytic infection in lung epithelial cells (13). Fourth, by using cidofovir to inhibit viral replication, we showed that treatment initiated several days after intranasal infection did not interfere with the establishment of latency in the spleen (13). Finally, Moser et al. (14) used a replication-deficient virus blocked for the expression of an immediate early viral gene to show that latency could be established in the lung following intranasal infection, and we showed that latency could be established in the spleen after i.p. administration of a replication-deficient virus blocked at the stage of late viral gene expression (13). Together, these results demonstrate that there is no direct link between the lytic infection and the onset of latency and indicate that the latent phase of infection must be targeted directly to reduce or prevent long-term latency.

The requirement for targeting latency directly adds severe limitations to the subunit vaccine approach because latency-specific epitopes in γHV68 infection have been difficult to define, and questions remain whether the latent stage of the infection is immunogenic. To circumvent these problems, several labs have explored vaccination strategies involving live attenuated viruses, taking advantage of the availability of mutant viruses that are incapable of establishing latency or that are capable of establishing a latent infection with various defects in reactivation (15–18). The new finding that viral replication is not absolutely required for the establishment of latency has led to renewed interest in replication-deficient mutants (12–14, 19). The rationale for this approach is

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that the vaccinating virus can establish latency in the absence of lytic replication and elicit an immune response against the latent stages of infection. However, because of the inability to replicate and hence reactivate, the vaccinating virus would pose no threat to the host. Ideally, immunity generated during the establishment of latency would confer protection against challenge with wild-type virus.

Two previous studies with replication-deficient viruses blocked at different stages of the viral life cycle have been reported. Moser et al. used a replication-deficient virus with a translational stop codon inserted in ORF50, an immediate early viral gene that functions as a major transactivator of the lytic program (14, 20, 21). Infection with this mutant virus failed to establish latency in the spleen and failed to confer protective immunity against a subsequent challenge with wild-type virus. The absence of protection was consistent with the finding that infection by the mutant virus was not immunogenic, a conclusion supported by an absence of activated T cells and B cells in mice infected with the ORF50 mutant virus. A second study analyzed protection elicited by a replication-deficient virus with a frameshift deletion introduced in an early gene encoding ORF6, the ssDNA binding protein (19). Infection with this virus also failed to establish latency in the spleen; however, persistent infection was established in cells in the peritoneum. This mutant could protect against subsequent challenge with wild-type virus, although the mechanism of protection was not explored.

We reasoned that a virus blocked at a later stage of the life cycle might be more immunogenic. Therefore, in the current study, we used a replication-deficient virus generated by insertion of triple stop codons into the N terminus of ORF31 (22). ORF31 is a protein of unknown function that is conserved between the β- and γ-herpesviruses but for which there is no known mammalian homologue. The mutant virus (ORF31STOP) expressed immediate early and early genes but was deficient in late viral protein expression and failed to produce infectious viral particles in the absence of trans rescue (13, 22). The data show that i. p. infection with ORF31STOP virus established latency in the spleen, was highly immunogenic, and conferred substantial protection against challenge with wild-type virus, although sterilizing immunity was not achieved. In vivo analysis of mice infected with ORF31STOP provided insight into the requirements for the establishment of latency and the immunogenicity of a replication-deficient virus, principles underlying the use of live, attenuated viruses for effective γ-herpesvirus vaccine development.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory or obtained from the Trudeau Institute Animal Breeding Facility. Mice were housed under specific pathogen-free conditions before γHV68 infection at 6–10 wk of age and in animal biosafety level 3 containment after infection. All animal procedures were approved by the Institutional Animal Care and Use Committee at Trudeau Institute.

Virus stocks, infections, and vaccination

Clone WUMS of γHV68 (23) was propagated in NIH-3T3 cells. The generation of the replication-deficient ORF31STOP γHV68 viruses, BAC$^+$ ORF31STOP, and BAC$^-$ ORF31STOP have been described (22). The mutant viruses were propagated in the complementary T-Rex mouse fibroblast cell line in DMEM medium supplemented with 10% FBS, penicillin, streptomycin (Sigma-Aldrich), and doxycyline (BD Biosciences). Wild-type γHV68 and ORF31STOP virus titers were determined by plaque assay on NIH-3T3 and T-Rex cells, respectively. The absence of reversion either in vivo or during in vitro propagation of the virus was confirmed by the presence of the unique *Bam*HI restriction site integrated into the mutant virus (13, 22), the inability of mutant virus to grow on NIH-3T3 cells, and the absolute dependence on the T-Rex complementary cell line for plaque formation. Female C57BL/6 and BALB/c mice were anesthetized with 2,2,2-tribromoethanol (200 mg/kg) before intranasal (i.n.)[3] infection with 400 PFU wild-type γHV68 or 3×10^4 PFU BAC$^-$ ORF31STOP. Mice were infected via i.p. inoculation with either 10^5 PFU BAC$^-$ ORF31STOP or 10^6 PFU BAC$^+$ ORF31STOP. Except for the comparative data presented in Table I, all data presented were obtained with the BAC$^-$ ORF31STOP, referred to as ORF31STOP throughout the manuscript. For vaccination studies, 5- to 6-wk-old female C57BL/6 mice were vaccinated with either 10^5 PFU ORF31STOP virus i.p. or 3×10^4 PFU ORF31STOP i.n. Mice administered with PBS i.n. served as nonvaccinated controls. Thirty days following vaccination, mice were challenged with 400 PFU wild-type γHV68 virus, i.n.

Table I. *Effect of BAC sequences on the ability of ORF31STOP to establish infection in splenic B cells, macrophages, and dendritic cells*

	Reciprocal Frequency[a]			
	WT[b]	BAC$^-$ ORF31 STOP[c]	WT[d]	BAC$^+$ ORF31 STOP[e]
B cell[f]	114	287	54	14
	54	345		
Macrophage[g]	585	9,264	111	55,510
	748	2,049		
DC[h]	150	836	111	<60,000[i]
	114	1,706		

[a] Reciprocal frequencies at 14 days post infection (dpi) were determined by linear regression analysis of limiting dilution nested PCR assay (LDA/PCR) data.
[b] Mice were infected i.p. with 10^5 PFU wild-type γHV68. Results of two independent experiments are shown.
[c] Mice were infected i.p. with 10^5 PFU BAC$^-$ ORF31STOP. Results of two independent experiments are shown.
[d] Mice were infected i.p. with 10^6 PFU wild-type γHV68.
[e] Mice were infected i.p. with 10^6 PFU BAC$^+$ ORF31STOP.
[f] B cells were sorted based on CD19 expression.
[g] Macrophages were sorted as CD11b$^+$splenocytes.
[h] DC (dendritic cells) were sorted as CD11c$^+$ (CD11b$^+$ or CD11b$^-$) splenocytes.
[i] The limit of detection was 1/60,000 in this assay.

Tissue preparation

Single cell suspensions from spleens were prepared by mechanical disruption and straining through nylon mesh. Lymphocytes were isolated from lung tissue following Collagenase D digestion (5 mg/ml; Roche) and Percoll gradient centrifugation.

Plaque assay

The concentration of lytic virus in lung tissue was determined using a standard plaque assay on NIH-3T3 mouse fibroblasts (24). Lung tissue obtained at various times post infection was mechanically homogenized and incubated for 1 h with 3T3 cells at 37°C and then overlaid with carboxymethyl cellulose (Sigma-Aldrich). After 6 days of incubation at 37°C, monolayers were fixed with methanol, stained with 8% Giemsa, and plaques counted.

Infective center assay

The frequency of latently infected cells capable of spontaneous in vitro reactivation was assessed using an infective center assay, as previously described (25). Ten-fold serial dilutions (in triplicate) of splenocytes starting at 10^6 cells/well, were plated onto monolayers of NIH-3T3 mouse fibroblast cells. Monolayers were incubated overnight at 37°C and then overlaid with carboxymethyl cellulose. Plaques were quantitated 6 days later after methanol fixation and Giemsa staining. Samples were also assayed following one cycle of freeze/thaw to determine the contribution of lytic virus to the overall viral titers. The number of latently infected cells was then calculated as the difference between the total number of infected cells and the number of lytically infected cells.

Limited-dilution PCR analysis

The frequency of cells carrying viral genome was determined using an LDA/PCR for the γHV68 ORF50 gene as described (26, 27). Briefly,

[3] Abbreviations used in this paper: dpi, days post infection; LDA/PCR, limiting dilution nested PCR assay; i.n., intranasal.

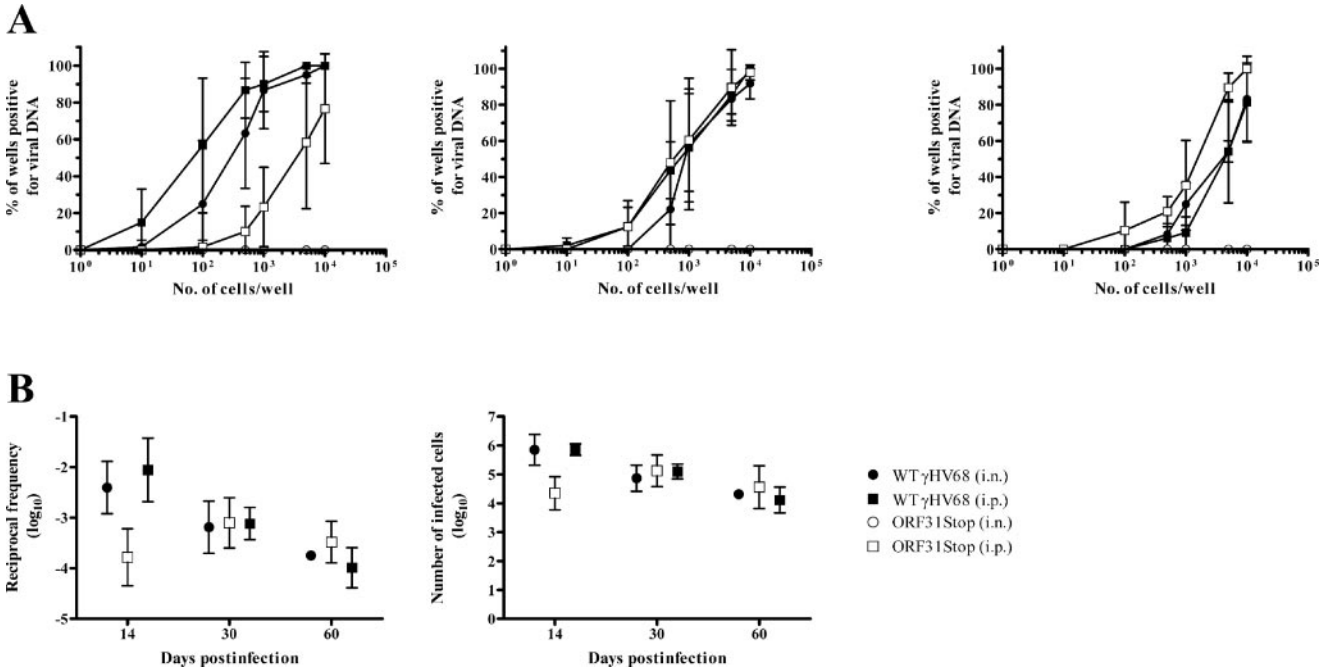

FIGURE 1. Establishment and maintenance of viral infection in the spleen by ORF31STOP is dependent on the route of infection. C57BL/6 mice were infected i.p. with 10^5 PFU of wild-type γHV68 (■) or (□); or i.n. with 400 PFU of wild-type γHV68 (●) or 3×10^4 PFU ORF31STOP (○) virus. *A*, LDA/PCR analysis of viral genome positive spleen cells at 14, 30, and 60 dpi. *B*, The reciprocal frequency of virus genome positive splenocytes was determined at 14, 30, and 60 dpi by linear regression analysis with a 95% confidence level. The absolute numbers of virus infected cells per spleen were then calculated using the reciprocal frequencies. Data represent the mean value of three to five mice per experimental group ± SD.

FACS sorted cells or total cells were resuspended in isotonic buffer and starting at 10^3 or 10^4 cells/well were diluted with uninfected NIH-3T3 cells, and then transferred into 96-well plates. Twelve replicate reactions were performed for each cell dilution per experiment. Subsequently, cells were lysed without DNA isolation and the ORF50 gene was amplified using nested primers. A 2-μl aliquot of the first PCR product was reamplified using nested primers and the final PCR product was electrophoresed on a 3% agarose gel and stained with ethidium bromide. The reciprocal frequency of cells carrying viral genome was determined using linear regression with a 95% degree of confidence.

ELISA

Virus-specific IgG titers in sera were determined by ELISA (27). Nunc ImmunoMaxisorp plates were coated with purified virions at a concentration of 0.5 μg/well. Following an overnight incubation at 4°C, plates were washed with PBS-Tween (0.05%) and subsequently blocked with PBS/BSA (3%) overnight at 4°C. Dilutions of sera, starting at 1/20, were prepared in PBS/0.05% Tween/0.5% BSA and added to the Ag coated plates. After an overnight incubation at 4°C, γHV68-specific IgG was detected using alkaline phosphatase conjugated goat anti-mouse IgG Ab (Sigma-Aldrich) and *p*-nitrophenyl phosphate (Sigma-Aldrich). The color development was detected by 405 nm absorbance readings.

Virus neutralization assay

Virus (10^3 PFU) was incubated with serial dilutions of heat inactivated serum samples in 96-well plates for 1 h at 37°C. Following the incubation, virus/sera mixtures were transferred to 96-well plates containing monolayers of fibroblasts. After 7 days of incubation at 37°C 5% CO_2, the monolayers were fixed and plaque formation revealed by staining with 8% Giemsa. Neutralization titers are expressed as the highest reciprocal dilution of sera required causing a 50% reduction in plaque number.

ELISPOT

Nitrocellulose-coated 96-well plates (Millipore) were coated at 4°C overnight with anti-mouse IFN-γ (10 μg/ml; BD Pharmingen), washed three times with PBS, and blocked for 1 h at 37°C with complete medium. Splenocytes from uninfected B6 mice were incubated for 2 h at 37°C in complete medium containing either $ORF6_{487-495}$ (1 μg/ml), $ORF61_{524-531}$ (1 μg/ml) to measure epitope-specific CD8 T cells, or 10^8 PFU wild-type γHV68 to assess virus-specific CD4 T cells (28). The splenocytes (irradiated at 3000R) were added as feeder cells (1×10^6/well) to duplicate wells containing 3-fold serial dilutions of T cells isolated from infected mice (starting at 1×10^5 cells/well). Control wells contained T cells with unpulsed feeder cells. Plates were incubated at 37°C for 48 h in complete medium with 10 U/ml human rIL-2. Secreted cytokine was detected using biotinylated rat anti-mouse IFN-γ (BD Pharmingen) and streptavidin-alkaline phosphatase (DakoCytomation). Plates were washed five times with PBS/0.05% Tween 20 after each incubation, and spots were visualized with 5-bromo-4-chloro-3-indolyl phosphate/NBT substrate (Sigma-Aldrich) and counted. The number of Ag-specific cytokine secreting cells was determined by subtracting the mean number of spots in wells with no peptide (<10–20/well) from the mean number of spots in wells with peptide-pulsed feeder cells (200 spots/well).

Flow cytometry analysis and cell purification

$CD19^+$ B cells, $CD19^+sIgG^+$ memory B cells, $CD11c^+$ ($CD11b^+$ or $CD11b^-$) dendritic cells, and $CD11b^+$ macrophages from the spleens of infected mice were sorted using a FACSVantage SE with DIVA option (BD Biosciences). Cells were treated with Fc block and stained with fluorochrome conjugated mAbs specific for CD19, CD11b, CD11c, $IgG_{1,2a,2b,3}$, and IgD. A sample of sorted cells was removed to confirm sort purity. Lung and spleen cells were stained with tetramers $ORF6_{487-495}/D^b$, $ORF61_{524-531}/K^b$, and $M2_{91-99}/K^d$ and then stained with PerCp-, PE-, FITC-conjugated Abs. All MHC class I-peptide tetramers were generated by the Trudeau Institute Molecular Biology Core. Monoclonal Abs specific for CD8, CD11b, CD11c, CD19, CD44, CD69, IgD, and $IgG_{1,2a,2b}$ were purchased from either BD Biosciences or eBiosciences. All data were collected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

In vivo CTL assay

The donor (target) splenocytes were incubated with peptides (10^{-5} M) at 37°C in 5% CO_2 for 5 h with occasional mixing. The peptides included $ORF6_{487-495}$, $ORF61_{524-531}$, or irrelevant $FluNP_{366-374}$ for C57BL/6 mice and $M2_{91-99}$ or irrelevant $FluHA_{518-528}$ for BALB/c mice. Cells were washed and labeled with 2 nM, 1 nM, and 0.5 nM CFSE (Molecular Probes) to obtain $CFSE^{high}$ ($FluNP_{366-374}$), $CFSE^{mid}$ ($ORF61_{524-553}$), and $CFSE^{low}$ ($ORF6_{487-495}$) groups, or $CFSE^{high}$ ($M2_{91-99}$) and $CFSE^{low}$ ($FluHA_{518-528}$) groups, respectively. Following CFSE labeling, cells were combined at equal ratios, washed three times in PBS, and resuspended in

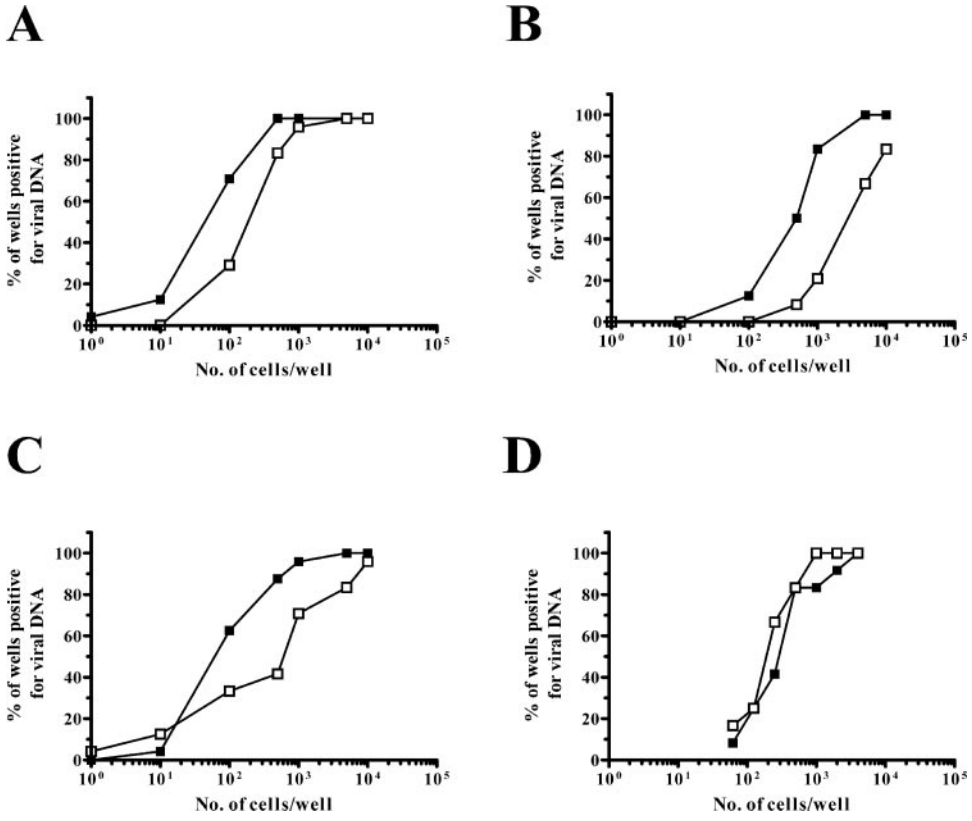

FIGURE 2. Intraperitoneal inoculation with ORF31STOP targets splenic B cells, macrophages, and dendritic cells and is maintained in class-switched memory B cells. C57BL/6 mice were infected i.p. with 10^5 PFU of wild-type γHV68 (■) or ORF31STOP (□). LDA/PCR to detect viral genome was performed on FACS sorted splenic B cells (*A*, $CD19^+CD11b^-CD11c^-$), macrophages (*B*, $CD19^-CD11b^+CD11c^-$), and dendritic cells (*C*, $CD19^-CD11c^+CD11b^+$ or $^-$) at 14 dpi, and memory B cells (*D*, $CD19^+sIgG^+IgD^-$) at 96 dpi. The data are representative of one (*D*) or two (*A–C*) experiments using pools of three to five mice.

PBS at a final concentration of 10^8 cells/ml. Ten million cells (100 μl) were injected i.v. into mice previously infected with γHV68 or ORF31STOP 30 days earlier, or into naive mice as a negative control. After 8 or 16–20 h, spleens were harvested from the recipient mice and single cell suspensions were prepared as above. $CFSE^{high}$, $CFSE^{mid}$, and $CFSE^{low}$ populations were identified and enumerated by flow cytometry. Percent-specific lysis was calculated by using the following formula: percent-specific lysis = [1 − (ratio for infected mice/ratio for naive mice)] × 100, where "ratio" refers to the number of relevant peptide pulsed/irrelevant peptide pulsed.

Statistical analyses

Where indicated, the Student's *t* test was used to determine statistical significance. All statistical analyses were performed using Prism software (GraphPad).

Results

Latency is established and maintained in splenic B cells following intraperitoneal infection with a replication-deficient virus

We have previously characterized infection with a replication-deficient mutant of γHV68 (ORF31STOP) (22) and shown that following i.p. inoculation, cells harboring viral genome could be detected in the spleen, whereas following i.n. infection no genome-positive splenic cells were detectable (13). Because the original studies were conducted with virus containing BAC sequences (BAC^+ ORF31STOP) that have been shown to attenuate the virus in vivo (29), we repeated the studies using a virus in which the BAC sequences had been removed (BAC^- ORF31STOP) (22). As shown in Fig. 1, and consistent with our previous results using BAC^+ ORF31STOP (13), cells harboring viral genome were detected in mice infected i.p. with either wild-type γHV68 or BAC^- ORF31STOP. The frequency of genome-positive cells was lower in mice infected with the replication-deficient mutant compared with the wild-type virus at 14 dpi, but the frequencies were equal by day 30. The frequencies remained comparable at

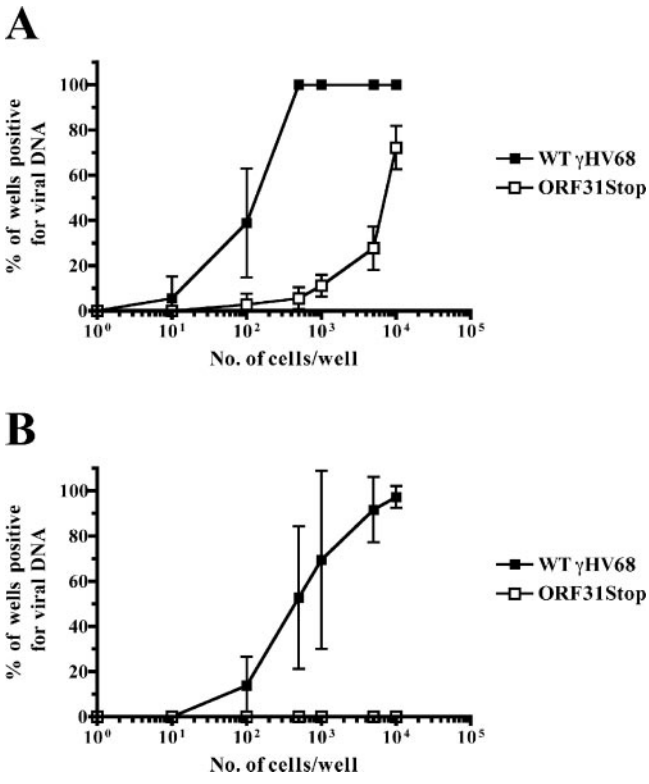

FIGURE 3. Latency is established but not maintained in lung B cells after i.n. ORF31STOP infection. C57BL/6 mice were infected intranasally with 400 PFU wild-type γHV68 (■) or 3×10^4 PFU ORF31STOP (□). $CD19^+$ B cells were isolated from lung tissues by positive enrichment and the frequencies of B cells harboring viral genome at 5 dpi (*A*) and 15 dpi (*B*) was determined by LDA/PCR. Data represent the mean values of three mice per group ± SD.

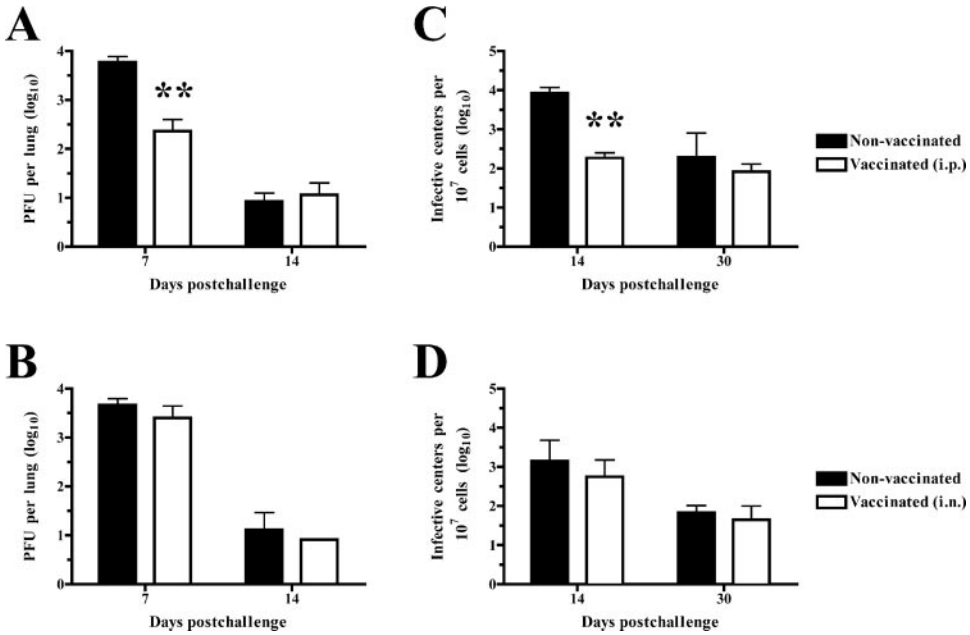

FIGURE 4. Intraperitoneal vaccination with ORF31STOP confers substantial protection against wild-type virus challenge. C57BL/6 mice were mock vaccinated or vaccinated with ORF31STOP either via the i.n. (3×10^4 PFU) or i.p. (10^5 PFU) route. Thirty days post vaccination, mice were i.n. challenged with wild-type γHV68 (400 PFU). Titers of lytic virus in the lungs of i.p. (*A*) or i.n. (*B*) vaccinated mice were determined by plaque assay at 7 and 14 days after challenge. Titers of latent virus in spleens from mice vaccinated by the i.p. (*C*) or i.n. (*D*) route were determined using the infective center in vitro reactivation assay at 14 or 30 days after challenge. Data represent the mean values ± SE of two independent experiments with four to five mice per group. **, *p* value (<0.05) was determined by using Student's *t* test.

60 and 90 dpi, the last timepoint analyzed (Fig. 1 and data not shown). Also consistent with previous results, no genome-positive cells were detected following i.n. infection. These data confirm our original observation using BAC^+ ORF31STOP demonstrating that viral replication is not essential for the effective colonization of the spleen following i.p., but not i.n., infection (13).

It has been previously shown that latency in the spleen is established in B cells, macrophages, and dendritic cells (30, 31), and is preferentially maintained in class-switched memory and germinal center B cells (32–34). To further characterize infection by ORF31STOP and to determine the impact of BAC sequences, we assessed which cell types harbored viral genome at early and late time points after i.p. infection with wild-type or ORF31STOP viruses (BAC^+ or BAC^-). Splenic macrophages, dendritic cells, and B cells were isolated from mice at 14 dpi and the frequencies of genome-positive cells were determined by LDA/PCR. The data in Fig. 2 show that all three cell types harbor viral genome 14 dpi with BAC^- ORF31STOP, although the frequencies were somewhat lower than cells isolated from mice infected with wild-type γHV68. Analyses of long-term viral reservoirs at 96 dpi in class-switched ($sIgG^+$) memory and germinal center B cells revealed that the frequencies were comparable following infection with BAC^- ORF31STOP and wild-type γHV68 (Fig. 2*D*), and only slightly reduced (~5-fold) among total B cells (data not shown). Thus, the BAC^- ORF31STOP virus established infection in the expected reservoirs (B cells, macrophages, and dendritic cells) and attained access to the long-lived memory B cell population in which latency has been shown to be maintained long-term. In contrast, analyses of the cellular reservoirs of infection in the spleen following i.p. inoculation with BAC^+ ORF31STOP showed striking differences in the pattern of infection. Infection was established predominantly in B cells, with 500-fold reduced frequencies of viral genome in macrophages, and no virus detected in dendritic cells at 14 dpi (Table I). In addition, the infection in B cells was not sustained, with the levels of virus below the limits of detection by 90 dpi (data not shown). Thus, BAC-associated attenuation of viral infection severely impacted the reservoirs and maintenance of latency. Therefore, all subsequent experiments were conducted with BAC^- ORF31STOP, referred to simply as ORF31STOP for the rest of the experiments.

Latency is established but not maintained in lung B cells following intranasal infection with a replication-deficient virus

We had previously reported that splenic latency was not established following i.n. infection with ORF31STOP, but had not determined whether lung B cells were infected (13). In the current

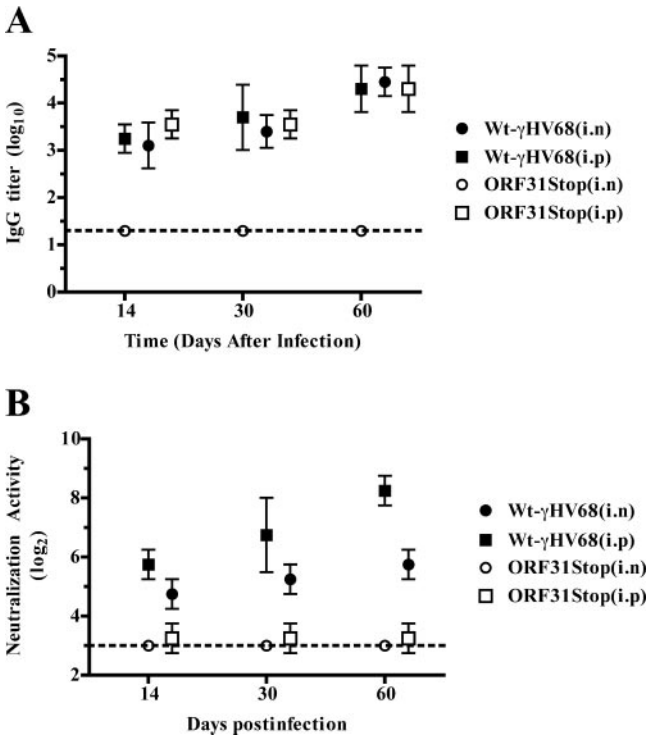

FIGURE 5. Impaired Ab responses following infection with ORF31STOP. C57BL/6 mice were infected i.p. with 10^5 PFU of wild-type γHV68 (■) or ORF31STOP (□), or i.n. with 400 PFU of wildtype γHV68 (●) or 3×10^4 PFU ORF31STOP (○). Serum samples were collected at indicated time points. Virus specific total IgG (*A*) and neutralizing activity (*B*) titers were analyzed as described in the *Materials and Methods*. The dotted line in *A* reflects the background of the assay and the dotted line in *B* indicates the limit of sensitivity of the assay based on the lowest dilution used. Data points represent the mean values of four mice ± SD.

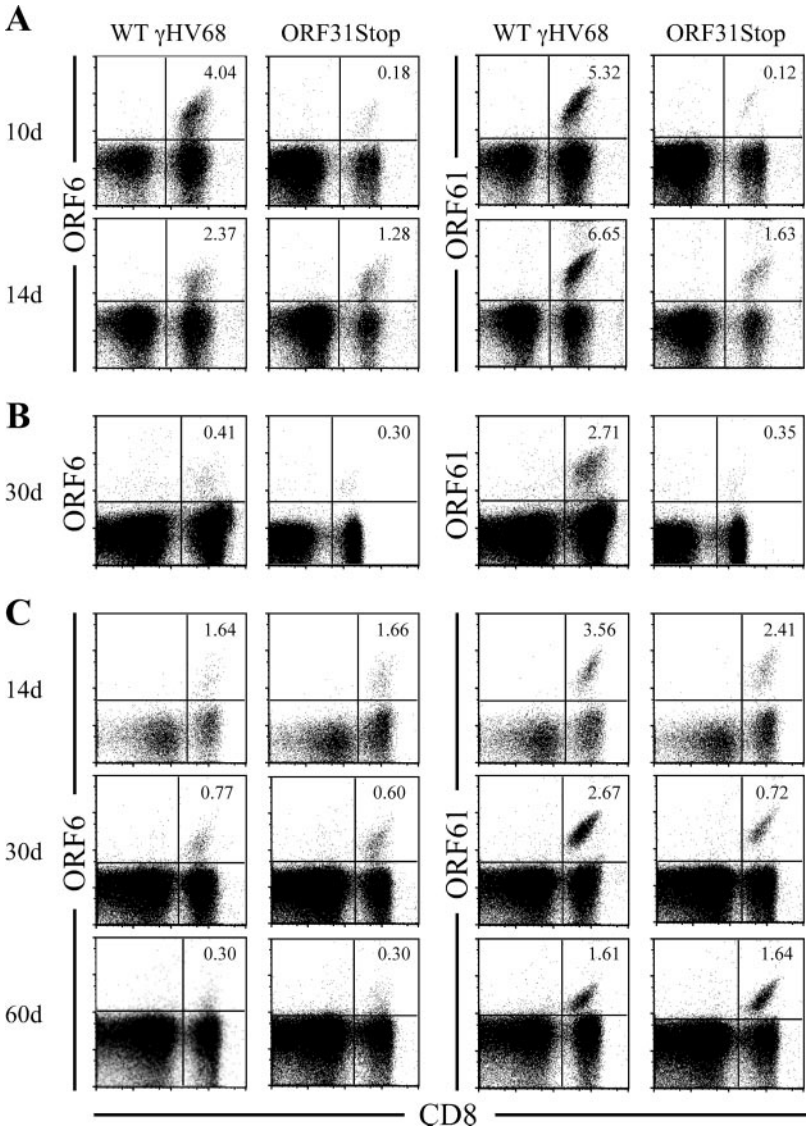

FIGURE 6. Infection with ORF31STOP elicits virus-specific CD8 T cells. C57BL/6 mice were infected with wild-type γHV68 or ORF31STOP either i.n. or i.p. The frequencies of CD8 T cells specific for two immunodominant lytic epitopes, $ORF6_{487-495}$ and $ORF61_{524-531}$, were determined via tetramer staining of $CD44^{high}$ cells. *A*, Following i.n. infection with 400 PFU wild-type γHV68 or 3×10^4 PFU ORF31STOP, frequencies of ORF6- and ORF61-specific CD8 T cells in lung tissue were analyzed at 10 and 14 dpi, and (*B*) frequencies in the spleen were analyzed at 30 dpi. *C*, Following i.p. infection with 10^5 PFU wild-type γHV68 or ORF31STOP, frequencies of ORF6- and ORF61-specific CD8 T cells in the spleen were determined at 14, 30, and 60 dpi. Numbers inside dot plots represent the average percentage values, ± SD, of Ag specific CD8 T cells for each group (four mice per group) at indicated time points. Data are representative of three independent experiments.

studies, we analyzed the frequency of virus in lung B cells at 5 and 15 days post i.n. infection. Latency was readily detected at both timepoints in mice infected with wild-type γHV68 (reciprocal frequencies of 91 and 1433 at 5 and 15 dpi, respectively). Whereas low frequencies of genome-positive lung B cells were detected at 5 days following i.n. infection with ORF31STOP, the infection was not sustained as the frequencies of genome-positive cells dropped to below the limit of detection at 15 dpi (reciprocal frequencies of 25,318 and <1/56,962 at 5 and 15 dpi, respectively) (Fig. 3). Thus we conclude that i.n. infection with ORF31STOP does not establish long-lasting latency in lung B cells.

Intraperitoneal but not i.n. infection with ORF31STOP protects against challenge with wild-type virus

A key question is whether infection with the ORF31STOP virus confers protection against a subsequent wild-type γHV68 challenge, as a replication-deficient virus capable of eliciting protective immunity would be a powerful vaccination strategy for the γ-herpesviruses. To test the protective efficacy of the mutant virus, mice were infected i.p. or i.n. with ORF31STOP virus and challenged intranasally with wild-type γHV68 30 days later. Lytic virus titers were assessed in the lung by a standard plaque assay and splenic latency was assessed by the in vitro reactivation assay. Note that the vaccinating replication-deficient virus will not score in either of these assays, so the assays are specific for the challenge wild-type virus.

Prior i.p. infection with ORF31STOP resulted in a major reduction in the peak titers of challenge wild-type lytic virus in the lung (Fig. 4*A*) and a ~2-log reduction in the peak numbers of latently-infected spleen cells 14 days following challenge with wild-type virus (Fig. 4*C*). However, by 30 dpi there was no statistical difference between the latent load of wild-type virus in the vaccinated and unvaccinated mice. In contrast, mice intranasally vaccinated with ORF31STOP did not reduce the lytic titers in the lung (Fig. 4*B*) or peak numbers of latently-infected cells in the spleen following wild-type challenge (Fig. 4*D*).

Despite the failure of either route of inoculation to provide sterilizing immunity and to prevent the establishment of latency, the striking differences in the degree of viral control following i.p. and i.n. administration of ORF31STOP suggested that the different routes of administration resulted in differences in the elicited immune response. Identification of these differences may provide insight into immune control mechanisms required for protection. Therefore, we assessed both humoral and cellular immunity induced by i.p. and i.n. infection with ORF31STOP.

Infection with ORF31STOP fails to elicit virus neutralizing Abs

Abs have been shown to play an important role in immunity to γHV68 (15, 27, 35) and ORF31STOP is defective in late viral protein expression (Q. Jia and R. Sun, unpublished observations). Therefore, it was of interest to compare the humoral immune response elicited by infection with ORF31STOP and wild-type virus in terms of virus-specific IgG (Fig. 5*A*) and in vitro neutralizing activity (Fig. 5*B*). The titers of virus-specific IgG generated following i.p. infection with ORF31STOP were comparable to those observed following infection with wild-type virus via either route. However, virus-specific IgG was not detected following i.n. ORF31STOP infection (Fig. 5*A*). Although i.p. infection with the ORF31STOP resulted in virus-specific IgG titers equivalent to those observed with wild-type infection, sera from ORF31STOP-infected mice failed to neutralize virus in vitro (Fig. 5*B*). These data suggest that both the inability of the ORF31STOP virus to replicate, as well as the route of infection, had profound effects on the nature of the virus-specific Abs elicited following infection.

Infection with ORF31STOP virus elicits strong cellular immunity

Cellular immunity mediated by CD8 T cells plays an important role in the initial clearance of lytic virus, and both CD4 and CD8 T cells have been shown to contribute to immune control of the latent stage of infection (24, 36–39). We assessed cellular immunity elicited by ORF31STOP compared with wild-type γHV68 infection, using three different assays. First, we examined tetramer-positive T cells specific for two well-characterized lytic epitopes in C57BL/6 mice derived from ORF6 ($ORF6_{487-495}/D^b$), which encodes a ssDNA binding protein, and ORF61 ($ORF61_{524-531}/K^b$), which encodes a ribonucleotide reductase (40), in both the lung and spleen following i.n. or i.p. infection with ORF31STOP and wild-type virus. At 10 dpi following i.n. infection, there was a 22- and 44-fold reduction in the frequency of activated CD8 cells specific for the ORF6 and ORF61 epitopes, respectively, in ORF31STOP-infected mice relative to wild-type γHV68-infected mice, whereas these differences were reduced at 14 dpi (Fig. 6*A*). Importantly at day 30, the time of viral challenge in the protection experiments (Fig. 4), the response to ORF6 was relatively comparable following infection with the ORF31STOP and wild-type viruses, whereas the response to ORF61 in the ORF31STOP-infected mice remained somewhat reduced (Fig. 6*B*). Similar analyses were conducted on CD8 splenic T cells at 14, 30, and 60 days following i.p. infection. The data in Fig. 6*C* show that i.p. infection with ORF31STOP elicited comparable frequencies of ORF6- and ORF61-specific CD8 T cells despite the inability of this virus to replicate.

Second, we used ELISPOT analyses to quantitate the frequencies of epitope-specific CD8 T splenic T cells capable of making IFN-γ following i.p. infection with ORF31STOP and wild-type mice. The ELISPOT data confirm the findings from the tetramer analysis of CD8 T cells (Fig. 7, *A* and *B*) and also allowed us to assess the frequency of splenic CD4 T cells elicited by the two viruses in terms of IFN-γ secretion (Fig. 7*C*) (28). There was no statistically significant difference between numbers of virus-specific CD4 T cells elicited by wild-type and ORF31STOP infections.

Third, we also conducted in vivo cytotoxicity analysis to compare the ability of ORF31STOP and wild-type virus to elicit CTL specific for ORF6 and ORF61. By labeling targets pulsed with different peptides with three concentrations of the CFSE dye, we were able to simultaneously assess cytotoxicity of the two γHV68-specific epitopes, as well as a negative control epitope from influenza virus, within individual mice (Fig. 8*A*). The results show that targets coated with ORF6 and ORF61 peptides were efficiently killed in mice infected i.p. with either ORF31STOP or wild-type γHV68 30 days earlier (Fig. 8*B*). In contrast, i.n.-infected mice showed comparable killing of ORF6-coated targets, but a statistically-significant reduction in the killing of ORF61-coated targets (Fig. 8*C*). Thus, the in vivo CTL data exactly mirrored the tetramer staining data (Fig. 6*B*).

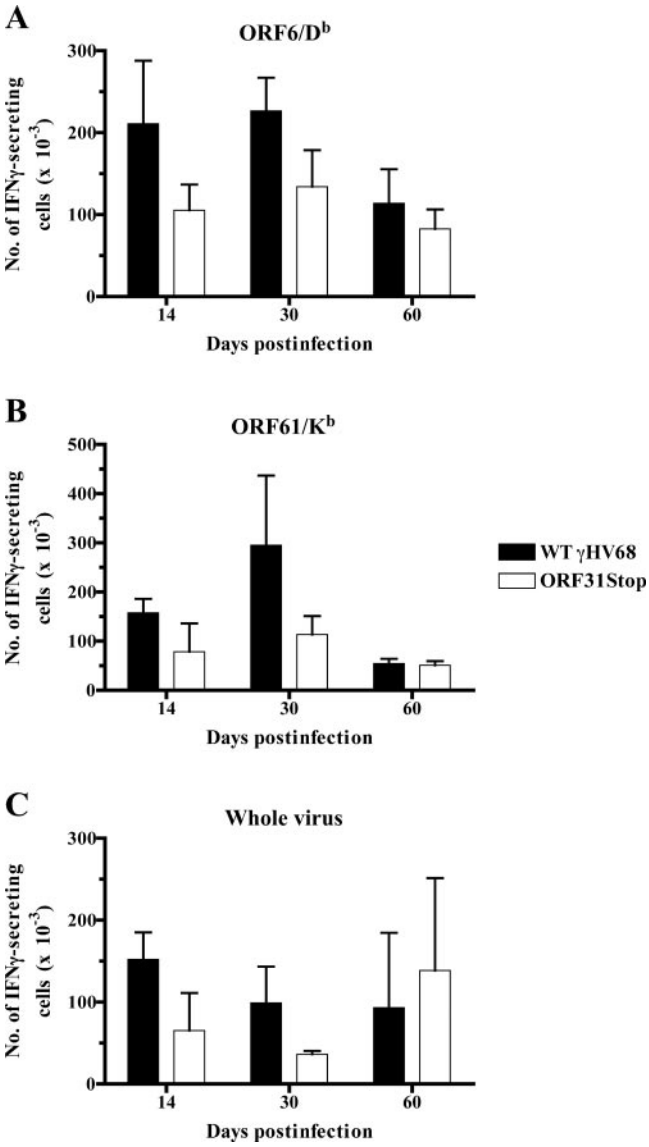

FIGURE 7. Comparable numbers of IFN-γ-secreting virus-specific CD8 and CD4 T cells are elicited following i.p. infection with wild-type γHV68 and ORF31STOP. C57BL/6 mice were infected i.p. with 10^5 PFU wild-type γHV68 or ORF31STOP virus. CD8 splenic T cells were enumerated by an IFN-γ ELISPOT assay in response to APCs pulsed with $ORF6_{487-495}$ (*A*) or $ORF61_{524-531}$ (*B*). CD4 splenic T cells were enumerated by IFN-γ ELISPOT assay in response to APCs infected with wild-type γHV68 (*C*). Results presented are the mean of three samples ± SD.

Using this assay, we also investigated whether i.p. infection with ORF31STOP elicited a response to the latent epitope in the M2 protein ($M2_{91-99}/K^d$), identified in BALB/c mice (36). Challenge studies in BALB/c mice gave patterns of partial protection analogous to the results shown in Fig. 4 for C57BL/6 mice (data not shown). Interestingly, following infection of BALB/c mice with ORF31STOP, there was partial and highly variable killing (between 10 and 70% cytotoxicity) of M2-pulsed targets (Fig. 9). This

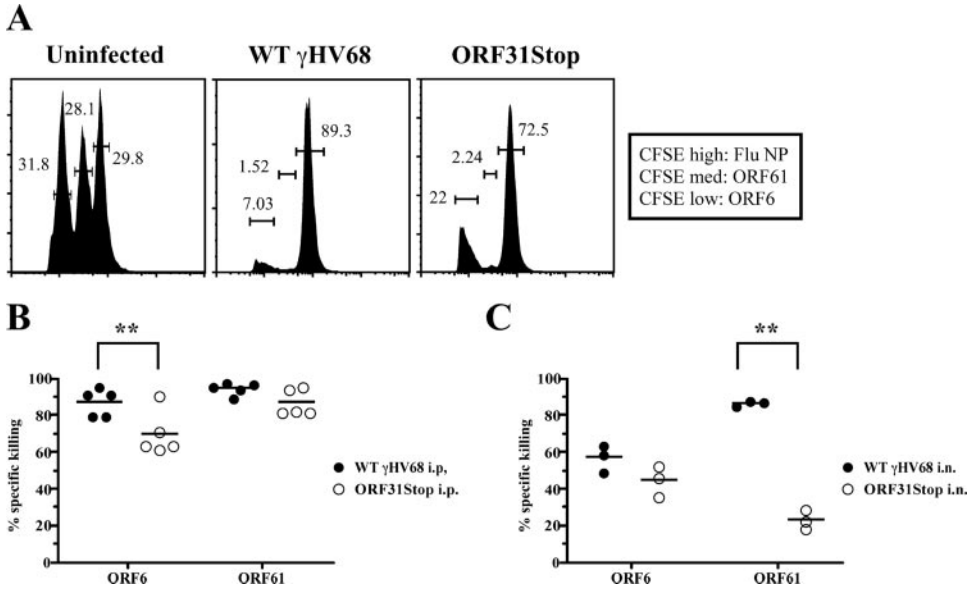

FIGURE 8. The route of infection influences epitope-specific cytotoxicity elicited by ORF31STOP. C57BL/6 mice were assessed for virus-specific cytotoxicity activity at 30 days following i.n. infection with 400 PFU wild-type γHV68 or 3×10^4 PFU ORF31STOP or following i.p. infection with 10^5 PFU wild-type γHV68 or ORF31STOP via an in vivo CTL assay, as described in *Materials and Methods*. Splenic B cells from naive C57BL/6 mice were loaded with peptides specific for $FluNP_{366-374}$, γHV68 $ORF61_{524-553}$, and $ORF6_{487-495}$, and then labeled with graded concentrations of CFSE before i.v. injection into naive or infected mice. Specific lysis was calculated 16 h later. Representative flow cytometry data are shown from analysis of individual mice (*A*). The percent specific cytotoxicity of individual mice 30 days following i.p. (*B*) and i.n. (*C*) infection is plotted ± SD. **, *p* value (<0.05) was determined by using Student's *t* test.

quantitative variation in cytotoxicity was independent of the in vivo incubation time, as analysis of mice sampled between 8 and 20 h after injection of peptide-pulsed targets showed no correlation between degree of cytotoxicity and incubation time (Fig. 9*B*). Taken together, these data show that i.p. infection with a replication-deficient virus elicited vigorous cellular immunity by both CD4 and CD8 T cells against lytic epitopes, whereas there was a deficiency in the response elicited to an epitope associated with the latent infection.

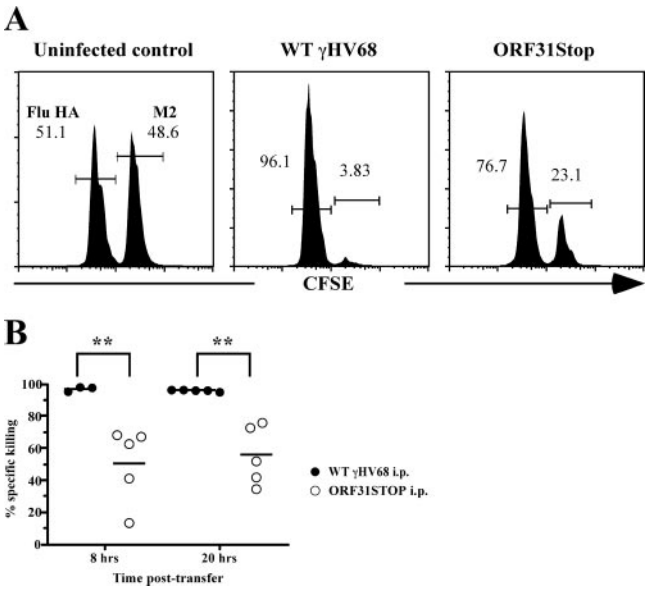

FIGURE 9. Infection with ORF31STOP elicits a weak and highly variable CTL response against a latent viral epitope. BALB/c mice were infected i.p. with 10^5 PFU of either wild-type γHV68 or ORF31STOP. Cytotoxicity was assessed at 30 dpi via an in vivo CTL assay, as described in *Materials and Methods*. Splenic B cells from naive BALB/c mice were loaded with peptides specific for influenza virus hemagglutinin (FLU $HA_{518-528}$) and γHV68 $M2_{91-99}$, and then labeled with graded concentrations of CFSE before injection into naive or infected mice. Specific lysis was calculated 8 and 20 h later. *A*, Representative flow cytometry data from analysis of individual mice. *B*, Percent specific cytotoxicity of individual mice is plotted ± SD. **, *p* value (<0.05) was determined by using Student's *t* test.

Discussion

In this study, we have analyzed the infectivity, immunogenicity and protective efficacy of a replication-deficient murine γ-herpesvirus, ORF31STOP. The data show that the presence of BAC sequences in mutant viruses alters in vivo infectivity, as previously demonstrated (29). Comparative analysis of mice infected with ORF31STOP (BAC^-) and wild-type γHV68 showed that i.p., but not i.n., inoculation resulted in the establishment and maintenance of viral infection in splenic B cells comparable to the latency established by infection with wild-type virus. Intraperitoneal infection was immunogenic and generated strong cellular immunity against lytic epitopes. In contrast, the response to a latency-associated epitope in the M2 gene was variable and generally decreased compared with that elicited by infection with wild-type γHV68. In addition, humoral immunity elicited by ORF31STOP was defective. No virus-specific Abs were detected following i.n. infection and the immune serum of i.p. infected mice was devoid of neutralizing activity. These differences in immunogenicity of ORF31STOP infection correlated with differences in protective efficacy against subsequent challenge with wild-type γHV68. Importantly, i.p., but not i.n., infection with ORF31STOP conferred substantial protection against subsequent challenge with wild-type virus, resulting in an almost 2-log reduction in both lytic and early latent titers. However, the establishment of long-term latency was not prevented.

Prerequisites for the development of prophylactic vaccines for the γ-herpesviruses are to define the requirements for viral spread and the establishment of latency, to determine the immunogenicity

of each stage in the infection, and to acquire a fundamental understanding of the components of immunity capable of mediating protection. Our studies with ORF31STOP have provided insight into these three requirements.

First, regarding the establishment and dissemination of latency, our studies showed that the infectivity of ORF31STOP depended greatly on the route of inoculation. Transient infection was established in lung, but not splenic, B cells following i.n. inoculation, whereas stable splenic latency was established following i.p. administration. Although B cells have been implicated in the spread of latent infection from the lung to the spleen (41, 42), our data show that the infection of lung B cells following i.n. inoculation with ORF31STOP was insufficient for the establishment of latency in the spleen. This may be because the infection of lung B cells with ORF31STOP does not represent true latency capable of transferring infection, a possibility supported by the observation that infection of lung B cells following ORF31STOP infection was transient. Alternatively, because latency amplification in the spleen may involve viral reactivation (43), it is possible that small numbers of infected B cells migrate to the spleen, but there is no amplification of latency due to the inability of replication-deficient ORF31STOP to reactivate in the spleen. These data differ with those previously reported following i.n. infection with the ORF50 replication-deficient virus, where it was found that virus in the lung was sustained (14).

We had initially hypothesized that the establishment of latency in the spleen following i.p., but not i.n., infection with ORF31STOP was a consequence of delivering the virus directly to a target organ of long-term viral latency, consistent with a role for viral replication in the dissemination of latency from the lung (13). However, this straightforward interpretation is not supported by the disparate results obtained by others, using different replication-deficient viruses (14, 19). For example, the replication-deficient G50.STOP virus established infection in lung B cells following i.n. administration. However, infection in the spleen was not observed after either i.n. or i.p. infection, and this result was independent of viral dose (14). In another example, a replication-deficient virus with a mutation in the ORF6 gene established infection in peritoneal cells but not spleen cells following i.p. infection (19). A likely possibility is that the ability of mutant viruses to establish splenic infection is indicative of how early in the life cycle the blocks in viral replication are (see below). Another possible explanation for differential infectivity is that the presence of BAC sequences in the ORF6 STOP virus may have resulted in an inability to establish latency in the spleen, as our studies comparing infections with ORF31STOP with and without BAC sequences revealed striking differences in the cellular reservoirs of latency. Removal of BAC sequences was associated with the establishment of latency in splenic B cells, macrophages, and dendritic cells, the previously-identified cellular reservoirs of early latency in the spleen following infection with wild-type γHV68 (30, 31, 34), whereas the presence of BAC sequences resulted in latency largely restricted to B cells (Table I).

Second, our studies with ORF31STOP infection have contributed to our understanding of the immunogenicity associated with each stage of γHV68 infection. In contrast to previous studies with replication-deficient viruses blocked at the immediate early and early stages of the viral life cycle, infection with ORF31STOP was found to be strongly immunogenic. It was somewhat surprising that infection with a replication-deficient virus led to such a vigorous response to lytic epitopes. There are two nonmutually exclusive possibilities to explain this finding. One possibility is that the delivery of a large bolus of ORF31STOP as the inoculum may be sufficiently antigenic to elicit a cellular immune response without viral replication. Another possibility is that epitopes from ORF6, ORF61, and other early Ag gene products may be available for antigenic presentation despite the inability of this virus to generate infectious particles. The availability of viral gene products for Ag presentation would be strongly influenced by the stage at which the viral life cycle is blocked.

Third, our studies using ORF31STOP are relevant for defining the requirements of protective immunity against γ-herpesviruses. Based on observations that CD8 T cells are effectively induced by infection, and are highly efficient at eliminating lytic virus and controlling immunosuppression-induced lymphoproliferative disease (3, 44, 45), virus-specific CD8 T cells are assumed to be a key component of protective immunity. This notion is supported by previous subunit vaccination studies in which both lytic and latent viral epitopes conferred protective immunity in mice by reducing viral loads and lowering the peak levels of latency (7–9). However, conventional wisdom is that T cell immunity can limit but not prevent infection, as T cells are not likely to be primed and present in sufficient numbers at the site of viral entry to provide sterilizing immunity. Therefore, at most, they are likely to reduce the lytic phase of infection. This is consistent with results from subunit vaccination approaches (7–10) and the current study, in which the lytic loads are dramatically reduced (Fig. 4). The differences in epitope specificity of cellular immunity elicited by i.n. and i.p. infections probably reflect differential infectivity, rather than epitope specificity of protective cellular immunity, as previous epitope-specific vaccination protocols revealed no difference in the long-term protection afforded by ORF6- or ORF61-specific T cells alone (7, 8).

The role of cellular immunity in controlling the establishment of latency is uncertain, as it has not been established that latency-specific epitopes are presented during the initial infection. An epitope associated with the M2 protein has been shown to be a latency-associated epitope (36), but it is possible that M2 is expressed during the amplification stage rather than the initiation of latency, as M2-specific T cells can't be detected early after infection in the lung and have a very transient kinetics of expression during the peak levels of latency in the spleen (46). In this regard, it is intriguing that i.p. infection with ORF31STOP elicited T cells specific for the M2-associated epitope, as detected by the in vivo cytotoxicity assay, although the cytotoxicity was partial and highly variable.

Expression of early latent epitopes may be prevented by viral immune evasion molecules such as K3, which causes down-modulation of MHC class I proteins (47), ORF73, which inhibits T cell epitope presentation (5), or M3, which is a chemokine binding protein and interferes with cellular trafficking essential to the initiation of an effective immune response (48–50). It is possible that mutant viruses with combined defects in replication and expression of immune evasion molecules will allow for the expression of such latency-associated Ags during the establishment of latency and allow the development of protective immune responses directed against bona fide latent Ags. For example, M3, which encodes a chemokine-binding protein, is an important immune evasion protein. Infection with an M3-deficient virus resulted in a severe reduction of latency that was largely reversible by CD8 T cell depletion. Thus, it is possible that vaccination with an M3-deficient, replication-deficient virus would allow generation of robust protective cellular immunity.

Abs, particularly those that neutralize, are also assumed to be an important factor in protective immunity to the γ-herpesviruses. Vaccines targeting EBV gp350 are protective against lymphoma in cottontop tamarinds (51). Similarly, vaccination with γHV68 gp150, a positional homologue of the major neutralizing EBV

epitope gp350 (23), reduced the latent load, but did not prevent the establishment of latency (52). This result is consistent with the reports of a lack of dependence of the establishment of latency on the inoculating dose of lytic virus (11). Recently, the importance of gp150 as a major neutralization target for γHV68 has been questioned (5), so further definition of additional neutralizing epitopes may be necessary for a complete understanding of the role of neutralizing Ab in protective γHV68 immunity (53, 54). In addition, there is generally not a clear correlation between neutralization in vitro and protection in vivo (55), and the importance of neutralizing Ab is especially unclear for the herpesviruses, which spread mainly through cell/cell contact (56). Finally, Ab has effector functions other than neutralization. For example, it is likely that Ab plays a role in controlling viral spread and viral reactivation, as gp150 and gp48 have been shown to promote virion release and intercellular viral spread (57–59). Since both humoral and cellular immunity have been shown to be involved in controlling distinct stages of γ-herpesvirus infection, and the importance of neutralizing Abs for viruses that spread mainly by cell-cell contact is unclear, we cannot conclude that the absence of neutralizing Abs following i.p. infection with ORF31STOP contributed to the incomplete protection against challenge with wild-type virus. Understanding the role of Abs in protective immunity will require a detailed analysis of Ab epitopes and Ab-mediated effector functions, in addition to neutralization, during various stages of γHV68 infection.

In conclusion, these studies have exploited a replication-deficient γHV68 virus to increase our understanding of the mechanisms underlying viral infectivity and dissemination. In addition, our analysis of a replication-deficient virus suggests that a successful vaccine against latency must involve both arms of the adaptive immune response. Importantly, these data affirm that the use of mutant γ-herpesviruses blocked at late stages in the viral replication cycle remains an attractive experimental approach for elucidating fundamental principals associated with the establishment, dissemination, and immunogenicity of γ-herpesvirus latency, which will be applicable for the development of rational vaccination and therapeutic strategies for the human γ-herpesviruses.

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Disclosures

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