Attenuation of the Adaptive Immune Response in Rhesus Macaques Infected with Simian Varicella Virus Lacking Open Reading Frame 61

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Simian varicella virus (SVV) is a neurotropic alphaherpesvirus that causes chickenpox during primary infection and establishes latency in sensory ganglia. Infection of rhesus macaques (RM) with the homologous simian varicella virus (SVV) recapitulates hallmarks of VZV infection. We have shown that an antisense transcript of SVV open reading frame 61 (ORF61), a viral transactivator, was detected most frequently in latently infected RM sensory ganglia. In this study, we compared disease progression, viral replication, immune response, and the establishment of latency following intrabronchial infection with a recombinant SVV lacking ORF61 (SVVΔORF61) to those following infection with wild-type (WT) SVV. Varicella severity and viral latency within sensory ganglia were comparable in RMs infected with SVVΔORF61 and WT SVV. In contrast, viral loads, B and T cell responses, and plasma inflammatory cytokine levels were decreased in RMs infected with SVVΔORF61. To investigate the mechanisms underlying the reduced adaptive immune response, we compared acute SVV gene expression, frequency and proliferation of dendritic cell (DC) subsets, and the expression of innate antiviral genes in bronchoalveolar lavage (BAL) samples. The abundance of SVV transcripts in all kinetic classes was significantly decreased in RMs infected with SVVΔORF61. In addition, we detected a higher frequency and proliferation of plasmacytoid dendritic cells in BAL fluid at 3 days postinfection in RMs infected with SVVΔORF61, which was accompanied by a slight increase in type I interferon gene expression. Taken together, our data suggest that ORF61 plays an important role in orchestrating viral gene expression in vivo and interferes with the host antiviral interferon response.

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Varicella zoster virus (VZV) is a neurotropic human alphaherpesvirus that causes chickenpox during primary infection and establishes latency in sensory ganglia, including the trigeminal and dorsal root ganglia. Reactivation of VZV leads to herpes zoster (HZ; shingles), which is estimated to affect 1 million individuals each year in the United States and can result in significant morbidity and occasionally mortality in aged and immunocompromised individuals (1–4). Reactivation of VZV is generally believed to be due to a decline in T cell immunity (5–10); however, the viral genes that control the switch between latent and lytic replication remain unknown. This is due in part to the fact that VZV is strictly a human pathogen, and animal models of VZV infection recapitulate only certain aspects of pathogenesis. We have previously shown that rhesus macaques (RMs) infected with simian varicella virus (SVV) display the hallmarks of VZV infection in humans, including generalized varicella, development of cellular and humoral immunity, and establishment of latency (11).

Utilizing this model, we previously found that SVV open reading frame 61 (ORF61) was the most prevalent transcript detected during latency in sensory ganglia (12). Interestingly, SVV ORF61 sense transcripts predominate in acutely infected neural ganglia, while antisense transcripts predominate in latently infected ganglia (11, 13). These data imply that ORF61 plays a role in the establishment and/or maintenance of SVV latency. SVV ORF61 encodes a 54.1-kDa polypeptide that is homologous to VZV ORF61 and to other alphaherpesvirus proteins located in similar regions of the viral genome and possessing transcriptional regulatory activity, including herpes simplex virus type 1 (HSV-1) ICP0, equine herpesvirus 1 (EHV-1) ORF63, bovine herpesvirus (BoHV-1) bICP0, and pseudorabies virus (PRV) EP0 (14–19).

Homology between SVV ORF61 and other alphaherpesvirus ICP0-related proteins is predominantly localized to a conserved RING finger motif at the amino terminus, which is important for potential E3 ubiquitin ligase activity (16, 20, 21). SVV ORF61 also contains a nuclear localization signal at the carboxyl terminus of the protein (16). SVV ORF61 transactivates its own promoter and the promoters of SVV immediate-early ORF62, early ORF28, and ORF29, and late ORF68 in transfected Vero cells (16). SVV ORF61 was shown to be nonessential for SVV replication in vitro, though SVVΔORF61 replicates 2- to 5-fold less efficiently and produces smaller plaques than WT SVV in CV-1 cells (16).

In this study, we sought to investigate the role of SVV ORF61 during acute and latent infection, whereby RMs were inoculated intrabronchially with either wild-type (WT) SVV or recombinant SVV lacking ORF61 (SVVΔORF61). Our analysis of the pathogenesis of SVVΔORF61 in vivo revealed that despite similar peak viral loads, the adaptive immune response was attenuated in RMs infected with SVVΔORF61. Further analysis revealed the expression of multiple viral transcripts was significantly reduced in bronchoalveolar lavage (BAL) cells of SVVΔORF61-infected RMs. In addition, we saw an increase in the frequency of plasmacytoid dendritic cells (pDCs) and type I interferon (IFN) gene expression in BAL fluid at 3 days postinfection (dpi) in RMs infected with SVVΔORF61.
MATERIALS AND METHODS

Ethics statement. All rhesus macaques were housed at the Oregon National Primate Research Center Institutional Animal Care and Use Committee. All rhesus macaques were housed at the Oregon National Primate Research Center and were handled in accordance with good animal practices as defined by the Office of Laboratory Animal Welfare. Animals were euthanized at 84 to 103 days postinfection. Sensory ganglia, including trigeminal ganglia (TG) and cervical, thoracic, and lumbar-sacral dorsal root ganglia (DRG-C, DRG-T, and DRG-L/S, respectively), were flash frozen and stored at −80°C until analysis.

Comparative genome analysis of SVVΔORF61 and SVV WT DNA. A microarray hybridization-based method was used to compare SVVΔORF61 genomic DNA (test) to WT SVV (reference) DNA (CGS 385K mutation mapping array phase 1; NimbleGen Systems, Inc., Madison, WI). The design of the microarray used published sequence data for SVVΔORF61 to WT SVV. In addition to the deletion of ORF61, there are 4 nucleotide substitutions within the SVVΔORF61 genome. (A) Schematic representation of the SVV genome highlighting the SVV ORFs (arrows) that contain sequence changes. Sequence variation results in different hybridization intensities, as indicated by the hybridization ratio between SVVΔORF61 and WT SVV and signal potential nucleotide changes. (B to E) Regions containing sequence variation were amplified by PCR and directly sequenced. Sequencing identified that (B) the putative noncoding region before ORF C contains a single-nucleotide substitution from A to G resulting in a silent mutation; (D) within ORF62/71, a transition occurs from T to C results in a silent mutation (note that the nucleotide and position number refers to the genomic position); and (E) the putative noncoding region between the OriS and ORF63 contains a substitution from A to T. Nucleotide substitutions are indicated by boldface and italics.

FIG 1 Comparative genomic hybridization and sequence analysis comparing SVVΔORF61 to WT SVV. In addition to the deletion of ORF61, there are 4 nucleotide substitutions within the SVVΔORF61 genome. (A) Schematic representation of the SVV genome highlighting the SVV ORFs (arrows) that contain sequence changes. Sequence variation results in different hybridization intensities, as indicated by the hybridization ratio between SVVΔORF61 and WT SVV and signal potential nucleotide changes. (B to E) Regions containing sequence variation were amplified by PCR and directly sequenced. Sequencing identified that (B) the putative noncoding region before ORF C contains a single-nucleotide substitution from A to G resulting in a silent mutation; (D) within ORF62/71, a transition occurs from T to C results in a silent mutation (note that the nucleotide and position number refers to the genomic position); and (E) the putative noncoding region between the OriS and ORF63 contains a substitution from A to T. Nucleotide substitutions are indicated by boldface and italics.

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CD20 (primer 1) and 5′-ACGGTTGCTGGATGGATCATCA-3′ (primer 2) (Fig. 1D), and 5′-ATTGTTGCTGGATGGATCATCA-3′ (primer 1) and 5′-AT GCCATGTCGCTTTCA-3′ (primer 2) (Fig. 1E).

RNA isolation and amplification and cDNA synthesis. Total RNA was isolated from cells of BAL fluid collected during acute infection (3 dpi) and latently infected sensory ganglia (>84 dpi) using the Isol-RNA lysis reagent (5 PRIME) method. Samples were DNase treated using the Turbo DNA-free kit (Life Technologies, Grand Island, NY). RNA was amplified using global PCR and T7 RNA polymerase-based amplification (adapted from references 24, 25, and 26). Briefly, first-strand cDNA was synthesized from 1 μg of total RNA using T7 oligo(dT)-T primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Second-strand synthesis was performed using Taq DNA polymerase (New England Biolabs, Ipswich, MA) and a degenerate oligonucleotide primer (DOP) (500 ng/μl; CCGACTCGAGNNNNNNATGTGG) using the following PCR program: 94°C for 3 min, 30°C for 2 min, heat from 30 to 72°C with a rate increase of 0.2°C/s, and a hold at 72°C for 4 min. Global PCR then was performed with the addition of T7 oligo(dT) and DOP primers, followed by 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 4 min, which was followed by a hold at 72°C for 6 min. The cDNA was isolated using phenol-chloroform extraction and concentrated with the DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA). The cDNA was amplified using the T7 MEGAscript kit (Ambion), and the amplified RNA (aRNA) was purified using the RNeasy Minikit (Qiagen, Valencia, CA). Ten μg of aRNA was reverse transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA).

RT-qPCR detection of SVV gene expression. The cDNA was diluted 1:10 and analyzed by quantitative real-time reverse transcriptase PCR (RT-qPCR) using primer and probe sets specific for each viral ORF as described above. A list of the primer and probe sequences designed using Primer Express software (Applied Biosystems) were previously described (12). SVV ORF copy numbers were normalized to the reference gene, glutathione synthetase (GSS; primer 1, 5′-GTGCTGAAGCCCCAGAGA GA-3′; primer 2, 5′-CTTCCTACGTCTCCAGCTTGT-3′). Copy numbers are reported as the averages of triplicate RT-qPCR results for each sample and are within 25% standard deviations for the population.

DNA extraction and qPCR. DNA was extracted from heparinized whole blood (WB), BAL fluid, and portions of frozen ganglia using an ArchivePure DNA Cell/Tissue kit (5 PRIME, Gaithersburg, MD) according to the manufacturer’s protocol. SVV DNA viral loads in WB, BAL fluid, and sensory ganglia were measured by qPCR using Maxima Probe/ROX qPCR Master mix (2×; Fermentas, Glen Burnie, MD) and primers/taqMan probe specific for SVV ORF21. SVV ORF21 is a single-copy gene of the SVV genome that can be used to establish viral loads, thereby facilitating comparison across studies. Other single SVV ORFs have been tested for viral load analysis with comparable results. Following an initial 10-min step at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C were completed, followed by a hold at 72°C for 6 min. The cDNA was isolated using phe-nol-chloroform extraction and concentrated with the DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA). The cDNA was amplified using the T7 MEGAscript kit (Ambion), and the amplified RNA (aRNA) was purified using the RNeasy Minikit (Qiagen, Valencia, CA). Ten μg of aRNA was reverse transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA).

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Whole-genome analysis of SVVΔORF61. SVVΔORF61 is a large (amino acids 5 to 464) ORF61 deletion virus generated using the SVV cosmid recombination system (16). Genetic manipulation of a viral genome can introduce random mutations or genomic re-organization; therefore, the SVVΔORF61 viral genome was comprehensively analyzed via comparative genomic hybridization (CGH) and directly compared to wild-type (WT) SVV (28, 29). Using this technique, any differences in genomic sequence between SVVΔORF61 and WT SVV results in variations in hybridization intensities to corresponding segments represented on the array, giving an altered hybridization ratio between SVVΔORF61 and WT SVV (Fig. 1A). CGH analysis revealed that in addition to the region encompassing ORF61, which is absent from SVVΔORF61, 4 additional areas displayed variations from WT SVV, indicating differences in nucleotide sequence at these loca-
tions. These regions were subsequently amplified via PCR and directly sequenced, resulting in the identification of 4 nucleotide substitutions that produce 2 silent mutations within coding regions and 2 substitutions within noncoding regions of the SVVΔORF61 genome. Specifically, we identified a mutation of nucleotide 391 from A to G in the putative noncoding region before ORF2, located between the terminal (TRL) and the internal (IRL-A) repeat regions (Fig. 1B). Due to a transition of nucleotide 35353 from A to G, a silent mutation occurs within ORF22 (Fig. 1C), while a transition of nucleotide 106546 from T to C produces a silent mutation within ORF62/71 (Fig. 1D). Lastly, the putative noncoding region between the OriS and ORF63 contains a mutation of nucleotide 110356 from C to T (Fig. 1D). Therefore, the deletion of ORF61 did not result in changes in the coding regions of the viral genome that can affect protein products produced by this virus.

Disease severity, viral load, and gene expression in rhesus macaques infected with SVVΔORF61. Rhesus macaques (RMs) were infected with WT SVV or SVVΔORF61 at 4 × 10^5 PFU intrabronchially (n = 4 per group; sex and age matched). We investigated the role of ORF61 in vivo by measuring disease progression, viral replication, immune response, and establishment of latency. We collected blood (PBMC) and bronchoalveolar lavage (BAL) cells at various days postinfection (dpi), and sensory ganglia were collected at necropsy (>84 dpi). All infected RMs displayed hallmarks of SVV infection (11), including development of rash lasting approximately 10 days. There were no quantitative or qualitative differences in rash severity or duration between the cohorts. SVV viral loads were measured by quantitative real-time PCR in BAL and whole-blood samples. Viral loads from BAL fluid peaked at 3 dpi in both WT- and SVVΔORF61-infected RMs and then decreased to levels near or below our limit of detection by 14 to 17 dpi (Fig. 2A). At 3 dpi, viral loads were comparable between the two groups, but at 7 dpi SVVΔORF61-infected RMs had a significantly (P < 0.05) lower SVV genome copy number in BAL fluid than the WT (101 ± 43 compared to 1,193 ± 949 genome copy numbers in 100 ng of DNA). As we previously reported (11, 22), SVV viral loads in whole blood were significantly lower than those in BAL fluid. Nevertheless, we were able to detect SVV DNA in whole blood at 7 dpi in RMs infected with WT SVV but not in RMs infected with SVVΔORF61 (Fig. 2B). Therefore, the ability of SVVΔORF61 to replicate in vivo was reduced compared to that of WT SVV.

Because SVV ORF61 is a viral transactivator (16) and a decrease in viral genome copy number was observed at 7 dpi, we evaluated gene expression of a subset of SVV ORFs in BAL fluid at day 3 postinfection (Table 1). Total RNA was isolated from 2.5 × 10^6 cells from BAL fluid, and 1 μg of total RNA was amplified and analyzed by quantitative real-time reverse transcriptase PCR (RT-qPCR) using primer and probe sets specific for each viral ORF (12). We evaluated SVV gene expression of immediate-early (IE), early (E), and late (L) genes. Specifically, we measured transcripts associated with transcriptional activators ORF4 (IE2), ORF61 (IE3), ORF62 (IE4), and ORF63 (IE5); DNA replication proteins ORF28 (DNA polymerase), ORF29 (single-stranded DNA binding protein), and ORF55 (component of DNA helicase-primase complex); capsid proteins ORF20, ORF23, ORF40 (major capsid protein), and ORF41; tegument proteins ORF9, ORF11, ORF12, ORF21, ORF22, ORF46, ORF64 (phosphoprotein), and ORF65 (phosphoprotein); envelope protein ORF39; and glycoproteins ORF5 (gK), ORF9A (gN), ORF14 (gC), ORF31 (gB), ORF37 (gH), ORF50 (gM), ORF60 (gL), ORF67 (gI), and ORF68 (gE). Our analysis revealed that gene expression in cells from BAL fluid of this subset of viral ORFs is significantly decreased in SVVΔORF61-infected RMs compared to that in WT SVV (P < 0.03). The decrease in the expression of viral transactivators was especially evident for ORF62, where expression was below our limit of detection in three of four animals. These data reveal a critical role for ORF61 in regulating SVV transcription in vivo.

B cell and antibody response to SVVΔORF61. To determine if RMs infected with SVVΔORF61 elicit an immune response similar to those of animals infected with WT SVV, we measured the magnitude and kinetics of the B cell response as well as the generation of SVV-specific IgM and IgG antibody titers postinfection. The expansion of B cells can be measured by flow cytometry based on expression of Ki67, a nuclear protein involved in DNA replication (30). SVV infection induces strong proliferation of B cells, which is indicated by an increase in the frequency of Ki67-positive

![Graph A](http://jvi.asm.org/jvi10295_f2a.png)

**Fig 2.** Viral loads are decreased in the lungs and whole blood at day 7 postinfection in RMs infected with SVVΔORF61. SVV DNA viral load in BAL fluid (A) and whole blood (B) was measured by quantitative PCR using primers and probe specific for SVV ORF21 from WT SVV-infected (closed circle) and SVVΔORF61-infected (open circle) RMs. Data are averages ± standard errors of the means (SEM). *, P < 0.05; ***, P < 0.001. The dashed line indicates the limit of detection.
ORF61 modulates host responses to SVV infection

We next measured SVV-specific IgM (Fig. 3C) and IgG (Fig. 3D) antibody endpoint titers in plasma using standard ELISA. In contrast to RMS infected with WT SVV, RMS infected with SVVΔORF61 generated a modest increase in IgM titers during acute infection. Specifically, the average IgM titer of RMS infected with WT SVV increased from 241 ± 88 (7 dpi) to 2,486 ± 797 (14 dpi), while the average IgM titer of RMS infected with SVVΔORF61 increased from 91 ± 11 (7 dpi) to 196 ± 56 (14 dpi). Although the kinetics of IgG production were similar during SVVΔORF61 and WT SVV infection, endpoint titers were significantly decreased by approximately 1 log in RMS infected with SVVΔORF61 from day 14 postinfection through necropsy. Production of SVV-specific antibodies was significantly reduced in RMS infected with SVVΔORF61 despite an earlier peak within the memory B cell population. This is most likely due to the shorter duration of the proliferative burst.

T cell response to SVVΔORF61. After antigen encounter, naive T cells become activated, proliferate, and differentiate into central memory (CM; CD28+CD95+) and effector memory (EM; CD28−CD95−) T cells. To compare the kinetics and magnitude of the T cell response between RMS infected with SVVΔORF61 and WT SVV, we measured the frequency of Ki67-positive T cells within CM and EM subsets in BAL fluid (Fig. 4A and B) and PMBC (Fig. 4C and D). WT SVV infection induced strong T cell proliferation, as shown by an increase in Ki67-positive T cells from days 3 to 17 postinfection. In cells from BAL fluid, the peak of T cell proliferation was similar, but the duration of proliferation was shorter in RMS infected with SVVΔORF61 than with WT SVV, resulting in significantly lower Ki67 expression at 10 and 14 dpi. To assess differences in the magnitude of the proliferative burst, we measured the area under the curve (AUC). Proliferation of CD4 CM (P < 0.03) as well as CD8 EM (P < 0.05) in cells from BAL fluid between 0 and 21 dpi was significantly reduced in SVVΔORF61-infected RMS. Within PMBC, the proliferation kinetics of CD4 T cells was reduced, although only the decreased proliferation of CD4 EM T cells was statistically significant at 14 dpi in RMS infected with SVVΔORF61 and in overall magnitude (AUC P < 0.005). CD8 T cell proliferation was delayed, peaking 10 dpi instead of 7 dpi in SVVΔORF61-infected RMS.

Additionally, we measured the frequency of SVV-specific T cells within each subset by quantifying the number of IFN-γ- and TNF-α-producing cells using intracellular cytokine staining (ICCS) after stimulation with either SVV lysate or an SVV overlapping peptide pool covering ORF4, ORF31, ORF61, and ORF63. Our controls for ICCS included stimulation of cells from BAL fluid and PMBCs with uninfected cell lysate and DMSO, which did not result in production of IFN-γ and/or TNF-α above background levels. Moreover, the primary rhesus fibroblasts used to generate the SVV lysate express undetectable levels of major histocompatibility complex (MHC) class I and II. Thus, T cell responses detected following stimulation with SVV lysate are specific to SVV and are not alloreactive in nature.

We stimulated both BAL fluid (Fig. 5A and B) and PMBC (Fig. 5C and D) isolated from infected RMS at different dpi. Figure 5 depicts the percentage of responding SVV-specific CD4 CM and EM T cells in SVV lysate (Fig. 5A and C) and CD8 CM and EM T cells in the SVV peptide pool (Fig. 5B and D) in BAL fluid. While the percentage of SVV-specific responding CD4 CM T cells increased from 7 to 21 dpi in both groups, RMS infected with SVVΔORF61 generated a significantly reduced frequency of cells responding to SVV lysate at days 21 and 28 postinfection. In RMS infected with SVVΔORF61, the percentage of responding CD4 EM T cells was also significantly reduced at 28 dpi. Our previous studies indicate that CD8 T cells show modest responses to lysate, likely due to the absence of free virus in SVV lysate (SVV is highly cell associated) or to the low efficacy of cross-presentation of MHC class I-associated viral antigens following pinocytosis/phagocytosis by antigen-presenting cells (31). Therefore, we determined the frequency of SVV-specific CD8 T cells following stimulation with peptide pools covering ORF4, ORF31, ORF61, and ORF63. These ORFs were selected based on the immunoge-
nicity of their VZV homologs in humans (32–34). The percentage of SVV-specific CD8 CM T cells was significantly reduced at 14 and 21 dpi in RMs infected with SVV/H9004 ORF61. The frequency of SVV-specific CD8 EM T cells increased in a similar fashion from day 0 to day 14 postinfection and then remained relatively stable in RMs infected with either virus.

In PBMC from RMs infected with SVV/H9004 compared to RMs infected with WT SVV, we detected reduced frequencies of responding CD4 CM and EM T cells at 21 dpi to lysate (Fig. 5C), but the differences did not reach statistical significance. Similarly, the frequency of CD8 CM and EM T cells responding to SVV peptides (Fig. 5D) at 21 dpi was lower in RM infected with SVVΔORF61 than with WT SVV, but these differences also did not reach statistical significance.

**Plasma cytokine levels during SVVΔORF61 infection.** We next measured plasma cytokine levels in RMs infected with SVVΔORF61 and WT SVV by multiplex ELISA (Fig. 6). Plasma levels of the inflammatory cytokines IFN-γ and IL-15 and the regulatory cytokines IL-6 and IL-1Ra peaked at 7 dpi in RM infected with WT SVV. In contrast, plasma levels of these cytokines remained stable at preinfection levels in RM infected with SVVΔORF61 throughout the acute phase. We also measured MCP-1, sCD40L, IL-8, and IL-12/23(p40), which showed no change with infection or between groups (data not shown). Production of lower plasma cytokine levels correlates with the dampened adaptive immune response measured in SVVΔORF61-infected RMs.

**Modulation of dendritic cell recruitment and response by SVVΔORF61.** Dendritic cells (DCs) play a critical role in the antiviral immune response by secreting antiviral factors, including type I interferons, and by priming the adaptive immune response. To determine if the differences in the T and B cell responses observed in SVVΔORF61-infected RMs were due to differences in DC populations, we measured the frequency and proliferation of myeloid (mDC; CD11c+) and plasmacytoid (pDC; CD123+) and CD123+CD11c+ DCs by flow cytometry of BAL fluid (Fig. 7). During acute infection with WT SVV or SVVΔORF61 in the lungs, mDC frequencies in BAL fluid decrease from 0 to 10 dpi while CD123+CD11c+ DC frequencies increase (Fig. 7B and D). Although the kinetics and amplitude of the frequency changes are similar, mDC proliferation occurs earlier and reaches a higher peak in SVVΔORF61- than WT SVV-infected RM (Fig. 7E). Proliferation of CD123+CD11c+ DCs also occurred earlier in SVVΔORF61-infected RM but reached peak levels similar to those of WT SVV-infected RM at 7 dpi (Fig. 7G). Finally, the frequency of pDCs increased significantly only in BAL fluid of
SVVΔORF61-infected RMs at 3 dpi (Fig. 7C). Accordingly, both the peak and magnitude of pDC proliferation were significantly increased at 3 and 7 dpi only in SVVΔORF61-infected RMs (AUC $P < 0.01$) (Fig. 7F). In summary, overall DC proliferation in BAL fluid occurred earlier in SVVΔORF61-infected RMs with a larger proliferative burst in mDCs and pDCs. Moreover, the frequency of pDCs increased only in RMs infected with SVVΔORF61. These data suggest that the loss of ORF61 results in an improved antiviral DC response in BAL fluid in vivo.

Expression levels of innate immune genes during acute infection. Previous studies have shown that VZV ORF61 interferes with the innate immune response. Specifically, VZV ORF61 degrades activated IRF3 (35) and inhibits the NF-κB pathway in DCs (36). Given the increased frequency and proliferation of pDCs in BAL fluid of SVVΔORF61-infected RMs at 3 dpi, we measured the expression of type I interferons (IFN-α and IFN-β), interferon-stimulated genes (ISG54 and ISG56), and IL-6, which requires NF-κB activation, in BAL cells from RMs infected with WT SVV or SVVΔORF61 collected at 3 dpi (Fig. 8). Expression levels overall were increased in BAL fluid of RMs infected with SVVΔORF61 compared to WT SVV, though only differences in IFN-β expression reached statistical significance. Our data suggest that the in-
nace of SVV latency in sensory ganglia.

ORF61 is not required for maintenance of SVV latency. We did not measure a significant difference in the number of viral ORFs detected during infection with SVV by RT-qPCR (12). We did not measure a significant difference in the number of viral ORFs detected during infection with SVV by RT-qPCR (12). We did not measure a significant difference in the number of viral ORFs detected during infection with SVV by RT-qPCR (12).


detection of latent viral genome copy number and gene expression. SVV DNA viral loads in sensory ganglia were measured by quantitative PCR using primers and probe specific for SVV ORF21 (Table 2). We detected SVV DNA within the sensory ganglia of RMs infected with SVV, indicating that ORF61 is not necessary for establishment of SVV within sensory ganglia. The viral loads reported in Table 2 reflect SVV genome copy numbers in a portion of the ganglia and therefore are not representative of the entire organ. We next measured the gene expression of a subset of viral ORFs (A, B, 4, 10, 61, 64, 65, 66, 67, and 68) previously detected in the sensory ganglia of RMs latently infected with SVV by RT-qPCR (12). We did not measure a significant difference in the number of viral ORFs detected during latency between SVVΔORF61- and WT SVV-infected RMs (data not shown), suggesting that ORF61 is not required for maintenance of SVV latency in sensory ganglia.

Discussion

SVV infection of rhesus macaques recapitulates the hallmarks of VZV infection, thereby providing a robust animal model to elucidate host-pathogen interactions during VZV infection. Previous studies showed that SVV ORF61 transactivates the hallmark of VZV infection, thereby providing a robust animal model to elucidate host-pathogen interactions during VZV infection. Previous studies showed that SVV ORF61 transactivates the hallmark of VZV infection, thereby providing a robust animal model to elucidate host-pathogen interactions during VZV infection.
activates its own promoter 3-fold, ORF62 5-fold, ORF28 10-fold, ORF29 7-fold, and ORF68 (gE) 14-fold (16). Early studies also showed that VZV ORF61 transactivates multiple VZV gene promoters and enhances the infectivity of VZV DNA (40, 41). More recently, another group showed decreased gene expression in vitro when VZV ORF61 protein was limited, specifically VZV ORF4, ORF47, ORF62, ORF63, ORF66, and ORF68 (42). They were also able to show that in a skin xenograft model, reduced VZV ORF61
expression correlated with decreased virulence in skin (42). Similarly, our transcriptional analysis within BAL fluid at 3 dpi in RMs infected with SVV/H9004 ORF61 demonstrates that ORF61 is important for the expression of multiple SVV ORFs encompassing all kinetic classes in vivo (Table 1). Importantly, the expression of ORF61 is critical for the expression of SVV transcriptional activators, including ORF4, ORF62, and ORF63, during SVV acute infection. Therefore, it remains unknown whether ORF61 directly interacts with other viral ORFs or whether reduced viral gene expression is due to decreased expression of the other SVV transactivators. The second possibility is supported by data from a primary guinea pig enteric neuron model where VZV ORF63 localization to the nucleus was shown to be dependent on the coexpression of VZV ORF61 (43). Taken together, our data reveal an important role for SVV ORF61 in orchestrating viral transcription in vivo.

Further, we report that infection of RMs with SVV lacking ORF61 leads to an overall dampened adaptive immune response that is characterized by decreased (i) B cell and T cell proliferative burst, (ii) SVV-specific IgM and IgG titers, (iii) frequency of SVV-specific T cells, and (iv) plasma inflammatory cytokine levels. Although we used a peptide library that included ORF61 along with ORF4, ORF31, and ORF63, the reduced frequency of SVV-specific T cells detected in SVV/H9004 ORF61-infected RMs is not due to the inability of T cells from these animals to respond to ORF61. This statement is supported by a decreased response to SVV lysate by CD4 T cells (Fig. 5A) and reduced T cell proliferation (Fig. 4) in RMs infected with SVV/ORF61. It is possible that reduced transcript quantities in BAL fluid contributed to the decreased frequency of SVV-specific T cells by lowering the amount of SVV proteins available for processing and presentation.

Several additional functions for VZV ORF61 have been described, including (i) influencing the phosphorylation of cellular JNK/stress-activated protein kinase and p38/mitogen-activated kinase (44); (ii) dispersal of promyelocytic leukemia protein (PML) nuclear bodies (NBs) or nuclear domain 10 (ND10) (45–47); (iii) disrupting the IFN-β pathway via IRF3 degradation (35); and (iv) inhibition of NF-κB activation in DCs (36). These actions likely modulate cell survival and the proinflammatory response

### FIG 8 Expression of innate immune genes in BAL fluid at 3 dpi. IFN-α, IFN-β, IL-6, ISG54, and ISG56 gene expression was measured by RT-qPCR in RMs infected with WT SVV (closed circles) or SVV/ORF61 (open circles). Fold change values represent expression from 0 to 3 dpi for each RM.

### TABLE 2 SVV viral load in sensory ganglia

<table>
<thead>
<tr>
<th>Virus, RM no., and sample type</th>
<th>Avg. copy no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SVV</td>
<td></td>
</tr>
<tr>
<td>19559 DRG-C</td>
<td>87</td>
</tr>
<tr>
<td>21211 DRG-C</td>
<td>93</td>
</tr>
<tr>
<td>23217 DRG-C</td>
<td>ND</td>
</tr>
<tr>
<td>26290 DRG-C</td>
<td>20</td>
</tr>
<tr>
<td>22199 DRG-C</td>
<td>103</td>
</tr>
<tr>
<td>24257 DRG-C</td>
<td>34</td>
</tr>
<tr>
<td>29405 DRG-C</td>
<td>123</td>
</tr>
<tr>
<td>29431 DRG-C</td>
<td>343</td>
</tr>
<tr>
<td>SVV/ORF61</td>
<td></td>
</tr>
<tr>
<td>22199 DRG-C</td>
<td>103</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>26290 DRG-C</td>
<td>74</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>ND</td>
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<tr>
<td>29431 DRG-T</td>
<td>ND</td>
</tr>
<tr>
<td>29431 DRG-L/S</td>
<td>97</td>
</tr>
<tr>
<td>29431 DRG-L/S</td>
<td>34</td>
</tr>
</tbody>
</table>

*DRG-C, cervical dorsal root ganglia; DRG-T, thoracic dorsal root ganglia; DRG-L/S, lumbar/sacral dorsal root ganglia.

*Average copy number per μg of DNA. ND, not detected.
during infection. To explore the possibility that the absence of SVV ORF61 resulted in an improved innate immune response, we compared DC subset frequencies and proliferation in BAL fluid during acute infection. DCs are a key component of the innate immune response and play a major role in the priming of the adaptive immune response. Infection with SVVΔORF61 resulted in increased frequencies and proliferation of pDCs in the BAL fluid compared to WT SVV infection (Fig. 7C and F). Although initial frequencies of mDC and CD11c–CD123– DC differed between the two groups, infection-induced changes exhibited similar kinetics and magnitude. Despite these similarities, we measured a larger proliferative burst within mDCs and earlier proliferation of the CD11c–CD23+ DCs in RM s infected with SVVΔORF61. These data suggest that SVV ORF61 interferes with the antiviral DC response.

Given that pDCs produce large amounts of type I interferons, which are critical antiviral compounds upon stimulation and activation (48, 49), we investigated the expression levels of key innate immune genes. Our data show that in the absence of SVV ORF61 protein, the levels of gene expression of the cytokines IFN-α, IFN-β, IL-6, and the interferon-stimulated genes 54 and 56 are increased, indicating that SVV ORF61 has a potential role in evading the interferon response, which is consistent with data from studies of VZV ORF61 (35) and HSV-1 ICP0 (50–58). Only differences in IFN-β gene expression were statistically significant, although a trend for the other genes was measured.

Interestingly, IL-6 gene expression in BAL cells at 3 dpi was higher in RMs infected with SVVΔORF61. In contrast, IL-6 protein levels in plasma were significantly reduced at 7 dpi in RMs infected with SVVΔORF61. This differential impact of ORF61 on IL-6 expression most likely is due to the tissue and time postinfection analyzed. Initially in the lungs, the loss of ORF61 results in improved innate immune response with higher levels of IL-6 putatively produced by DCs. The increased DC response together with a decrease in viral transcription leads to a reduced antigen load and dampened adaptive immune response, which is indicated by lower IL-6 production by T cells at 7 dpi.

In summary, compared to WT SVV, in the BAL microenvironment at day 3 postinfection SVVΔORF61 displays (i) reduced SVV gene expression, (ii) increased pDC frequency and proliferation, and (iii) higher gene expression of innate immune cytokine gene expression. This combination of factors potentially results in the reduced antigen load and dampened adaptive immune response. Our studies raise additional important questions. Was the decrease in viral transcription due to enhanced innate immunity, which interfered with viral replication, or to the absence of a major transactivator, or both? Another critical question is whether SVV ORF61 interferes with innate immunity directly or indirectly by transactivating additional genes that interfere with innate immunity, such as ORF63, which disrupts the IFN-α antiviral response during VZV infection (59).

SVVΔORF61 was able to establish latency in the sensory ganglia of rhesus macaques, and the latent transcriptome was comparable to that of controls. The VZV ORF61 deletion mutant was also able to establish latency in a rodent model (60). In future experiments, we hope to investigate the ability of SVVΔORF61 to reactivate from latently infected mouse trigeminal ganglia (61–63).

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