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Adaptive Immune Responses following Senecavirus A Infection in Pigs

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ABSTRACT Senecavirus A (SVA), an emerging picornavirus of swine, causes vesicular disease (VD) that is clinically indistinguishable from foot-and-mouth disease (FMD) in pigs. Many aspects of SVA interactions with the host and the host immune responses to infection, however, remain unknown. In the present study, humoral and cellular immune responses to SVA were evaluated following infection in pigs. We show that SVA infection elicited an early and robust virus-neutralizing (VN) antibody response, which coincided and was strongly correlated with VP2- and VP3-specific IgM responses. Notably, the neutralizing antibody (NA) responses paralleled the reduction of viremia and resolution of the disease. Analysis of the major porcine T-cell subsets revealed that during the acute/clinical phase of SVA infection (14 days postinfection [p.i.]), T-cell responses were characterized by an increased frequency of $\alpha\beta$ T cells, especially $CD4^+$ T cells, which were first detected by day 7 p.i. and increased in frequency until day 14 p.i. Additionally, the frequency of $CD8^+$ and double-positive $CD4^+$ $CD8^+$ T cells (effector/memory T cells) expressing interferon gamma (IFN-γ) or proliferating in response to SVA antigen stimulation increased after day 10 p.i. Results presented here show that SVA elicits B- and T-cell activation early upon infection, with IgM antibody levels being correlated with early neutralizing activity against the virus and peak B- and T-cell responses paralleling clinical resolution of the disease. The work provides important insights into the immunological events that follow SVA infection in the natural host.

IMPORTANCE Senecavirus A (SVA) has recently emerged in swine, causing outbreaks of vesicular disease (VD) in major swine-producing countries around the world, including the United States, Brazil, China, Thailand, and Colombia. Notably, SVA-induced disease is clinically indistinguishable from other high-consequence VDs of swine, such as FMD, swine vesicular disease, vesicular stomatitis, and vesicular exanthema of swine. Despite the clinical relevance of SVA-induced VD, many aspects of the virus infection biology remain unknown. Here, we assessed host immune responses to SVA infection. The results show that SVA infection elicits early B- and T-cell responses, with the levels of VN antibody and $CD4^+$ T-cell responses paralleling the reduction of viremia and resolution of the disease. SVA-specific $CD8^+$ T cells are detected later during infection. A better understanding of SVA interactions with the host immune system may allow the design and implementation of improved control strategies for this important pathogen of swine.

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Senecavirus A (SVA), an emerging picornavirus and the only member of the genus *Senecavirus* in the family *Picornaviridae* (International Committee on Taxonomy of Viruses, 2017), is a causative agent of vesicular disease (VD) in pigs (1–3). Notably, SVA-induced VD is clinically indistinguishable from other high-consequence VDs of swine, including foot-and-mouth disease (FMD), vesicular stomatitis, vesicular exanthema of swine, and swine vesicular disease (1–3). Historically, SVA has been associated with sporadic cases of VD in the United States and Canada (4, 5). Recently, however, an increased number of cases of SVA has been reported in the United States (6–8), and the virus has emerged in other major swine-producing countries around the world, including Brazil (9, 10), China (8, 11), Thailand (12), and Colombia (13), causing numerous outbreaks of VD in pigs.

Infection with SVA likely occurs via the oronasal route (1–3), and after a short incubation period (3 to 5 days), animals present with lethargy and lameness, which are usually followed by the development of vesicles on the snout, oral mucosa, and/or feet (1–3). SVA induces a short-term viremia (from ~1 to 10 days postinfection [p.i.]), and the clinical phase of the disease usually subsides within 10 to 14 days p.i. Infectious virus is excreted in oral and nasal secretions and/or feces for up to 21 days p.i. (3). Additionally, viral RNA is detected in tissues (especially the tonsils) of SVA-inoculated animals several weeks (~3 weeks) after resolution of the clinical disease (3). These observations indicate a complex interaction of SVA with the host immune system.

Humoral responses mediated by neutralizing antibodies (NA) seem to play a critical role in the control of picornavirus infection (14). Virus-specific NA are detected early upon infection by several picornaviruses, including SVA (3), and are essential to control viremia, limit virus spread to tissues, delay and/or reduce disease severity, and prevent reinfection(s) (15). Neutralization of picornaviruses is mediated through antigenic sites located mainly within the external viral capsid proteins (VPs; VP1, VP2, and VP3). Linear and conformational antigenic sites forming discontinuous arrangements within all three external capsid proteins (VP1, VP2, and VP3) and epitopes containing residues from multiple VPs have been described in various members of the family *Picornaviridae* (16–19).

The role of T cells in the control of picornavirus infection, however, is not yet completely understood, and several picornaviruses are known to evade host cellular immune responses (20–24). Foot-and-mouth disease virus (FMDV), for example, is thought to evade porcine cellular immune responses by inducing severe lymphopenia and lymphoid depletion during acute infection (25). Notably, despite the apparent inhibitory effect of the virus on cellular responses, pigs mount robust T-cell-dependent antibody (IgG) and memory B-cell responses within a few weeks of infection, suggesting an efficient stimulation of $CD4^+$ T cells (25–28). While the role of $CD8^+$ T cells is not completely understood, infection of cattle with FMDV seems to induce activation of these cells during acute infection (29). Since SVA has only recently emerged as an important pathogen of swine, many aspects of the virus infection biology and the immunological mechanisms involved in the control of infection remain unknown.

In the present study, we evaluated SVA-specific humoral and cellular immune responses during infection in pigs. NA and SVA-specific IgM and IgG antibody responses were evaluated. Additionally, recall T-cell responses were assessed by intracellular cytokine staining (ICS) and proliferation assays.

RESULTS

SVA infection results in robust and early NA responses. To evaluate SVA-specific host immune responses, 15-week-old pigs were inoculated with SVA strain SD15-26 and monitored for characteristic clinical signs of the disease, levels of viremia, viral load in tissues, and circulating NA levels. SVA infection resulted in lethargy and lameness, followed by the development of vesicular lesions on the snout and/or feet of inoculated

animals (Fig. 1A). Lesions were first observed as erythematous areas in the skin (indicated by arrows in Fig. 1A, day 4 postinfection [p.i.]), which progressed to vesicles 0.5 to 3 cm in diameter (Fig. 1A, day 7 p.i.). Between days 5 and 10 p.i. the vesicles ruptured, resulting in an erosion in the skin, with most lesions being completely resolved by day 14 p.i. (snout lesion sites are indicated by arrows in Fig. 1A, day 14 p.i.). No clinical signs or lesions were observed in noninfected control animals ($n = 4$) during the study.

Viremia was evaluated in serum samples by real-time reverse transcriptase quantitative PCR (RT-qPCR) (Fig. 1B) and virus isolation (Table 1). SVA RNA was detected in serum from all infected animals between days 3 and 7 p.i., with peak viremia levels being detected on day 3 p.i. (Fig. 1B). By day 10 p.i., only one out of four animals remained viremic. All animals cleared the virus from the blood by day 14 p.i. (Fig. 1B). Despite the relatively high levels of SVA RNA detected in serum, infectious virus was isolated from five out of six animals only on day 3 p.i., and no infectious virus was recovered from any other serum sample collected at later time points p.i. (Table 1). Interestingly, despite clearance of the virus from the blood after day 10 p.i., high SVA genome copy numbers were detected in the tonsil and mediastinal lymph node on day 14 p.i. (Fig. 1E).

High levels of virus-neutralizing (VN) antibodies were detected in all SVA-infected animals as early as day 5 p.i. (Fig. 1C). Peak VN antibody titers were detected on day 7 p.i. and remained elevated until the end of the experiment on day 14 p.i. Increasing levels of NA paralleled the resolution of the VD in SVA-infected animals (Fig. 1A and C). Most importantly, a negative correlation (Spearman's rank correlation coefficient [r] = −0.62, 95% confidence interval [CI] = −0.002 to −0.2793, $P = 0.0012$) between VN antibody titers and SVA genome copy numbers in serum was observed in infected animals (Fig. 1D). These observations suggest a critical role for NA in the control of acute SVA infection and disease in infected pigs.

SVA-specific IgM antibody responses correlate with NA levels. To further evaluate the humoral response elicited by SVA infection, specific IgM and IgG antibody levels were assessed by indirect immunofluorescence assay (IFA) (Fig. 2). SVA-specific IgM was first detected in the serum from SVA-infected animals on day 5 p.i., and all SVA-infected animals presented moderate titers by day 7 p.i. (Fig. 2A). IgM peaked on day 10 p.i. and was present at similar titers in all SVA-infected animals on day 14 p.i. SVA-specific IgG was first detected in the serum from SVA-infected animals on day 7 p.i. (Fig. 2B). IgG titers peaked on day 10 and remained elevated up to day 14 p.i. (Fig. 2B). Notably, the kinetics of SVA-specific IgM antibodies paralleled the NA responses in infected animals (Fig. 1C and 2C). Most importantly, IgM antibody levels presented a moderate but significant positive correlation with VN antibody titers (Fig. 2C) ($r = 0.51$, 95% CI = 0.1769 to 0.7387, $P < 0.004$), whereas no significant correlation between IgG and VN responses was observed (Fig. 2D) ($r = 0.30$, 95% CI = −0.1193 to 0.6391, $P = 0.15$). These results indicate that early NA activity is likely due to the presence of neutralizing IgM antibodies in serum.

Consistent with the early antibody responses, histological examination of the mediastinal lymph nodes of SVA-infected animals revealed a pronounced follicular hyperplasia, with numerous germinal centers—indicative of B-cell proliferation—observed on days 3 and 7 p.i. (Fig. 2E). Interestingly, lymphoid hyperplasia was present, albeit it was less pronounced on day 14 p.i. (Fig. 2E).

VP2- and VP3-specific IgM antibody responses correlate with early NA levels following SVA infection. To evaluate the contribution of individual external capsid proteins (VP1, VP2, and VP3) to SVA-specific IgM and IgG antibody responses, serum samples were tested by fluorescent microsphere immunoassay (FMIA). As shown in Fig. 3A, anti-SVA IgM responses were detected early upon infection (at about day 5 p.i.) and peaked between days 7 and 10 p.i. (Fig. 3A). Notably, IgM responses were directed primarily against VP2 and VP3 (Fig. 3A, middle and right, respectively), while the levels of IgM against VP1 in infected animals were variable and did not differ from those in

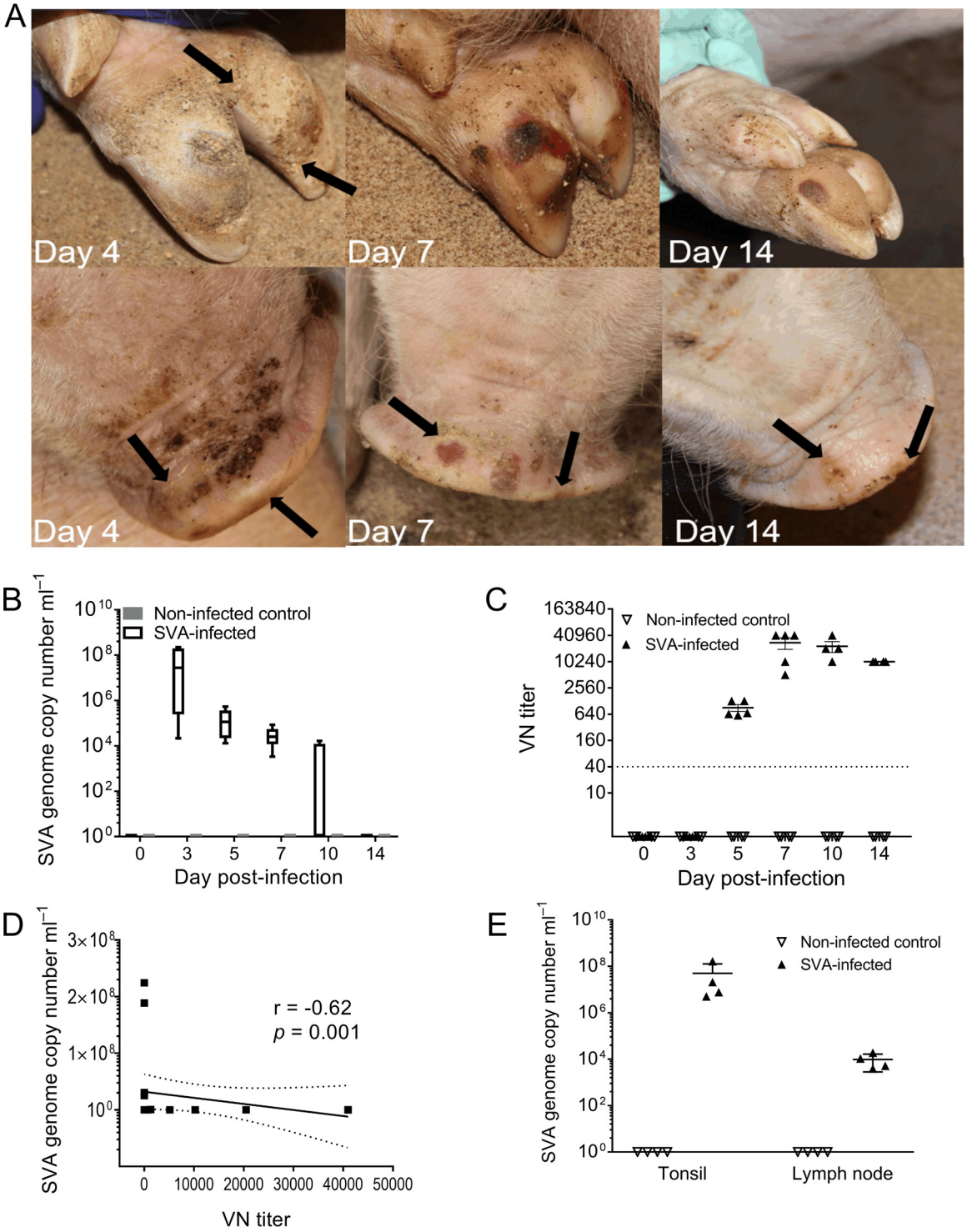

FIG 1 Clinical and virological findings and neutralizing antibody responses following SVA infection. (A) Progression of SVA-induced VD in pigs. Animals were inoculated oronasally with SVA strain SD15-26. Vesicular lesions on the foot (top) and snout (bottom) were first observed around day 4 p.i. as erythematous areas in the skin (left; lesion sites are indicated by arrows), which progressed to vesicles by day 5 to 7 p.i. (middle); the lesions usually ruptured between days 5 and 10 p.i. and were resolved by the end of the experiment on day 14 p.i. (right). (B) Viremia dynamics. The amount of viral RNA in serum was assessed by RT-qPCR throughout the duration of the experiment. Data are expressed as the $\log_{10}$ genome copy number per milliliter. The results are shown as a box-whisker plot. (C) Virus-neutralizing (VN) antibody responses elicited by SVA infection. (D) Correlation of the virus-neutralizing antibody responses of individual animals in relation to viremia levels after SVA infection ($r = -0.6214$, 95% CI $= -0.0012$ to -0.2793, and $P < 0.0012$ for days 3 to 14). (E) SVA RNA detection in tonsil and lymph node tissues of SVA-infected animals on day 14 p.i. Bars represent means ± SEMs.

TABLE 1 Virus isolation in serum samples from SVA-infected and noninfected control animals

	CPE/IFA result for animal:									
	Noninfected control				SVA infected					
dpi[c]	1	2	3	4	11	12	13	14	15	16
0	−[a]/−[b]	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
3	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+	+/+	+/+
5	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
7	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
10	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
14	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−

[a]Negative (−) or positive (+) result for cytopathic effect (CPE).
[b]Negative (−) or positive (+) result for indirect immunofluorescence assay (IFA).
[c]dpi, day postinfection.

noninfected control animals at any of the time points evaluated (Fig. 3A). Anti-SVA IgG antibodies appeared slightly later, being initially detected between days 10 and 14 p.i. (Fig. 3B). Similar to the IgM responses, SVA-specific IgG antibodies were directed primarily against VP2 and VP3 (Fig. 3B, middle and right, respectively), with VP1- and VP2-specific IgG antibody levels differing from those in the control group only on day 14 p.i.

The association of VP-specific IgM and/or IgG antibodies with the overall VN antibody responses detected after SVA infection was evaluated. Notably, IgM responses to VP2 and VP3 presented a high correlation with VN antibody levels ($r = 0.78$, 95% CI = 0.5174 to 0.9141, and $P < 0.0001$ for VP2 and $r = 0.88$, 95% CI = 0.7176 to 0.9546, and $P < 0.0001$ for VP3) (Fig. 3C), whereas no significant correlation between VN antibody titers and the presence of IgG antibodies against SVA VPs was observed (data not shown). These results suggest that VP2 and VP3 may contain major neutralizing epitopes of SVA. The high correlation between the IgM responses and NA levels following SVA infection further suggests that the early SVA-specific IgM responses may play an important role in the control of SVA viremia by the host.

To assess the duration of SVA-specific IgG and IgM antibody responses, samples from a previous experiment performed by our group (3) were analyzed using the VP FMIAs. As shown in Fig. 4, the levels of IgM against all three capsid proteins decreased after day 14 p.i., being undetectable by day 35 p.i. (Fig. 4A). IgG responses against VP1 and VP3 presented similar kinetics (Fig. 4B). Interestingly, variable levels of VP2-specific IgG antibodies were detected until day 35 p.i. (Fig. 4B). These results corroborate findings from previous studies that showed lower but sustained IgG antibody responses against SVA up to 38 to 60 days p.i. (3, 30) and further suggest that VP2-specific IgG antibodies might be responsible for the neutralizing activity detected in SVA-infected animals at later times p.i. (3, 30).

SVA infection elicits IFN-γ-producing cells early upon infection. We next sought to evaluate SVA-specific T-cell responses by an intracellular cytokine staining (ICS) assay for interferon gamma (IFN-γ) expression. Upon singlet selection and live/dead cell discrimination, IFN-γ expression by different T-cell subsets, including total T cells ($CD3^+$), γδ T cells ($CD3^+$ γδ T-cell receptor positive [TCR^+]), αβ T cells ($CD3^+$ γδ TCR negative [TCR^-]), and the αβ T-cell subsets $CD4^+$ T cells ($CD3^+$ $CD4^+$), $CD8^+$ T cells ($CD3^+$ $CD8^+$), and $CD4^+$ $CD8^+$ T cells ($CD3^+$ $CD4^+$ $CD8^+$), was evaluated (Fig. 5A). Analysis of these major porcine T-cell subsets revealed that during the acute/clinical phase of SVA infection (0 to 14 days p.i.), T-cell responses were characterized by an increased frequency of SVA-specific αβ T cells. The levels of $CD4^+$ T cells expressing IFN-γ (Fig. 5B) were significantly higher by day 7 p.i. and increased in frequency until day 14 p.i. (Fig. 5B). Additionally, the frequency of $CD8^+$ and double-positive $CD4^+$ $CD8^+$ T cells (effector/memory T cells) expressing IFN-γ in response to SVA antigen stimulation increased after day 10 p.i. (Fig. 5).

Next, the analysis was extended to a later phase of SVA infection (after resolution of the disease; day 35 p.i.). For this, a second set of peripheral blood mononuclear cells

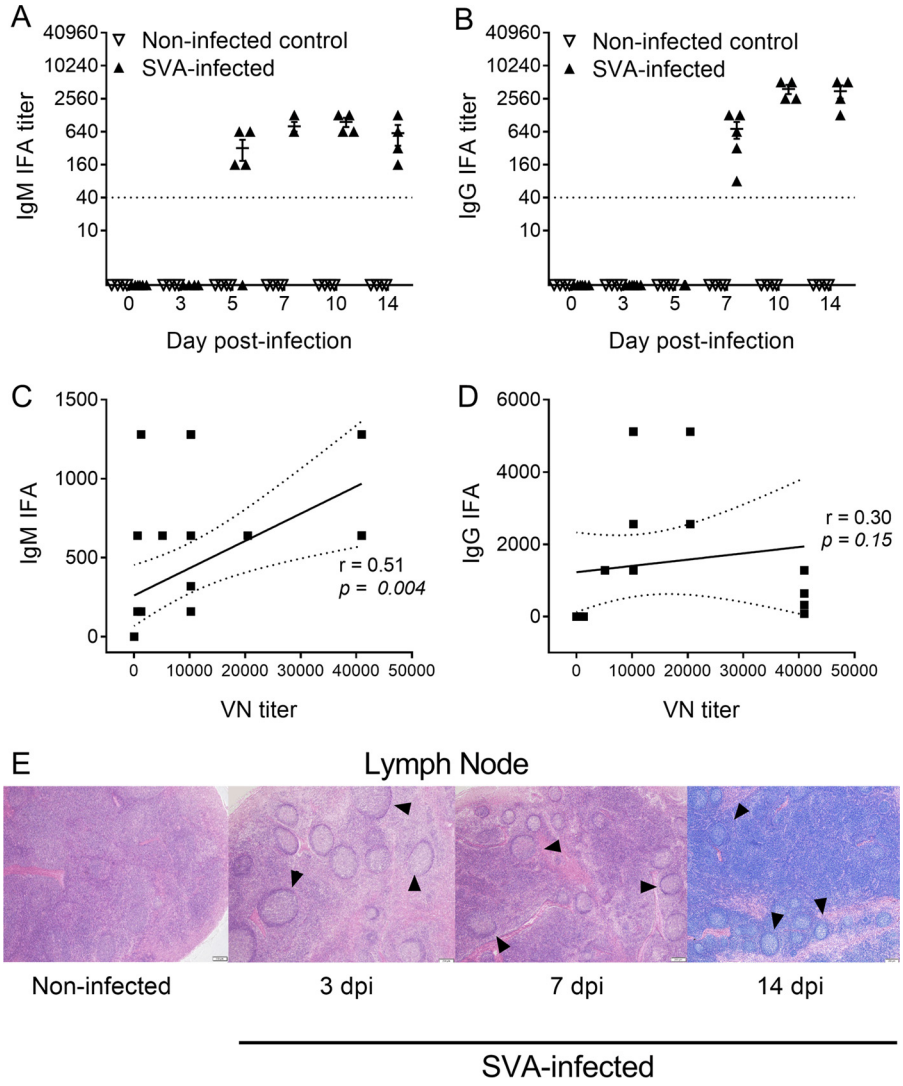

FIG 2 SVA-specific IgG and IgM antibody responses following infection in pigs. (A) Indirect immunofluorescence assay (IFA) results demonstrating the serum IgM antibody responses elicited by SVA infection. (B) IFA demonstrating the serum IgG antibody responses elicited by SVA infection. (C) Correlation of the virus-neutralizing responses of individual animals to the total anti-SVA IgM IFA titers upon SVA infection ($r = 0.51$, 95% CI = −0.122 to −0.6375, $P = 0.004$ for days 3 to 14 p.i.). (D) Correlation of the virus-neutralizing responses of individual animals with the anti-SVA IgG IFA titers upon SVA infection ($r = 0.30$, 95% CI = −0.122 to −0.6375, $P = 0.15$ for days 3 to 14 p.i.). (E) Histological examination of the mediastinal lymph nodes of noninfected control and SVA-infected animals (days 3, 7, and 14 p.i.). Arrowheads point to germinal centers/active B-cell follicles.

(PBMCs) collected on days 0, 3, 7, 10, 14, and 35 p.i. during a previous SVA pathogenesis study (3) was subjected to IFN-γ ICS assay, as described above. Similar T-cell responses to SVA were observed during the acute phase of infection in the two independent experiments (Fig. 5B and 6). Although recall stimulation with UV-inactivated SVA (uvSVA) resulted in early IFN-γ production (day 3 p.i.) by T cells (gated on $CD3^+$), no clear polarization toward any of the T-cell subsets evaluated was observed at this time point (Fig. 6). A robust increase in the frequency of $\alpha\beta$ T cells expressing IFN-γ was observed between days 7 and 14 p.i., with $CD4^+$ T cells being the main cell subset expressing IFN-γ (Fig. 5B and 6). Similarly, when samples from day 35 p.i. were restimulated *in vitro*, the frequency of $CD4^+$, $CD8^+$, and double-positive $CD4^+$ $CD8^+$ T cells expressing IFN-γ was increased (Fig. 6). These results indicate that cellular immune responses to SVA are elicited early after infection, with $\alpha\beta$ T cells being the predominant cell population responding to infection. Additionally, the T-cell responses de-

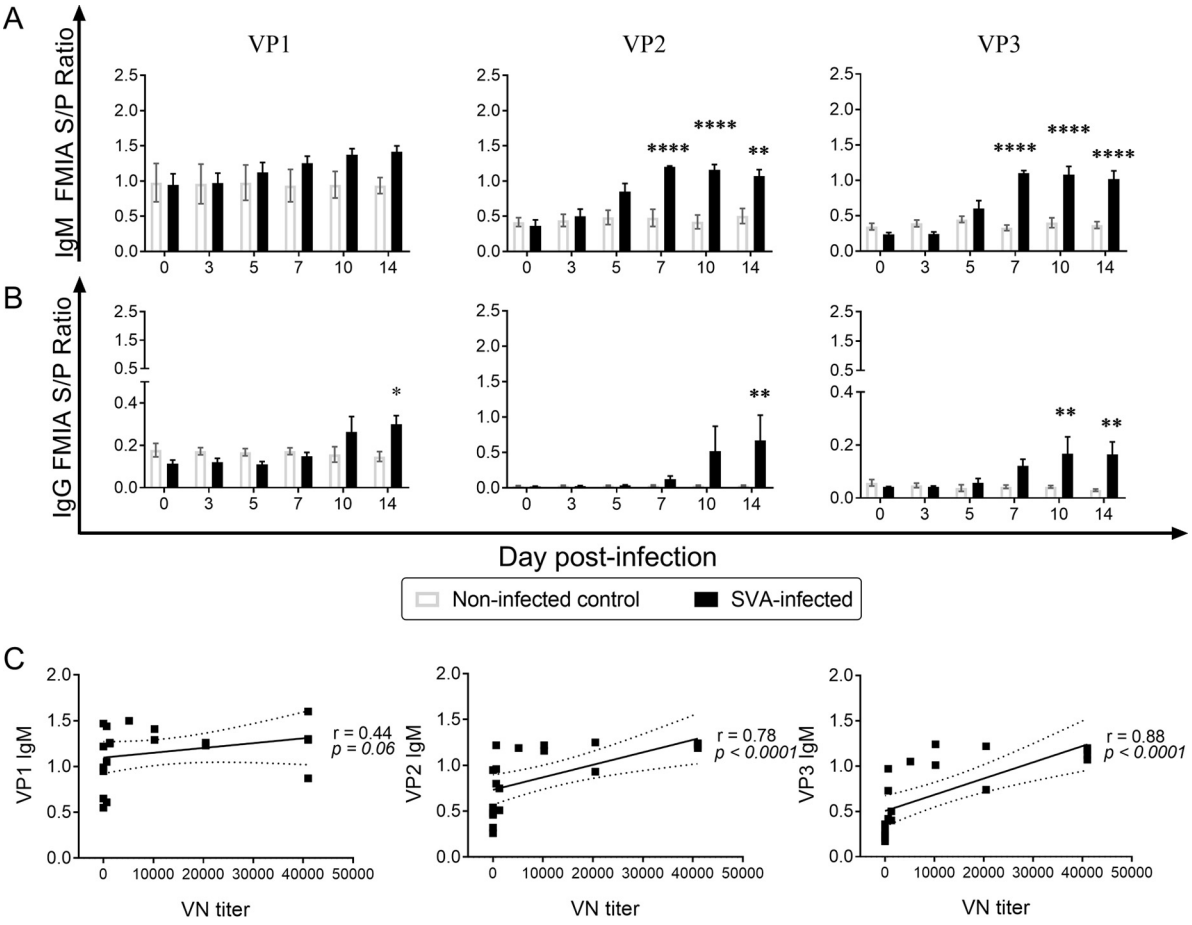

FIG 3 SVA infection-elicited IgM responses directed against the VP2 and VP3 capsid viral proteins are correlated with virus-neutralizing (VN) antibody titers. (A) Kinetics of anti-VP1, VP2, and VP3 IgM responses assessed by FMIA in SVA-infected pigs and noninfected controls. (B) Kinetics of the anti-VP1, VP2, and VP3 IgG responses assessed by FMIA in SVA-infected pigs and noninfected controls. Data in panels A and B represent sample-to-positive (S/P) ratios. Bars represent sample means ± SEMs. *P* values were determined by Sidak's multiple-comparison test. *, $P < 0.05$; **, $P < 0.001$; ****, $P < 0.0001$. (C) Correlation of the IgM responses to either VP1 (left) ($r = 0.44$, 95% CI = −0.0548 to 0.7458, $P = 0.06$ for days 3 to 14 p.i.), VP2 (middle) ($r = 0.78$, 95% CI = 0.5174 to 0.9141, $P < 0.001$ for days 3 to 14 p.i.), or VP3 (right) ($r = 0.88$, 95% CI = 0.7176 to 0.9546, $P < 0.0001$ for days 3 to 14 p.i.) in SVA-infected pigs in relation to VN antibody responses.

tected on day 35 p.i., which mainly consisted of $\alpha\beta$ T cells ($CD4^+$, $CD8^+$, and $CD4^+$ $CD8^+$ T cells), indicate that SVA induces memory T cells that readily respond to recall stimulation *in vitro*. Although SVA elicited the expression of IFN-γ by both $CD4^+$ and $CD8^+$ T cells, the frequency of $CD4^+$ T cells was higher than that of $CD8^+$ T cells, with the former also being detected earlier after infection (Fig. 5 and 6).

SVA infection elicits T-cell-specific proliferation early upon infection. SVA-specific T-cell responses were also evaluated by the carboxyfluorescein succinimidyl ester (CFSE) dilution assay to determine the specific T-cell subsets proliferating upon infection (Fig. 7). As described above for the IFN-γ ICS, upon singlet selection and dead cell exclusion, proliferation by the major swine T-cell subsets was evaluated. Similar to the results observed for IFN-γ expression, significant SVA-specific proliferation was observed for $\alpha\beta$ T cells as early as day 7 p.i., with increasing frequencies of these cells being detected up to day 14 p.i. (Fig. 7B). The early proliferative responses by $\alpha\beta$ T cells were predominantly confined to $CD4^+$ T-cell subsets (both $CD4^+$ and $CD4^+$ $CD8^+$ T-cell subsets). While $CD8^+$ T-cell populations also expanded after recall stimulation (Fig. 7B), significant differences between SVA-infected and control animals were observed only at later time points p.i. (day 14 p.i.). Interestingly, the $\gamma\delta$ T-cell population also proliferated following recall SVA stimulation, with significant differences from control animals being observed between days 10 and 14 p.i. (Fig. 7B). It is important to

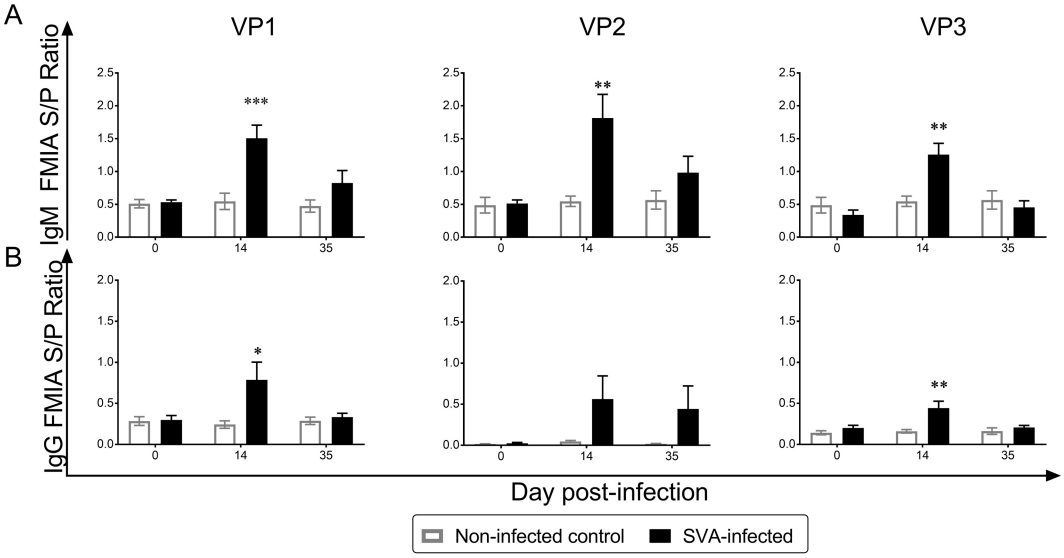

FIG 4 SVA VP-specific antibody responses after resolution of clinical disease. Anti-SVA VP1, VP2, and VP3 IgM (A) and IgG (B) responses assessed by FMIA on days 0, 14, and 35 p.i. are shown. *P* values were determined by Sidak's multiple-comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

note, however, that high levels of nonspecific proliferation by $\gamma\delta$ T cells were observed under unstimulated conditions, which may indicate that these cells were more prone to bystander activation, and the responses detected may not have been SVA specific. Overall, these findings confirm the results from the IFN-γ ICS analysis, indicating an early cellular immune response to SVA which mainly involves $\alpha\beta$ T cells. In this context, $CD4^+$ T-cell responses are more robust and develop earlier than $CD8^+$ T-cell responses.

External SVA capsid proteins contribute to SVA-specific T-cell response. To assess the contribution of the external SVA capsid proteins to cellular immune responses, we evaluated IFN-γ expression in response to VP1, VP2, and VP3 recall stimulation by flow cytometry. PBMCs collected on days 14 and 35 p.i. were stimulated with VP1, VP2, or VP3, and subjected to IFN-γ ICS (Fig. 8). SVA-infected animals presented specific T-cell responses to all three proteins, with the earlier responses (day 14 p.i.) being mainly directed to VP2 and VP3 (Fig. 8A). At day 35 p.i., similar frequencies of IFN-γ-expressing T cells were detected in response to VP1, VP2, and VP3 stimulation (Fig. 8B, bottom). Additionally, robust T-cell activation was observed on day 35 p.i., at levels similar to those detected at the peak of T-cell responses on day 14 p.i. (Fig. 8A). Interestingly, no clear polarization toward $CD4^+$ or $CD8^+$ T cells was observed when the cells collected on day 14 p.i. were restimulated with either of the VPs. Responses to VP2 on day 35 p.i., however, were mainly driven by $CD4^+$ T cells, with the frequencies of both $CD4^+$ and $CD4^+$ $CD8^+$ T cells being higher in SVA-infected animals than in noninfected controls. Together these results demonstrate that VP1, VP2, and VP3 contain T-cell epitopes and contribute to SVA-specific T-cell responses.

DISCUSSION

In this study, we evaluated the immunological responses to SVA following infection in pigs. Results here show that early host responses to SVA are characterized by the development of robust NA titers, and the increasing levels of SVA-specific NA paralleled both reduction of viremia and loss of infectivity of the virus present in serum (Fig. 9). These observations suggest that, similar to other picornavirus infections (14, 15, 31), NA may play an important role for the control of SVA infection in pigs.

Analysis of IgG and IgM antibody levels revealed an early IgM response against SVA that was highly correlated with the VN antibody response (first detected around days 5 to 7 p.i.). IgG antibodies, on the other hand, appeared slightly later and did not correlate with the early SVA-neutralizing activity. These results corroborate the findings

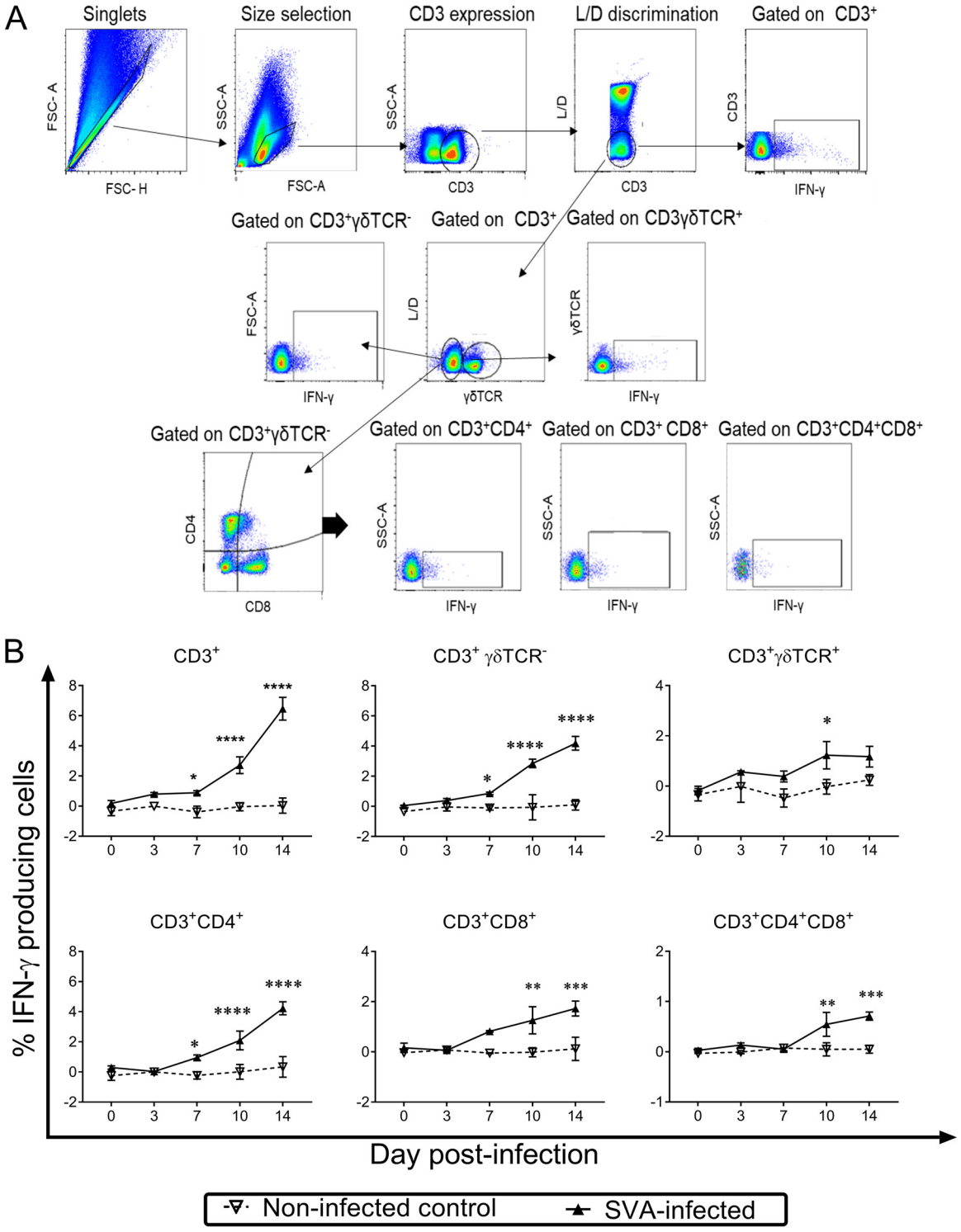

FIG 5 SVA infection elicits early IFN-γ production by T cells. Cryopreserved PBMCs isolated from control or SVA-infected pigs were thawed and allowed to rest for 6 h. Cells were then cultured in the presence of UV-inactivated SVA (MOI = 1), cRPMI, or ConA (5 μg/ml) plus PHA (5 μg/ml). At 4 h poststimulation, brefeldin A was added to the culture and the cells were further stimulated for another 12 h (for a total of 16 h culture/stimulation). After stimulation, αβ and γδ T cells were analyzed for virus-specific IFN-γ expression as measured by ICS. (A) Gating strategy. Following doublet exclusion and lymphocyte selection (on the basis of forward scatter [FSC] and side scatter [SSC] properties), live $CD3^+$ T cells were evaluated. Each subsequent panel shows only the population of interest that was selected from the gate in the previous plot, as indicated by arrows and plot titles and labels. (B) Collective results of flow cytometric analysis are shown for each population of cells evaluated from SVA-infected and noninfected control animals. Data represent group means ± SEMs. L/D, live/dead cell. *P* values were determined by Sidak's multiple-comparison test. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$; ****, $P < 0.0001$.

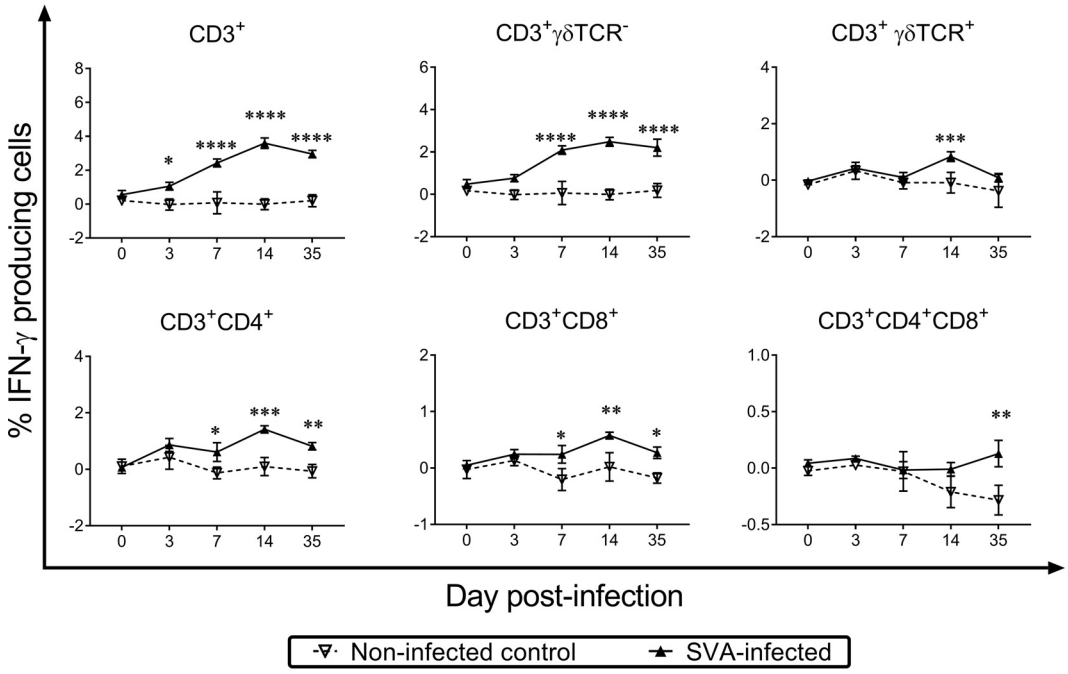

FIG 6 SVA-specific IFN-γ production by T cells following the resolution of clinical disease. Cryopreserved PBMCs isolated from control or SVA-infected pigs were thawed and allowed to rest for 6 h. The cells were then cultured in the presence of UV-inactivated SVA (MOI = 1), cRPMI, or ConA (5 μg/ml) plus PHA (5 μg/ml). At 4 h poststimulation, brefeldin A was added to the cultures and the cells were incubated for another 12 h (for a total of 16 h culture/stimulation). After stimulation, $\alpha\beta$ (CD3$^+$ $\gamma\delta$TCR$^-$) and $\gamma\delta$ T cells were analyzed for virus-specific IFN-γ expression by ICS. The gating strategy for data analysis is shown in Fig 5A. Data represent group means $\pm$ SEMs. *P* values were determined by Sidak's multiple-comparison test. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$; ****, $P < 0.0001$.

of a previous study showing that early IgM responses parallel the NA responses following SVA infection (3, 30). Additionally, evaluation of the IgM and IgG responses against the external SVA capsid proteins demonstrated a short-lived IgM response against VP1, VP2, and VP3. Similarly, the levels of IgG against VP1 and VP3 declined following resolution of the disease, with no antibodies against these two proteins being detected by day 35 p.i. Interestingly, low levels of VP2-specific IgG antibodies were detected by day 35 p.i. (Fig. 4). These observations suggest that the neutralizing activity observed at later times after SVA infection (day 38 or 60 p.i.) (3, 30) could potentially be a function of VP2-specific IgG antibodies. The possibility that VP1 and VP3 play a role in this NA response, however, cannot be formally excluded, as antibodies directed against conformational or complex epitopes involving more than one VP were likely not detected with the single-protein FMIAs used here. The high IgG levels detected by whole-virus IFAs or competitive enzyme-linked immunosorbent assays at late time points after SVA infection (3, 30) strengthen the hypothesis that conformational epitopes composed of antigenic motifs from more than one SVA VP may contribute to the late IgG and NA responses in SVA-infected animals.

Although similar levels of IgM against both VP2 and VP3 were detected (on days 7, 10, and 14 p.i.; Fig. 2A), the levels of anti-VP2 IgG on days 10 and 14 p.i. were approximately 2-fold higher than the respective anti-VP3 IgG levels (Fig. 3B). This may represent a unique feature of SVA, given that VP1 has been shown to be highly immunogenic in other picornaviruses, including enteroviruses (19, 32, 33), rhinoviruses (34, 35), and FMDV (36).

In addition to assessing SVA-specific humoral responses, SVA-specific T-cell responses were investigated during acute infection (days 0 to 14 p.i.) and after the resolution of clinical disease (day 35 p.i.). Recall stimulation of PBMCs with uvSVA revealed that specific T cells were induced early after infection (between days 3 and 7 p.i.), with SVA-specific T cells proliferating and secreting IFN-γ in response to restimu-

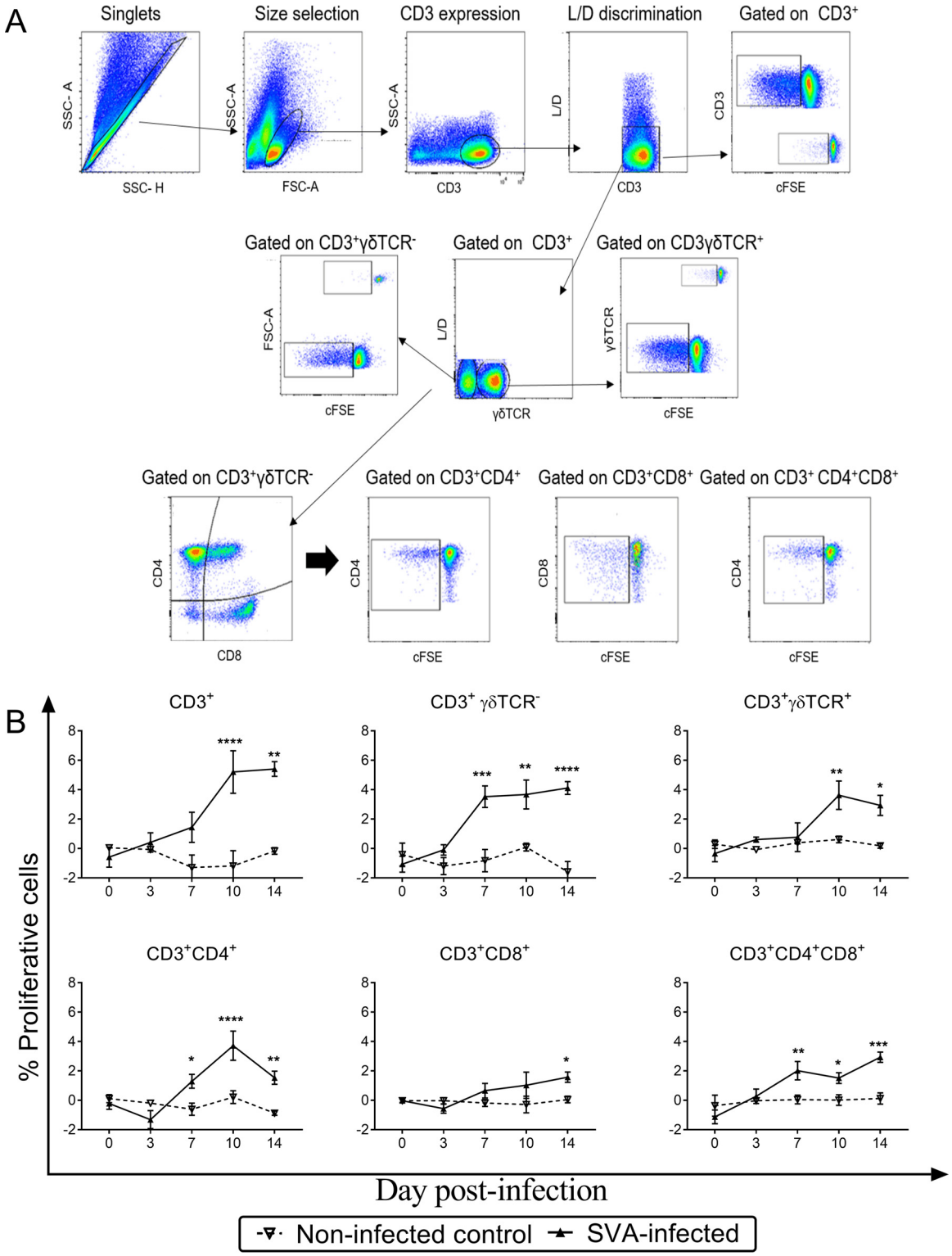

FIG 7 SVA infection elicits early T-cell proliferative responses. Cryopreserved PBMCs isolated from control or SVA-infected pigs were thawed, allowed to rest for 6 h, and labeled with CFSE. The cells were then cultured for 5 days with UV-inactivated SVA (MOI = 1), cRPMI, or ConA (5 μg/ml) plus PHA (5 μg/ml). After stimulation, $\alpha\beta$ T (CD3$^+$ $\gamma\delta$TCR$^-$) and $\gamma\delta$ T cells were analyzed for virus-specific proliferation by CFSE dilution assay. (A) Representative flow plots are shown to exemplify the gating strategy. Following doublet exclusion and lymphocyte selection (on the basis of forward scatter [FSC] and side scatter [SSC] properties), live CD3$^+$ T cells were evaluated (responses under a nonstimulated condition are depicted in the insets for T cells, $\alpha\beta$, and $\gamma\delta$ T cells). Each subsequent panel shows only the population of interest that was selected from the gate in the previous plot, as indicated by the arrows and plot titles and labels. (B) Collective results from flow cytometric analysis are shown for each population evaluated. Data represent group means ± SEMs. *P* values were determined by Sidak's multiple-comparison test. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$; ****, $P < 0.0001$.

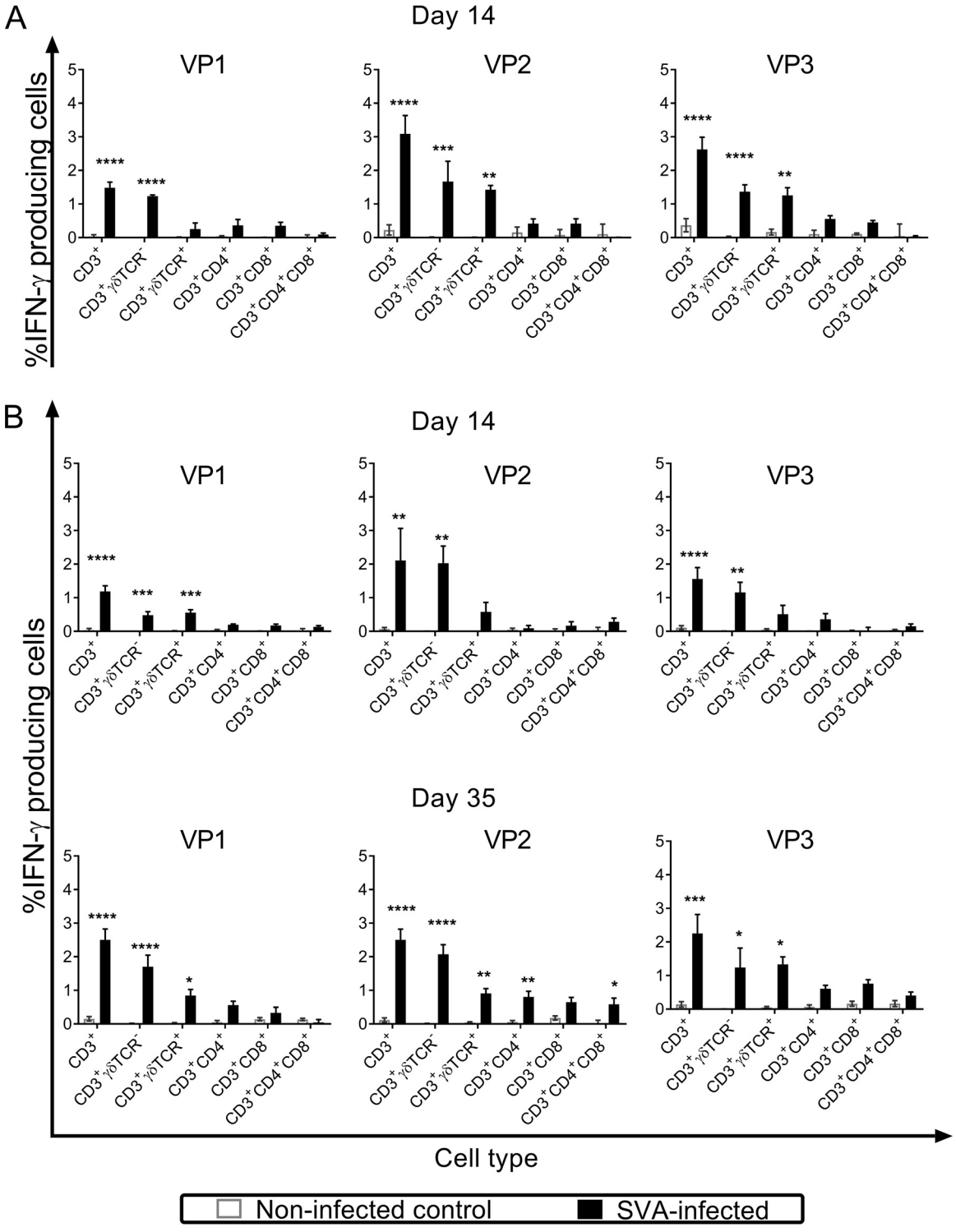

FIG 8 T-cell responses against VP1, VP2, and VP3 are elicited during SVA infection. Cryopreserved PBMCs isolated from control or SVA-infected pigs were thawed and allowed to rest for 6 h. The cells were then cultured in the presence of either VP1, VP2, or VP3 (5 μg/ml), cRPMI, or ConA (5 μg/ml) plus PHA (5 μg/ml). Brefeldin A was added to the cultures 4 h poststimulation, and the cells were incubated for another 12 h. After stimulation, $\alpha\beta$ ($CD3^+$ $\gamma\delta TCR^-$) and $\gamma\delta$ T cells were analyzed for virus-specific IFN-γ expression by ICS. (A) Collective results from flow cytometric analysis showing the percentage of IFN-γ-producing T cells from SVA-infected animals at day 14 p.i. in response to recall stimulation with VP1, VP2, or VP3. (B) Collective results from flow cytometric analysis showing the percentage of IFN-γ-producing T cells from SVA-infected animals at days 14 and 35 p.i. in response to recall stimulation with VP1 (left), VP2 (middle), or VP3 (right). Samples for panel B were collected in a previous SVA experiment (3). Data represent group means ± SEMs. *P* values were determined by Sidak's multiple-comparison test. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$; ****, $P < 0.0001$.

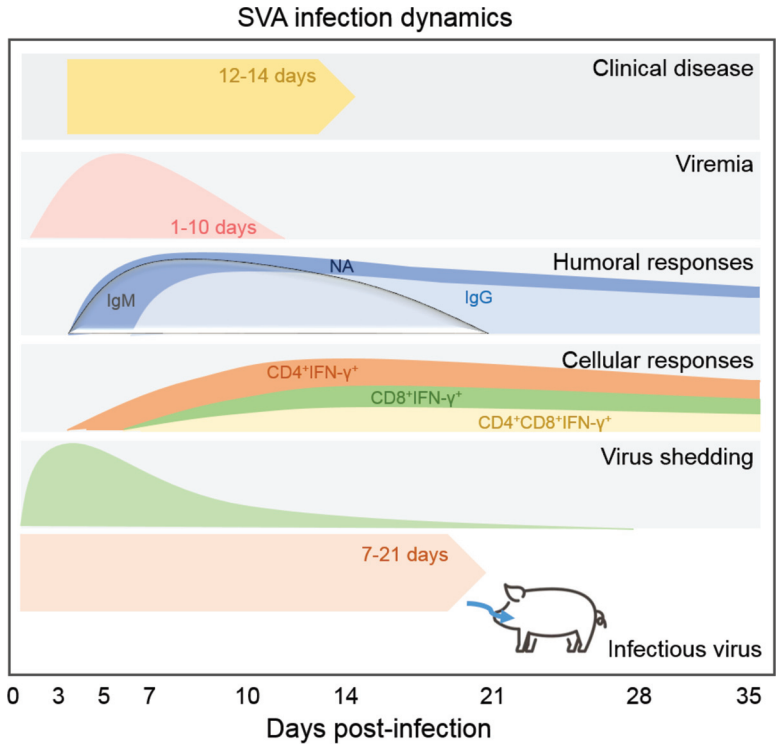

FIG 9 SVA infection dynamics in pigs. After a short incubation period (3 to 5 days), SVA-infected animals present clinical signs characterized by lethargy and lameness. Vesicular lesions develop on the snout and/or feet around days 3 to 5 p.i. and usually last for 10 to 12 days (~14 days p.i.). A short-term viremia follows SVA infection, with viral RNA being detected in serum between days 1 and 10 p.i. A robust neutralizing antibody response is detected around day 4 or 5 p.i., with high NA titers being detected up to 35 to 38 days p.i. Notably, the NA response parallels and correlates with decreased levels of viremia. SVA-specific T-cell responses are characterized by an increased frequency of $\alpha\beta$ T cells, especially $CD4^+$ T cells, producing IFN-γ. These cells are first detected around day 7 p.i. and increase in frequency up to day 14 p.i. The frequency of $CD8^+$ and double-positive $CD4^+$ $CD8^+$ T cells (effector/memory T cells) expressing IFN-γ or proliferating in response to SVA increases after day 10 p.i. Recall stimulation of T cells after resolution of the disease on day 35 p.i. showed an increased frequency of SVA-specific $CD4^+$, $CD8^+$, and double-positive $CD4^+$ $CD8^+$ T cells at this time point, suggesting the efficient induction of memory T-cell populations. Virus shedding in feces and nasal and oral secretions is detected up to 21 to 28 days p.i. Infectious virus was isolated from nasal secretions, feces, and oral secretions up to days 7, 14, and 21 days p.i., respectively. This figure contains data highlights from the present study and from previous studies on SVA infection and pathogenesis (1–3, 30).

lation *in vitro*. Similar to what has been described for FMDV (25), $CD4^+$ T-cell responses were detected earlier than $CD8^+$ T-cell responses, with increasing $CD4^+$ T-cell activity (IFN-γ secretion and proliferation) paralleling decreased levels of viremia. Additionally, $CD4^+$ T-cell responses also coincided with peak IgG, IgM, and neutralizing antibody levels, as might be expected, since these cells are critical for antibody production by B cells.

$CD8^+$ T-cell responses were also detected in SVA-infected animals, although the frequency of SVA-specific $CD8^+$ T cells was lower and these cells appeared later during infection (between days 10 and 14 p.i.). Peak $CD8^+$ T-cell activity coincided with disease resolution and clearance of the virus from secondary sites of replication (i.e., skin). Notably, although active T-cell responses were detected in SVA-infected animals, RT-qPCRs performed with tonsil and lymph node tissues from infected animals revealed the presence of viral RNA in those tissues on day 14 p.i. Interestingly, the levels of SVA RNA detected on day 14 p.i. in the tonsil and mediastinal lymph node were lower than those detected earlier during infection (days 3 and 7 p.i.). These observations suggest that both $CD4^+$ and $CD8^+$ T cells may contribute, at least to some extent, to the control of SVA infection. Future studies assessing local T-cell responses at sites of SVA replication will be critical to precisely define the effector function(s) of these cells and their relevance for the control of SVA infection.

Double-positive $CD4^+$ $CD8^+$ T cells are a unique T-cell subset of swine which comprise both activated and memory T cells (37–40). Results presented here show that SVA infection elicited $CD4^+$ $CD8^+$ T-cell responses, as evidenced by an increased frequency of this T-cell subset producing IFN-γ or proliferating in response to SVA recall stimulation. Additionally, low but significant $CD4^+$ $CD8^+$ T-cell responses were detected on day 35 p.i., after disease resolution. While early $CD4^+$ $CD8^+$ T-cell responses may represent activated T cells responding to SVA infection, the responses detected on day 35 p.i. likely represent SVA-specific memory T cells. The importance of this T-cell population for protection against reinfection with SVA awaits further experimentation.

The specificity of T-cell responses against the external SVA capsid proteins (VP1, VP2, and VP3) was evaluated. PBMCs collected on days 14 and 35 after SVA infection were restimulated with recombinant VP1, VP2, and VP3 proteins, and IFN-γ production by T cells was evaluated by ICS. Recall T-cell responses ($\alpha\beta$ and $\gamma\delta$ T cells) against all three proteins were detected, suggesting the presence of T-cell epitopes in VP1, VP2, and VP3. Notably, the frequencies of T cells detected in response to VP2 recall stimulation were slightly higher than those detected in response to VP1 and VP3. Most importantly, VP2-specific $CD4^+$ and $CD4^+$ $CD8^+$ T-cell responses were also higher than responses to the other two capsid proteins, suggesting that VP2 contains immunodominant T-cell epitopes of SVA. These results corroborate the findings of a study conducted with enterovirus 71, in which the response to VP2 was shown to dominate the $CD4^+$ T-cell responses compared to the responses to the other capsid proteins (41). In addition to the structural capsid proteins for which results are shown here, nonstructural proteins (including 2A, 2B, 2C, 3C, and 3D, which have been shown to contain T-cell epitopes in other picornaviruses [42]) may also contribute to the host T-cell responses to SVA.

The results presented here showed that despite active B- and T-cell responses to SVA, the virus RNA was not cleared from infected lymphoid tissues by day 14 p.i. Most importantly, earlier studies have demonstrated the presence of viral RNA in the tonsils and lymph nodes of experimentally infected pigs several weeks after complete clinical resolution of the disease (3) or in the tonsils of naturally infected animals (43). Although no infectious virus was isolated from tissues positive for SVA by RT-qPCR, these results indicate that the virus was not efficiently cleared from the lymphoid tissues of infected animals. Notably, other picornaviruses, including FMDV, encephalomyocarditis virus (EMCV), and poliovirus (PV), are known to persist and establish a carrier state in affected hosts (44–46). These observations and the potential implications of carrier animals to the epidemiology of VDs warrant additional studies to investigate if this is indeed the case for SVA.

This study provides important insights into the immunological events that follow SVA infection in swine (Fig. 9). Early immune responses are characterized by $CD4^+$ T-cell and NA activity, whereas later events involve the development of SVA-specific $CD8^+$ T cells (Fig. 9). Increasing levels of NA and $CD4^+$ T cells paralleled the reduction in viremia and, ultimately, decreased virus shedding and disease resolution (Fig. 9). These observations suggest that cooperation between humoral and cellular immune responses is likely important for the control of SVA infection. A better understanding of SVA interactions with the host immune system will provide important insights into the virus infection biology and may support the design of improved strategies to control this important pathogen of swine.

MATERIALS AND METHODS

Cells and virus. H1299 cells were obtained from the American Type Culture Collection (ATCC; CRL 5803) and cultured at 37°C with 5% CO_2 in RPMI 1640 medium (Corning, Corning, NY) supplemented with 10% fetal bovine serum (FBS; VWR, Radnor, PA), 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) (Corning, Corning, NY). SVA strain SD15-26 was isolated from a swab specimen collected from a vesicular lesion on a finishing pig (3). Low-passage SVA strain SD15-26 stocks (passage 4) were prepared, titrated in H1299 cells, and used in all experiments described below.

Animal inoculation. To investigate the immune responses following SVA infection, 10 SVA-negative pigs (15-week-old finishing pigs; weight, approximately 55 kg) were randomly allocated into two experimental groups, as follows: a noninfected control group ($n = 4$) or an SVA-infected group (SVA strain SD15-26; $n = 6$). Animals from the SVA-infected group were inoculated with 10 ml of a virus

suspension containing $10^{7.79}$ 50% tissue culture infective dose ($TCID_{50}$)/ml by the oral (5 ml) and intranasal (2.5 ml to each nostril) routes. Noninfected control animals were inoculated with RPMI 1640 medium by the oronasal route as described above. Animals were challenged on the day of arrival and monitored daily for clinical signs and lesions for 14 days. Two animals from the SVA-infected group were euthanized for tissue collection on days 3 and 7 p.i. All the remaining animals were euthanized on day 14 p.i. Animal experiments were conducted at the South Dakota State University (SDSU) Animal Resource Wing (ARW) and followed the protocols and guidelines approved by the SDSU Institutional Animal Care and Use Committee (approval number 16-002A).

Samples from a previous SVA pathogenesis study (3) were included in our analysis to evaluate the immunological responses at later times p.i. Animals from this study were challenged on the day of arrival and monitored for 38 days.

Sample collection and processing. Blood samples were collected on days 0, 3, 7, 10, and 14 p.i. Blood samples were collected by jugular venipuncture with Vacuette tubes containing clot activator (for serum) and heparin (for PBMCs) (Greiner Bio-One, Monroe, NC). Serum samples were aliquoted and stored at −20°C until they were processed. PBMCs were isolated from whole blood by density gradient centrifugation in Ficoll-Paque Plus separation medium (GE Healthcare, Little Chalfont, UK) and cryopreserved in 10% dimethyl sulfoxide (Biotium, Fremont, CA) solution in FBS (VWR, Radnor, PA) using a Mr. Frosty freezing container (Nalgene Labware, Penfield, NY). The vials were kept at −80°C for 24 h and then transferred and stored in liquid nitrogen until they were processed. The samples collected during a previous SVA pathogenesis study (3) were processed as described here. This sample set consisted of serum and PBMCs collected on days 0, 3, 7, 14, and 35 p.i. Tissue sections from mediastinal lymph nodes from SVA-infected and noninfected control animals were processed following standard histological procedures and stained with hematoxylin and eosin for histological examination.

Viral RNA extraction and RT-qPCR. Viral nucleic acid was extracted from serum samples, mediastinal lymph nodes, and tonsils using a MagMAX viral RNA/DNA isolation kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Detection of SVA RNA was performed using a commercial real-time reverse transcriptase quantitative PCR (RT-qPCR) kit targeting the SVA 3D polymerase gene (EZ-SVA; Tetracore, Rockville, MD). All SVA RT-qPCR assays were performed at the SDSU ADRDL.

Virus isolation and titrations. Virus isolation was performed in RT-qPCR-positive samples as previously described (3). Each sample was subjected to three blind passages, and the results were confirmed by using an indirect immunofluorescence assay (IFA) with an SVA-specific rabbit polyclonal antibody.

Virus neutralization assay and IFA. The neutralizing antibody responses elicited by SVA infection were assessed using a virus neutralization assay (3). Serial 2-fold dilutions of serum (1:40 to 1:40,096) were incubated with 100 to 200 $TCID_{50}$ of SVA strain SD15-26 for 1 h at 37°C. H1299 cells were added to each well (approximately 2×10^4 cells per well), and the plates were incubated at 37°C for 48 h. Cells were fixed with 3.7% formaldehyde and stained with an anti-SVA polyclonal antibody as previously described (3). Virus neutralization titers were read under a fluorescence microscope, and NA titers were expressed as the reciprocal of the highest dilution of serum capable of completely inhibiting SVA infection/replication. IFAs were performed with serum samples as previously described (3). SVA-specific IgM or IgG responses were assessed using an anti-swine IgM-fluorescein isothiocyanate (FITC) secondary antibody or an anti-swine IgG-FITC (both from Bethyl Laboratories, Montgomery, TX). Negative- and positive-control sera were included in all assays.

Expression of SVA capsid proteins. The external SVA capsid proteins (VP1, VP2, and VP3) were expressed using a prokaryotic expression system. SVA strain SD15-26 VP1-, VP2-, and VP3-coding sequences were chemically synthesized (GenScript, Piscataway, NJ) and cloned into the bacterial expression plasmid pET-28a (EMD Millipore/Novagen, Billerica, MA). DNA sequencing was used to confirm the identity and in-frame cloning of the SVA protein with a 6×His tag. The recombinant proteins were expressed in *Escherichia coli* BL-21 cells as 6×His-tagged fusion proteins and purified using nickel-charged agarose resin (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Affinity-purified recombinant proteins were refolded and used as antigens in fluorescent microsphere immunoassays (FMIA) and for PBMC stimulation *in vitro*. The VPs used for PBMC stimulation were subjected to protein dialysis in phosphate-buffered saline (PBS) overnight.

FMIA. A two-step carbodiimide-based procedure was used to couple the SVA VP1, VP2, and VP3 proteins to Luminex microspheres as previously described (47). Porcine serum binding to coupled microspheres was analyzed using a Bio-Plex 200 system (Bio-Rad, Hercules, CA). The median fluorescent intensity (MFI) for 100 microspheres was recorded for each well or sample. All MFI measurements were normalized by subtracting the background signal from uncoated beads and converted to sample-to-positive (S/P) ratios. IgM and IgG isotype-specific antibody levels were detected using SVA VP-coated microspheres and differentiated by using IgM- or IgG-specific secondary antibodies.

PBMC recall stimulation. Cryopreserved PBMCs were thawed and rested for 6 h in complete RPMI 1640 medium (cRPMI) at 37°C with 5% CO_2. RPMI 1640 medium was supplemented with 10% FBS (VWR), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Corning). For the proliferation assays, PBMCs were stained with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE; in PBS) according to the manufacturer's instructions (BD Biosciences). CFSE-stained cells were seeded at a density of 5×10^5 cells/well in 96-well plates and stimulated with the following: UV-inactivated SVA SD15-26 (multiplicity of infection [MOI] = 1), concanavalin A (ConA; 5 μg/ml) plus phytohemagglutinin (PHA; 5 μg/ml) (both from Sigma-Aldrich, St. Louis, MO), or cRPMI alone. Additionally, stimulation with recombinant SVA VP1, VP2, or VP3 (5 μg/ml) was performed with samples from days 14 and 35 p.i. After stimulation, the cells were incubated for 5 days at 37°C with 5% CO_2. For the intracellular cytokine staining (ICS) assays, PBMCs

were plated in 96-well plates (5×10^5 cells/well), rested for 6 to 8 h, and stimulated as described above. GolgiStop protein transport inhibitor (BD Biosciences) was added (10 μg/ml) 4 to 6 h after antigen stimulation, and the cells were incubated for an additional 12 h prior to flow cytometric analysis.

Antibodies and flow cytometry. Antigen-specific T-cell responses were assessed by flow cytometric analysis. T-cell phenotypes were determined using the following swine-specific antibodies: mouse anti-pig CD3 conjugated with Alexa Fluor 647 (ε chain specific; clone BB23-8E6-8C8, isotype IgG2a), anti-pig CD4 (clone 74-12-4, isotype IgG2b), anti-pig CD8α (clone 76-2-11, isotype Ig2a), and anti-pig $\gamma\delta$ TCR (clone PGBL22A) (all from the Washington State University MAb Center, Pullman, WA). Secondary antibodies included goat anti-mouse IgG2a-phycoerythrin (PE), goat anti-mouse IgG2a-FITC, and goat anti-mouse IgG2b-PE-Cy7 (all from Southern Biotech, Birmingham, AL) and goat anti-mouse IgG1 peridinin chlorophyll protein-Cy5.5 (BioLegend, San Diego, CA). Primary antibody concentrations were optimized by titration, and secondary antibodies were used at 5 μg/ml. LIVE/DEAD (BD Bioscience) fixable far-red dye was used to determine cell viability prior to the fixation and permeabilization. Surface staining was performed using PBS–5% FBS containing appropriate concentrations of antibodies in a volume of 50 μl/well. Each staining step consisted of a 30-min incubation with antibodies followed by a wash step. After surface staining, the cells were washed and fixed using BD Cytofix buffer (BD Biosciences) and then resuspended in PBS for acquisition. For ICS, cells were permeabilized using BD Perm/Wash buffer (BD Biosciences) and subsequently stained with an anti-porcine IFN-γ–PE antibody (clone P2G10; BD Biosciences) for 30 min. The cells were washed twice in Perm/Wash buffer and resuspended in PBS. Single-stain and fluorescence-minus-one (FMO) controls were included in all assays. All flow cytometry data were acquired with an Attune NxT flow cytometer (Thermo Fisher Scientific) and analyzed using FlowJo software (TreeStar, San Carlos, CA). Results were corrected for background proliferation/IFN-γ production by subtracting the frequency of cells that responded under mock stimulation condition. The percentage of responding cells was calculated as the percentage of total T cells (live $CD3^+$ cells).

Statistical analysis. Statistical analysis was performed by analysis of variance followed by Tukey's or Sidak's multiple-comparison test. Correlations were evaluated by the Spearman's rank correlation test. Statistical analysis and data plotting were performed using GraphPad Prism (version 6.0) software (GraphPad Software Inc., La Jolla, CA).

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