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Cytokine Protein Expression Levels in Tracheobronchial Lymph Node Homogenates of Pigs Infected with Pseudorabies Virus[▽]

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Pseudorabies virus (PRV) is a neurotropic alphaherpesvirus that produces fatal encephalitis in newborn pigs, respiratory disorders in fattening pigs, and reproductive failure in sows. Following primary infection of the respiratory tract, PRV can develop into a systemic infection with dispersion of the virus via the lymphatic system that involves mononuclear cells in tracheobronchial lymph nodes (TBLNs). The objectives of the present study were to evaluate the pathogenesis and to determine the early immune cytokine profiles in TBLNs following experimental infection with a feral swine PRV isolate at 1, 3, 6, and 14 days postinfection (dpi). Forty healthy pigs were purchased from a PRV-negative herd. Twenty pigs received the Florida strain isolate (FS268) of feral swine PRV intranasally, and 20 uninfected controls received a sham inoculum. Compared to the levels in the controls, the levels of alpha interferon (IFN-α), interleukin-1β (IL-1β), IL-12, and IFN-γ were increased in TBLN homogenates from PRV-infected pigs at 1 dpi, whereas the IL-18 levels were decreased from 3 to 6 dpi. The protein levels of IL-4 and IL-10 did not differ between the controls and the PRV-infected pigs at any time point. Flow cytometric analysis of TBLN homogenates of PRV-infected pigs and the controls revealed increases in the percentages of B cells at 6 dpi, CD4$^+$ cells at 14 dpi, and CD25 expression in TBLN homogenates (in the total mononuclear fraction and on B cells) in the PRV-infected pigs. Collectively, these findings demonstrate that a feral PRV in commercial swine can modulate the host's early immune response to allow the virus to establish an infection.

Pseudorabies virus (PRV) is a neurotropic alphaherpesvirus that produces fatal encephalitis in newborn pigs, respiratory disorders in fattening pigs, and reproductive failure in sows. There are three classes of swine in the United States, as defined in the USDA Veterinary Services Pseudorabies Virus Program Standards: feral swine that are wild and free roaming, transitional swine that are considered owned by someone and that may enter the food chain, and commercial swine that are raised in an environment in which there is no contact with feral swine. Studies of feral swine populations in the United States have demonstrated that PRV is indigenous (7), and it is believed to spread primarily through a venereal route rather than the respiratory route typical in domestic herds (34, 36). In contrast to the trigeminal ganglion latency of PRV in domestic swine, feral swine typically show latent infection of the sacral ganglia (35). Viral strain differences may explain the difference in tropism, as a comparison study of virus strains originating from either domestic or feral pigs found the feral strains to be markedly attenuated (18). Since the eradication of PRV from the U.S. commercial swine herd, there have been rare case reports of transitional swine becoming infected with feral swine PRV isolates (1a, 1b). The continued expansion of the feral swine population in North America presents an emerging threat to the PRV-free status of the U.S. commercial swine herd.

In general, the recognition and the subsequent elimination of herpesvirus-infected cells involve three major immune effector mechanisms: antibody-dependent lysis, major histocompatibility complex (MHC) class I-restricted cytolytic T lymphocyte (CTL)-mediated lysis, and natural killer (NK) cell-mediated lysis. PRV virions contain several envelope glycoproteins important for interactions between the virion and the host cell and constitute major targets for the immune response of the infected host (reviewed in references 27 and 28). Glycoprotein D (gD) is required for entry of the virus into host cells and is probably the most potent inducer of neutralizing antibodies (25). gC is the major attachment protein of PRV and a target of neutralizing antibodies (2), CTLs (31), and effector/memory CD4$^+$ CD8$^+$ T cells (21, 48). gB is required for entry and cell-to-cell spread and is highly conserved among herpesviruses. It represents a major constituent of the viral envelope and has been shown to induce complement-dependent and -independent neutralizing antibodies and helper T cells (21). gE is important for the neurovirulence of PRV and plays a role in transneuronal spread, although it is nonessential for viral replication in cell culture (29).

After acute infection, pigs develop specific antibody and T-cell responses against PRV (7, 8, 21, 46). Besides the induction of type I interferons (IFNs), gamma interferon (IFN-γ) production by peripheral blood mononuclear cells (PBMCs) is an indicator of antigen-specific cell-mediated immunity in swine with PRV and other viral infections (26, 40, 46, 47). IFNs not only play an important role as antiviral agents but also are interconnected regulators in the innate and adaptive immune responses (4). Administration of potent IFN-γ-eliciting cytokines, such as interleukin-12 (IL-12) and IL-18, with PRV antigens promotes the antigen-specific induction of IFN-γ and

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IL-12 yet suppresses PRV-specific antibody production and increases the susceptibility of pigs to subsequent challenge with virulent PRV (44).

In vitro studies have demonstrated that porcine IFN-α, IFN-β, and IFN-γ act synergistically to inhibit the replication of PRV (43). PRV avoids innate defenses via the active suppression of type I IFNs and downstream IFN-β-stimulated genes (6). Numerous studies have been performed to evaluate the protective efficacy of PRV vaccines (27, 28), and the association of vaccine efficacy with balanced IFN-γ and IL-4 memory responses has been described (3, 12, 17). Using the murine model, Bianchi et al. reported that both IFN-γ- and CD4$^+$-producing T cells play important roles in conferring protection against a lethal PRV infection (3). Studies evaluating the kinetics of cytokine production by leukocytes in the lung-draining lymph nodes of PRV-infected pigs, however, have not yet been reported, especially for feral PRV isolates. Accordingly, the goals of the present study were 2-fold: first, to evaluate the pathogenic effects of a feral swine PRV isolate and, second, to characterize the production of cytokines by cells of the tracheobronchial lymph node (TBLN) that drains infected lungs, one of the target organs in PRV-infected pigs.

MATERIALS AND METHODS

Virus, animals, and experimental design. Forty conventionally raised 4- to 5-week-old pigs, free of clinical disease, were purchased and tested negative for porcine circovirus 2, swine influenza virus, porcine respiratory and reproductive virus, and PRV. The pigs were allotted to two equal treatment groups, and each group was housed in an isolation room for about 1 week prior to the beginning of the experiment. On 0 days postinoculation (dpi), the pigs received an intranasal challenge with 3 ml of a sham inoculum (*n* = 20), prepared from swine testicle (ST) cell culture, or an inoculum (*n* = 20) of the Florida strain isolate (FS268) of feral swine PRV (34) at 1×10^6 50% cell culture infectious doses (CCID$_{50}$s) per pig. Five pigs from each group were euthanized and necropsied on 1, 3, 6, and 14 dpi.

Clinical signs, gross pathology, and pulmonary histopathology. Pigs were monitored daily for clinical signs, including rectal temperature and a clinical respiratory score, as described previously (19). Blood samples were collected on 0, 1, 3, 6, and 14 dpi. The rectal temperatures of pigs intended for necropsy on 14 dpi were recorded daily. Pig weights were recorded on 0 dpi and at necropsy. During the postmortem examination, gross lung lesions were evaluated by visual inspection and each lung lobe was scored to reflect the approximate volume or percentage of the lung tissue affected (19). Bronchoalveolar lavage fluid (BALF) and TBLNs were collected. The BALF was cultured for the presence of bacterial pathogens. Two milliliters of BALF was frozen at −80°C. A section of the TBLN was homogenized with a pellet pestle for flow cytometry analysis. Another section of TBLN was homogenized in tissue lysis buffer for cytokine analysis (16).

Antibody detection. For the PRV group, sera collected at 0 and 14 dpi were tested for antibody to the gB antigen of PRV by using the commercial HerdChek PRV gB blocking enzyme-linked immunosorbent assay (ELISA; IDEXX, Westbrook, ME), according to the manufacturer's directions. Test values were reported as the sample optical density value as a ratio of the positive control provided in the kit (S/N ratio). The cutoff S/N values for the PRV gB assay were as follows: negative, greater than 0.70; positive, less than or equal to 0.60; and suspect, greater than 0.60 and less than or equal to 0.70.

Virus detection. On 0, 1, 3, 6, and 14 dpi, all serum and BALF samples were tested for virus. Real-time PCR assays for the specific detection of the gB and gE genes of PRV were performed by the use of TaqMan chemistry, as described previously (24). The analytical sensitivity of the assays was approximately 0.1 PFU per reaction (24).

Virus isolation for the detection of PRV in the samples collected was performed with an ST cell line, as described previously (32). Briefly, each well of 24-well plates with 80 to 90% confluent monolayers of ST cells was inoculated with 100 μl of diluted medium in which the nasal swab specimen, BALF, or tissue homogenate had been placed. The inoculated cells were incubated at 37°C in a 5% CO$_2$ atmosphere for 72 h and monitored daily for the development of cytopathic effects.

Cytokine assays. After removal, approximately 1 g of TBLN was homogenized in 750 μl of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl, and 1 mM MgCl, pH 7.40, with a tissue homogenizer (Biospec Products, Bartlesville, OK). The homogenates were incubated on ice for 30 min and then centrifuged at 2,500 rpm for 10 min (16). The supernatants were collected, passed through a 0.45-μm-pore-size filter (Gelman Sciences, Ann Arbor, MI), and then stored at −20°C prior to the assessment of cytokine levels.

The total protein concentration in the supernatants was measured by use of a commercially available bicinchoninic acid protein kit (Sigma-Aldrich, St. Louis, MO), following the manufacturer's directions, on a NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Tumor necrosis factor alpha (TNF-α), IL-1β, IL-4, IL-6, IL-8, IL-10, and IL-12 protein levels were measured by use of the respective porcine DuoSet ELISA development kits (R&D Systems Inc., Minneapolis, MN), according to the manufacturer's protocol. IL-18 and IFN-γ protein levels were measured by use of the respective porcine ELISA kits (Biosource International, Inc., Camarillo, CA).

IFN-α protein was measured by a porcine IFN-α-specific ELISA with monoclonal antibody (MAb) F17 and MAb K9 (R&D Systems Inc.), as described previously (10). MAb K9 was conjugated with horseradish peroxidase (HRP) by use of a peroxidase labeling kit (Roche Molecular Biochemical, Indianapolis, IN). Immulon 2 flat-bottomed 96-well plates (Fisher Scientific, Houston, TX) were coated overnight at 4°C with MAb F17 at a concentration of 0.3 μg/well in coating buffer (100 mM carbonate buffer, pH 9.6; Sigma Inc., St. Louis, MO). After the wells were blocked with 1% nonfat dried milk and 0.05% Tween 20 in phosphate-buffered saline (PBS) for 1 h at 37°C, the plates were washed five times with 0.05% Tween 20 in PBS. Samples (50 μl) containing 50 μl of 1% nonfat dried milk and 0.05% Tween 20 in PBS were added to each well, and the plates were incubated for 2 h at 37°C. Following five washes, 100 μl of peroxidase-conjugated MAb K9 was added to each well. After 1 h of incubation at 37°C and five washes, 100 μl of tetramethylbenzidine substrate solution (KPL Inc., Gaithersburg, MD) was added to each well. After 30 min, the reaction was stopped with tetramethylbenzidine stop solution (KPL Inc.) and the optical density at 450 nm was measured with an ELISA plate reader. Quantified recombinant porcine IFN-α (rIFN-α; R&D Systems Inc.) was used as a standard, and the IFN-α concentrations were calculated on the basis of the concentrations on a standard curve. One unit per milliliter of rIFN-α was equivalent to 26 pg/ml.

Flow cytometry analysis. Cells from the TBLNs were phenotyped by flow cytometry, as described previously (38). Commercially available antibodies to the following markers were used: CD3 (2B3C), CD25 (PGBL25A), MHC class II (TH16B), B cell (BB6-11C9), CD4 (PT90A), CD8 (76-2-11), and γ/δ T-cell (PGBL22A) (VMRD Inc., Pullman, WA). Secondary antibodies (VMRD) targeted to murine primary antibodies included IgG1-fluorescein isothiocyanate (FITC), IgG2a-FITC, IgG2b-phycoerythrin (PE), IgG1-PE, IgG2a-PE, and IgM-PE. Data were acquired with CellQuest Pro software (BD Biosciences, San Jose, CA) on an LSR II flow cytometer (Becton Dickinson) and were analyzed with FlowJo software (TreeStar, Ashland, OR).

Statistical analysis. Differences in virus titers, body temperatures, and respiratory scores between the study groups were evaluated by analysis of variance (ANOVA). Two-way ANOVA and posttests were used for comparison of the cytokine titers. Spearman rank correlation tests were used to compare the individual virus titers, cytokine titers, body temperatures, and respiratory scores with the other parameters.

RESULTS

Clinical evaluation and gross pathology. PRV-inoculated pigs developed a mild clinical disease typical of PRV infection, as reflected by fever (Fig. 1A), anorexia, sneezing, increased respiration rates, and dyspnea that began at 2 to 3 dpi and that resolved by 14 dpi. Control animals did not display clinical signs or gross lung lesions consistent with PRV infection. Pigs inoculated with PRV had an increased ($P < 0.05$) rectal temperature from 1 to 6 dpi (Fig. 1A). No significant differences ($P > 0.05$) in daily weight gain (Fig. 1B) were found between pigs inoculated with PRV (average, 0.6 kg/day) and control pigs (average, 0.6 kg/day). Gross lung lesions in PRV-inoculated pigs mainly consisted of hilar multifocal areas of purple to red areas of consolidation. The lesions were most pronounced at 3 and 6 dpi (Fig. 1C). Bacteria typically associated
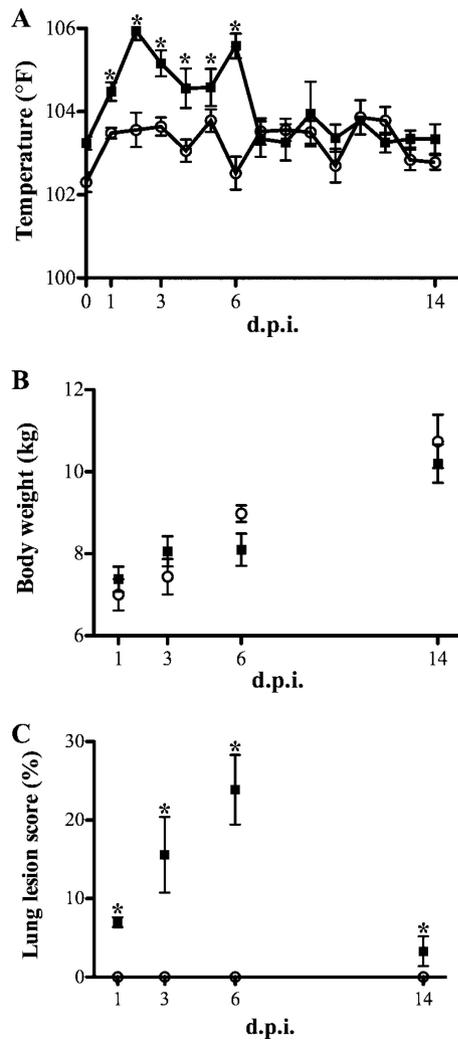
FIG. 1. Clinical evaluation and gross pathology. (A) Rectal temperature for control (○) and PRV-inoculated (■) pigs measured each day out to 14 dpi; (B) body weight measured prior to necropsy for control (○) and PRV-inoculated (■) pigs; (C) macroscopic lesion score for lungs at necropsy for control (○) and PRV-inoculated (■) pigs. Data are expressed as means ± standard errors ($n = 5$). ∗, $P < 0.05$.

with porcine respiratory disease were not isolated from the BALF of any of the pigs. Pig serum was negative for PRV gB antibody at 0 dpi (mean S/N ratio, 1.22 ± 0.03) and positive at 14 dpi (mean S/N ratio, 0.26 ± 0.2).

Quantitative PCR for virus nucleic acid and virus isolation. Table 1 summarizes the results of the virus detection assays. After intranasal infection, PRV was detected in the tonsil, lungs, BALF, and nasal swab of the inoculated pigs from 1 to 6 dpi; however, PRV was not detected in the brain, spleen, or sacral ganglia.

Cytokine assays. Cytokine protein level measurements were normalized to the total amount of protein per gram of TBLN. The ex vivo protein levels of the proinflammatory cytokines IFN-α and IL-1β were increased ($P = 0.06$ and $P < 0.05$, respectively) in the TBLN homogenates of PRV-inoculated pigs compared with those in the control pigs at 1 dpi (Fig. 2A and B), whereas the IL-6, IL-8, and TNF-α levels did not differ ($P > 0.05$) between the treatment groups at any time point. As with IFN-α and IL-1β, at 1 dpi the levels of the Th1 cytokines IL-12 and IFN-γ were increased ($P < 0.05$) in the PRV-inoculated pigs compared with the levels in the control pigs (Fig. 2C and D). In contrast, at 3 and 6 dpi the IL-18 level in the PRV-inoculated pigs was decreased ($P < 0.05$) compared with that in the control pigs (Fig. 2E). At 6 dpi, the IL-12 level in the PRV-inoculated pigs was also decreased ($P < 0.05$) compared with the level in the control pigs (Fig. 2C). Protein levels of the Th2/regulatory T cell cytokines IL-4 and IL-10 did not differ between the treatment groups at any time point (Fig. 2F and G).

Flow cytometry. Flow cytometric analysis of TBLN homogenates revealed increases ($P < 0.05$) in B-cell percentages (Fig. 3A) and a concomitant decrease in CD4+ cells at 6 dpi in PRV-infected pigs compared with the results for the control pigs (Fig. 3B). By 14 dpi, the percentages of CD4+ cells were increased ($P < 0.05$) in PRV-infected pigs compared with the percentages in the control pigs ($33.4\% \pm 1.2\%$ and $26.0\% \pm 2.5\%$, respectively). The mean fluorescence intensity of CD25 was increased ($P < 0.05$) both in total TBLN homogenates (Fig. 3C) and on B cells in TBLN homogenates (Fig. 3D) at 14 dpi in PRV-infected pigs compared with the mean fluorescence intensity for the control pigs. Differences ($P > 0.05$) between treatment groups in the numbers of B cells and CD4+ cells were not detected at 1 and 3 dpi. Differences ($P > 0.05$) between treatment groups in the numbers of CD4+ CD8+, γδ T-cell receptor-positive, CD8+, CD3+, or MHC class II-positive cells were not detected.

DISCUSSION

Feral swine PRV isolate FS268 was pathogenic in this study, inducing fever, anorexia, listlessness, and dyspnea following

TABLE 1. Distribution of PRV in tissues of intranasally inoculated pigs[a]

Tissue sampled	No. of pigs positive for PRV by the indicated assay/total no. of pigs tested on the following dpi:							
	1		3		6		14	
	RT-PCR	VI	RT-PCR	VI	RT-PCR	VI	RT-PCR	VI
Brain	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
Sacral ganglion	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tonsil	3/5	4/5	4/5	5/5	3/5	2/5	0/5	0/5
Lung	5/5	5/5	4/5	4/5	4/5	1/5	1/5	0/5
BALF	5/5	5/5	5/5	4/5	4/5	0/5	2/5	0/5
Nasal swab	4/5	5/5	5/5	5/5	5/5	5/5	0/5	0/5

[a] No PRV was detected in the tissue from control animals (data not shown). RT-PCR, reverse transcription-PCR; VI, virus isolation assay.
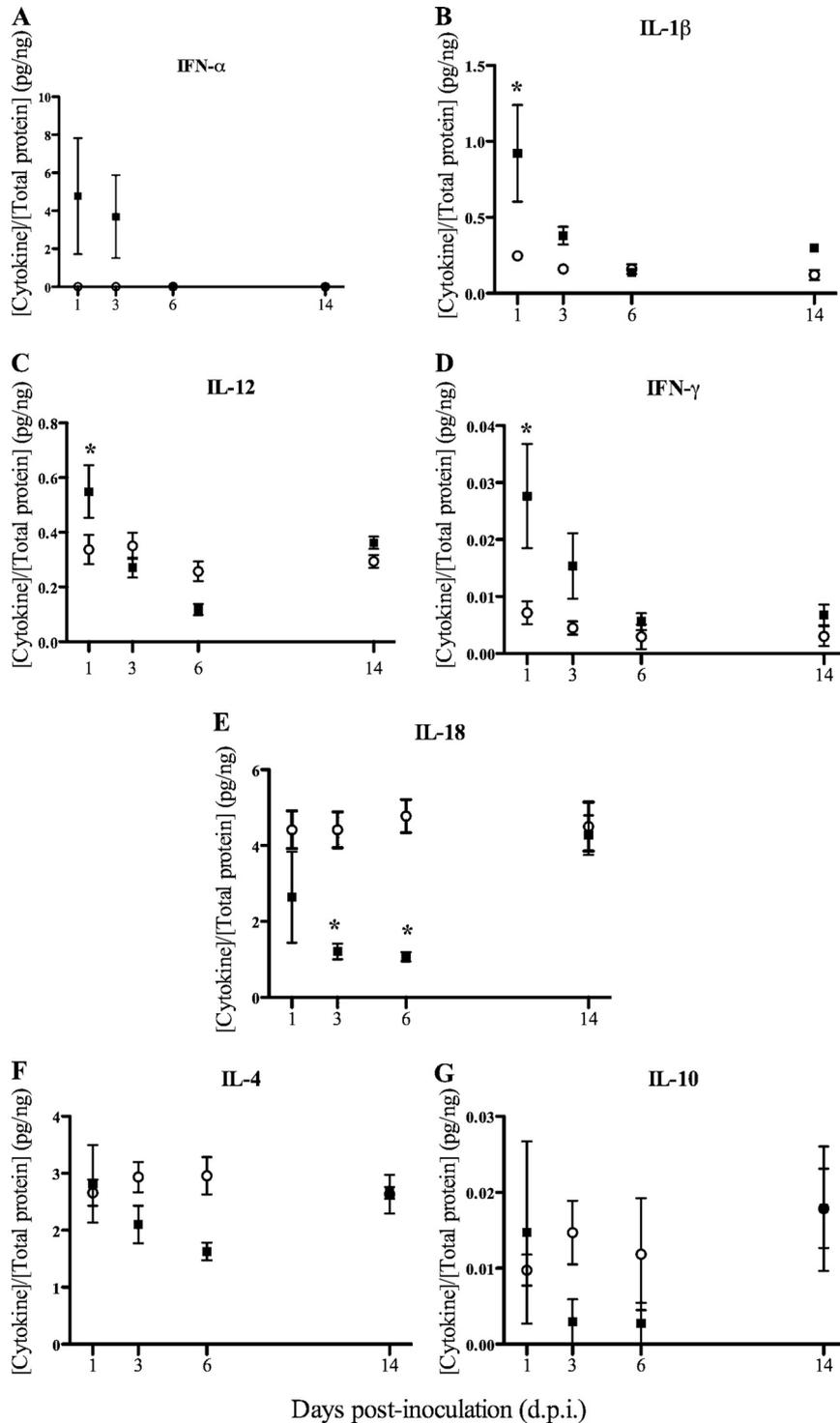
FIG. 2. Cytokine concentration in homogenates of TBLNs from pigs inoculated with PRV (■) or controls (○) normalized to the total protein concentration per gram of TBLN. Due to differences in the concentrations of the cytokines, the graphs use different scales. Means and standard errors of the means ($n = 5$) are shown. *, significantly different value than that for the control ($P < 0.05$).

experimental infection of young pigs. The presence of clinical signs was related to the detection of PRV in tissues associated with the respiratory tract. Likewise, the lack of central nervous system (CNS) clinical signs may be related to the absence of detectable virus in CNS tissues. The results of this study indi-

cate that while isolate FS268 may induce moderate to severe pneumonia, it may have a limited effect on the CNS, supporting the assumptions of others that feral swine PRV isolates may have a phenotype different from that of isolates from commercial swine. Few studies evaluating the kinetics of cyto-
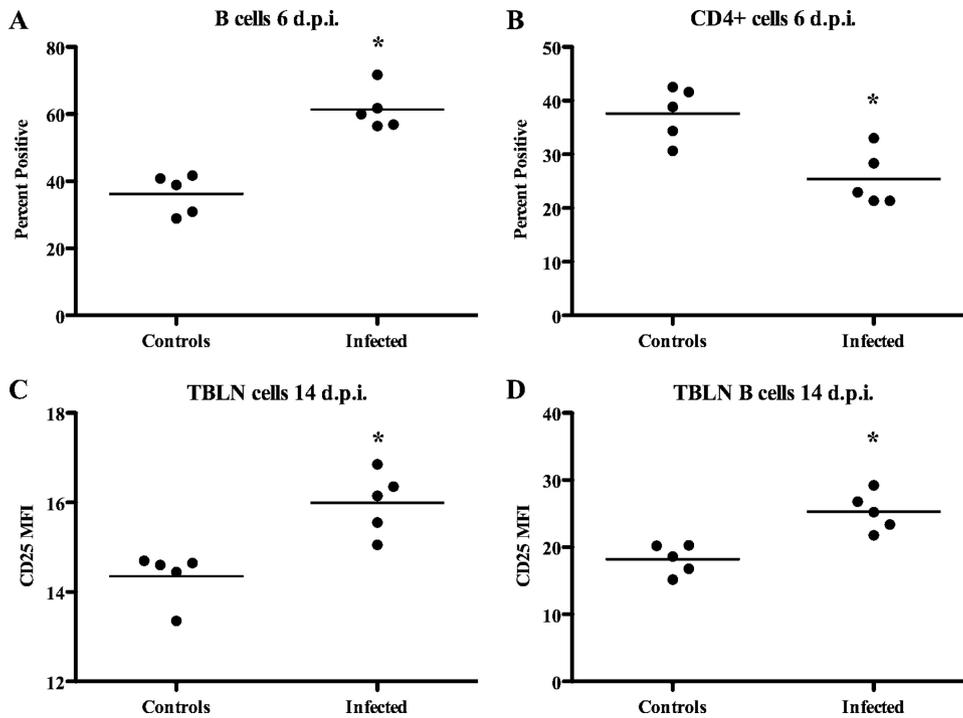
FIG. 3. Flow cytometric analysis of homogenates of TBLNs from pigs inoculated with PRV or controls. (A) B-cell percentages; (B) CD4$^+$-cell percentages; (C) mean fluorescence intensity (MFI) of CD25 for total TBLN homogenates; (D) mean fluorescence intensity of B cells in TBLN homogenates. $*$, significant difference ($P < 0.05$) from the values for the controls.

kine production in PRV-infected pigs have been reported (5, 7, 14, 21, 33, 42, 48). Previous studies used PBMCs in vitro or from pigs infected with attenuated (gE- or thymidine kinase-deleted) laboratory or wild-type PRV strains.

During the 14-day course of this study, the infected pigs began to recover clinically, and this was reflected in a variety of cellular and cytokine changes in the TBLNs. Overall, an early increase in proinflammatory cytokine levels was elicited by PRV in TBLNs, leading to an increase in the Th1 (IL-12 and IFN-γ) responses concurrently with increased percentages of B cells at 6 dpi and unchanged Th2 (IL-4 and IL-10) responses. It has previously been observed in PRV (domestic strain)-primed pigs that upon PRV reexposure of PBMCs in vitro, there is a significantly enhanced transcription of Th1-type cytokines (IL-2 and IFN-γ) but not of Th2-type cytokines (IL-4 and IL-10), but in naïve pigs there was no increased transcription of cytokines (14). The activation of B cells, as evidenced by the upregulation of CD25 (IL-2 receptor α chain marker), and their proliferation and expansion (likely with the support of Th cells) were observed at 14 dpi in our study. A significant decrease in the IL-18 response early following PRV infection could be a virulence mechanism and could be related to the persistence and latency of PRV, given that the IL-18-interacting Th1 cytokine (IL-12 and IFN-γ) responses were not sustained. IL-18 expression can be activated by Toll-like receptor signaling (1) or can be induced by IFN-γ via interferon consensus sequence-binding protein and activator protein 1 pathways (20), by inflammasome activation (39), or by autocrine signaling through the NF-κB pathway (15). In the presence of IL-12, IL-18 induces strong Th1-type immune responses (9, 23, 30, 41), primarily through the induction of IFN-γ expression by

T cells and natural killer cells (11, 45). Impairment of IL-18 expression, which is involved in the induction of antiviral cytokines, could represent a strategy that the virus uses to evade the immune response of the host.

A B-cell response is induced after PRV inoculation but alone is not a protective response (7). The proliferation of T cells after restimulation with PRV suggests that T cells are involved in protective immunity (21). These cells may produce IFN-γ and TNF-α (13). IFN-γ can be involved in the prevention of PRV replication either by a direct local antiviral effect or, indirectly, by the induction of proinflammatory cytokines, or by a combination of both mechanisms (17, 37). IFN-γ and TNF-α can also activate macrophages and natural killer cells, which are involved in the clearance of PRV (17). It has previously been demonstrated that CD8$^+$ T cells lysed PRV-infected cells and that CD2$^+$ CD4$^-$ and CD8$^-$ or CD8^{dull+} T lymphocytes (i.e., NK cells) lysed PRV-infected target cells (22, 48). More cytolytic cells may be found at specific sites in tissues involved in the infection process, such as mucosal tissues, tonsils, and draining lymph nodes (7). Lymphocytes that proliferate after pigs are inoculated with PRV probably migrate to the site of infection. This possibility is supported by the observation of a very localized influx of various T-lymphocyte subsets to the site of PRV infection, which was restricted to specific areas in the draining lymphoid tissues (5).

A change of lymphocyte subset frequency in TBLNs can be caused by an expansion or depletion of specific subsets after the inoculation of PRV. Our results showed that the proportion of various lymphocyte subsets in TBLNs changed after inoculation (Fig. 3C and D). This finding is in accordance with the observations of leukopenia after challenge with PRV and

lower percentages of total T and B lymphocytes (33, 42) and is in contrast to the finding that the lymphocyte subset proportion did not change after inoculation of an avirulent strain of PRV that induced protective immunity (7).

In conclusion, the TBLNs from PRV-infected pigs presented an altered cytokine protein expression profile. It was evident that the key cytokines responsible for driving the immune responses (IFN-α, IL-1β, IL-12, IL-18, and IFN-γ) showed changes in their levels of protein expression in the TBLNs of diseased pigs. These changes suggest a lack of induction of the IL-4 and IL-10 responses and a decrease in the IL-18 response. To our knowledge, this is the first *in vivo* study of the cytokine response to a feral pseudorabies virus strain in the TBLNs of pigs. Moreover, the feral swine strain of PRV used in this study caused mild clinical disease during the acute phase that included fever and lung lesions, which are associated with an inflammatory cytokine response. These resolved by 14 dpi, and there was an increase in the B-cell percentages in association with the induction of immune cytokines in the TBLNs. The presence of PRV-specific antibodies in serum at 14 dpi was consistent with the observation of increased B-cell percentages in TBLN homogenates.

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