

2-1-2004

# Lymphoid Hyperplasia Resulting in Immune Dysregulation Is Caused by Porcine Reproductive and Respiratory Syndrome Virus Infection in Neonatal Pigs

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# Lymphoid Hyperplasia Resulting in Immune Dysregulation Is Caused by Porcine Reproductive and Respiratory Syndrome Virus Infection in Neonatal Pigs

## Abstract

Amid growing evidence that numerous viral infections can produce immunopathology, including nonspecific polyclonal lymphocyte activation, the need to test the direct impact of an infecting virus on the immune system of the host is crucial. This can best be tested in the isolator piglet model in which maternal and other extrinsic influences can be excluded. Therefore, neonatal isolator piglets were colonized with a benign *Escherichia coli*, or kept germfree, and then inoculated with wild-type porcine reproductive and respiratory syndrome virus (PRRSV) or sham medium. Two weeks after inoculation, serum IgM, IgG, and IgA levels were 30- to 50-, 20- to 80-, and 10- to 20-fold higher, respectively, in animals receiving virus vs sham controls, although <1% was virus specific. PRRSV-infected piglets also had bronchial tree-associated lymph nodes and submandibular lymph nodes that were 5–10 times larger than colonized, sham-inoculated animals. Size-exclusion fast performance liquid chromatography revealed that PRRSV-infected sera contained high-molecular-mass fractions that contained IgG, suggesting the presence of immune complexes. Lesions, inflammatory cell infiltration, glomerular deposits of IgG, IgM, and IgA, and Abs of all three isotypes to basement membrane and vascular endothelium were observed in the kidneys of PRRSV-infected piglets. Furthermore, autoantibodies specific for Golgi Ags and dsDNA could be detected 3–4 wk after viral inoculation. These data demonstrate that PRRSV induces B cell hyperplasia in isolator piglets that leads to immunologic injury and suggests that the isolator piglet model could serve as a useful model to determine the mechanisms of virus-induced immunopathology in this species.

## Disciplines

Genetics and Genomics | Large or Food Animal and Equine Medicine | Veterinary Microbiology and Immunobiology | Veterinary Pathology and Pathobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

## Comments

This article is published as Lemke, Caitlin D., Joseph S. Haynes, Rodger Spaete, Deb Adolphson, Ann Vorwald, Kelly Lager, and John E. Butler. "Lymphoid hyperplasia resulting in immune dysregulation is caused by porcine reproductive and respiratory syndrome virus infection in neonatal pigs." *The Journal of Immunology* 172, no. 3 (2004): 1916-1925. doi: [10.4049/jimmunol.172.3.1916](https://doi.org/10.4049/jimmunol.172.3.1916). Posted with permission.

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This information is current as of October 10, 2017.

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*J Immunol* 2004; 172:1916-1925; ;

doi: 10.4049/jimmunol.172.3.1916

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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Lymphoid Hyperplasia Resulting in Immune Dysregulation Is Caused by Porcine Reproductive and Respiratory Syndrome Virus Infection in Neonatal Pigs<sup>1</sup>

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Amid growing evidence that numerous viral infections can produce immunopathology, including nonspecific polyclonal lymphocyte activation, the need to test the direct impact of an infecting virus on the immune system of the host is crucial. This can best be tested in the isolator piglet model in which maternal and other extrinsic influences can be excluded. Therefore, neonatal isolator piglets were colonized with a benign *Escherichia coli*, or kept germfree, and then inoculated with wild-type porcine reproductive and respiratory syndrome virus (PRRSV) or sham medium. Two weeks after inoculation, serum IgM, IgG, and IgA levels were 30- to 50-, 20- to 80-, and 10- to 20-fold higher, respectively, in animals receiving virus vs sham controls, although <1% was virus specific. PRRSV-infected piglets also had bronchial tree-associated lymph nodes and submandibular lymph nodes that were 5–10 times larger than colonized, sham-inoculated animals. Size-exclusion fast performance liquid chromatography revealed that PRRSV-infected sera contained high-molecular-mass fractions that contained IgG, suggesting the presence of immune complexes. Lesions, inflammatory cell infiltration, glomerular deposits of IgG, IgM, and IgA, and Abs of all three isotypes to basement membrane and vascular endothelium were observed in the kidneys of PRRSV-infected piglets. Furthermore, autoantibodies specific for Golgi Ags and dsDNA could be detected 3–4 wk after viral inoculation. These data demonstrate that PRRSV induces B cell hyperplasia in isolator piglets that leads to immunologic injury and suggests that the isolator piglet model could serve as a useful model to determine the mechanisms of virus-induced immunopathology in this species. *The Journal of Immunology*, 2004, 172: 1916–1925.

Evidence has accumulated that viral infections can produce dysregulation of the immune system and immunopathology (1–13). This includes polyclonal B cell activation (3, 4, 7, 8, 11), the appearance of autoantibodies (1, 2, 5, 6, 9, 12, 13), and kidney histopathology (14, 15). Most recently, it was reported that lymphocytic choriomeningitis virus (LCMV)<sup>3</sup> infection of mice induced polyclonal B cell activation that resulted in hypergammaglobulinemia and autoantibody production (13). The investigators speculated that the mechanisms underlying the induction of these immunopathologies during LCMV infection could be extrapolated to explain similar phenomena that result from many human and murine viral infections. However, the reports cited above are based on studies in species in which maternal and environmental factors cannot be rigidly controlled, leaving the question of a direct effect of viral infection ambiguous. In an effort to

address this deficiency in previous studies, we have used the isolator piglet model (16–18). The advantages of this model are that piglets receive no passive maternal Igs in utero, and because they are precocial, they can be reared colostrum deprived in specific pathogen-free autosows (19, 20) and in germfree (GF) isolator units (21). Although non-Ig regulatory factors such as cytokines are transferred in milk and colostrum, there is no evidence for their transplacental transport (22, 23). Thus, the effect of a virus on the immune system can be examined in the absence of maternal and environmental factors. Such studies are more controllable than in rodents and humans in which maternal regulatory factors are transferred in utero (22). This model also has the virtue that animals are outbred, so their responses are species, not strain, dependent.

Our laboratory is interested in how environmental factors affect the development of the neonatal immune system and has recently shown that colonizing bacteria are required (18). Therefore, we wondered whether a virus could have the same effect. Furthermore, we wondered whether viral infections could alone produce immunopathology, or whether colonizing bacteria and/or passive immunity were required. To test this in the isolator piglet model, we chose the porcine reproductive and respiratory syndrome virus (PRRSV), which is responsible for porcine reproductive and respiratory syndrome (PRRS). PRRS was first reported in the United States in 1987 (24). This syndrome is characterized by increased rates of reproductive failure in sows and respiratory infection, particularly in young piglets, and is now pandemic and considered to be the most important infectious disease of swine (National Pork Board; www.porkboard.org). PRRSV was isolated and characterized in 1992 (25, 26) and assigned to the family Arteriviridae, order Nidovirales (27). This family also includes lactate dehydrogenase-elevating virus (LDV), simian hemorrhagic fever virus, and equine arteritis virus.

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Received for publication July 29, 2003. Accepted for publication November 19, 2003.

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<sup>1</sup> This work was supported by the National Pork Board and the National Science Foundation (MCB 00-77237).

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<sup>3</sup> Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; GF, germfree; PRRS, porcine reproductive and respiratory syndrome; PRRSV, PRRS virus; LDV, lactate dehydrogenase-elevating virus; IC, immune complex; PCOL, PRRSV colonized; PGF, PRRSV GF; SCOL, sham colonized; CDR, complementarity-determining region; SE-FPLC, size-exclusion fast performance liquid chromatography; ANA, anti-nuclear Ab; BLN, bronchial lymph node; SMLN, submandibular lymph node; MLN, mesenteric lymph node; S/P, sample OD/positive reference OD.

The possibility that PRRSV infection produces immunopathology stems from studies showing that LDV infection of mice appears to induce nonspecific polyclonal activation of B cells, because serum Ig levels, especially IgG2a and IgG2b, were elevated in the absence of an antiviral response (3, 4, 7, 8, 10, 11). Polyclonal activation by PRRSV in tonsil and spleen of piglets was also suggested because of increased B cell numbers after infection (28). However, cell number and IgG levels are not criteria for polyclonality because single clones can give rise to increased numbers of B cells and raise Ig levels, as in the case of lymphomas (29). EBV infection is known to be associated with the development of Hodgkin's lymphoma, which involves the expansion of a monoclonal B cell population (30). Therefore, the possibility that increased B cell numbers observed during PRRSV infection (28) result from the expansion of a monoclonal B cell population cannot be excluded. In any case, the purported polyclonal activation of B cells in mice infected with LDV was associated with circulating immune complexes (IC) and autoantibodies (1, 2, 4, 7, 8, 10). The IC, which ranged in size from 150 to 300 kDa, contained mainly IgG2a and IgG2b. These were present in both neonatal and adult mice infected with LDV and could be detected in the absence of an antiviral humoral immune response (7, 8, 10). It was thus concluded that these IC did not contain viral proteins, and it was proposed that autoantigens were involved. However, the exact nature of the IC was not determined.

Autoantibodies that are specific for a range of autoantigens are also a prominent feature of LDV infection (1, 2). Interestingly, a transmissible agent, anti-Golgi apparatus-inducing agent, which was capable of causing increased production of autoantibodies against the Golgi apparatus in mice, was reported and ultimately identified as LDV. The production of Abs directed against Golgi Ags is, however, not unique to LDV infection. The clinical relevance of anti-Golgi apparatus Abs in humans is not well understood, but they were first identified in a patient with Sjögren's syndrome and have been associated with isolated cases of systemic lupus erythematosus (31). Later studies correlated the appearance of anti-Golgi apparatus Abs with a number of human viral infections, including HIV (5), EBV (6), and hepatitis B virus (9). More recently, infection of mice with LCMV was also shown to induce both polyclonal hypergammaglobulinemia and autoantibody production (13). Despite the common themes of polyclonal B cell activation and autoantibody production during many viral infections, evidence is often only suggestive, and the mechanisms responsible have yet to be elucidated. Furthermore, whether these outcomes are the direct effect of the virus acting alone or in conjunction with environmental factors remains unclear.

We report in this study that PRRSV infection of isolator piglets results in polyclonal B cell activation that is associated with extremely elevated levels of all serum Igs, lymphoid hyperplasia, circulating IC, and the presence of autoantibodies directed against Golgi Ags, dsDNA, and apparently vascular endothelium and basement membrane. Infection is also associated with kidney histopathology and IC deposition in glomeruli. Collectively considered, our results indicate that PRRSV infection can directly act on the immune system, resulting in lymph node hyperplasia, stimulating the appearance of autoantibodies through polyclonal B cell activation, and the formation of circulating IC that can altogether produce immunologic injury.

## Materials and Methods

### Animal studies

Piglets were recovered by closed hysterectomy from 112-day gravid outbred swine, placed in groups of four in GF, rigid-tub isolators, and reared on ESPLac (Pet Ag, Hampshire, IL) as previously described (21, 32). The

three treatment groups were as follows: 1) those colonized with 10<sup>9</sup> strain G58-1 *Escherichia coli* on day 3 of life and inoculated i.m. with PRRSV on day 7 (designated PRRSV colonized (PCOL)), 2) those maintained GF and inoculated i.m. with PRRSV on day 7 (designated PRRSV GF (PGF)), and 3) those colonized with 10<sup>9</sup> strain G58-1 *E. coli* on day 3 and inoculated with sham culture medium, prepared from mock-infected cell cultures, on day 7 (designated sham colonized (SCOL)). On day 28, piglets were given a second inoculation of PRRSV (PCOL and PGF) or sham medium (SCOL). Each treatment group included four animals. Previous studies have shown that four animals per group are sufficient to detect significant differences in treatment when isolator piglets are used (18).

Piglets were inoculated with strain NADC-8 of PRRSV (33). This strain was isolated from the serum of a 2-day-old moribund piglet from a naturally occurring case of PRRS in Iowa. Based on previous reports, i.m. inoculation of piglets was chosen (34, 35), because it was shown to result in viremia. The inocula contained 10<sup>4</sup> 50% tissue culture-infective dose of strain NADC-8.

Weekly blood samples were collected from the time of birth. On day 35, all piglets were euthanized by i.v. injection of pentobarbital (Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA). Tissues were grossly examined at necropsy and subsequently collected for preparation of RNA, conventional histopathology, and immunohistochemistry.

### Collection and processing of blood and tissue samples

Blood and tissue samples were collected and processed as previously described (18). Briefly, blood was collected in heparinized blood collection tubes (Vacutainer; BD Biosciences, Rutherford, NJ) and processed to recover both plasma and the leukocyte fraction. For this, blood was centrifuged at 1400 × *g* for 10 min at 4°C, and the plasma was removed and stored at -20°C. The buffy coat was then transferred to a 50-cc tube filled with ammonium chloride/potassium solution and incubated for 10 min to lyse the erythrocytes. The leukocytes were then pelleted, washed, and resuspended in 1 ml of TRI reagent (Molecular Research Center, Cincinnati, OH) for later isolation of RNA. Tissues were collected at the time of necropsy, frozen in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA), and stored at -20°C. Serum was also harvested at this time and stored at -20°C until tested for the presence of PRRSV and PRRSV-specific Ab (HerdChek; IDEXX Laboratories, Westbrook, ME). For the IDEXX test, sample OD/positive reference OD (S/P) ratios were calculated according to manufacturer's instructions, and a value of ≥0.4 was considered positive for PRRSV-specific Abs.

### Measurement of serum IgM, IgG, and IgA

Serum levels of IgM, IgG, and IgA were quantified by sandwich ELISA, as previously described (17, 32). In addition, the IgG concentration in day-28 and -35 serum was quantified using single radial diffusion. For this, 1.5% agarose gels with a homogeneous 1/30 dilution of rabbit anti-swine IgG (Fc-specific) serum (18) were prepared. Wells (2 mm in diameter) were made in the gels, and dilutions of day-28 and -35 serum samples and a reference standard serum were added. After overnight incubation at room temperature in a humidity chamber, diameters of IgG-anti-IgG precipitation halos were measured in two directions and averaged. The IgG concentrations were then determined against a reference standard curve.

### Quantification of IgG specific for PRRSV Ags

The proportion of serum IgG specific for PRRSV Ags was estimated by an Ab depletion method. Briefly, serum samples from PRRSV-infected and sham-inoculated piglets were appropriately diluted and incubated on plates coated with PRRSV Ags (HerdChek; IDEXX Laboratories) and then successively transferred to fresh microtiter wells until the IgG anti-PRRSV activity, determined according to the manufacturer's criteria, had been depleted. The amount of IgG remaining in the samples tested was quantitated by sandwich ELISA before and after depletion to determine the amount of IgG specific for PRRSV.

### Preparation of cDNA and spectratypic analysis

Total RNA was isolated from PBL or tissue, using the TRI reagent described above, according to the manufacturer's protocol. Spectrophotometric analysis quantified and verified the purity of the RNA. First-strand cDNA was prepared using 5 μg of total RNA and 1 μl of oligonucleotide mixture (IgG antisense CH2 (5 pmol/μl); IgM antisense CH2 (5 pmol/μl); IgA antisense CH3 (5 pmol/μl); IgD antisense hinge (5 pmol/μl); and random hexamers (10 pmol/μl)). The sequence of these antisense primers has been reported (17), except for the IgD primer. The sequence of the antisense IgD primer is 5'-GCTGGGAGCTGCCGAGAT-3'. The design of this 18-mer primer was based on the recently published sequence for

porcine IgD (36). This protocol produces cDNAs that are enriched for H chain transcripts. The complementarity-determining region (CDR)3 segments of the cDNAs were amplified by PCR using nested FR3 and  $^{32}\text{P}$ -labeled antisense FR4 primers that anneal to all porcine VDJ rearrangements, as previously reported (37, 38). PCR products were separated on 7.3% denaturing polyacrylamide gels, and visualized by autoradiography.

#### Detection of PRRSV and clinical scoring

Serum collected at the time of necropsy was stored at  $-20^{\circ}\text{C}$  until tested for the presence of PRRSV, as previously described (33). For qualitatively evaluating anorexia, piglets were given three milk meals per day, and those that did not consume their meals in their entirety were deemed anorexic.

#### Size-exclusion fast performance liquid chromatography (SE-FPLC)

SE-FPLC was performed with a Superose 6 column (Amersham Biosciences, Piscataway, NJ) in the context of a Waters (Milford, MA) HPLC system using a 510 pump, Rheodyne (Rohnert Park, CA) manual injector, Bio-Rad (Hercules, CA) cartridge guard column, and a 100- $\psi$  pressure restrictor. Samples were centrifuged at  $15,000 \times g$ , and the supernatant was filtered through a 0.45- $\mu\text{m}$  filter (Millex-HV; Millipore, Bedford, MA). Samples (100  $\mu\text{l}$ ) were injected, and separation was done at 150  $\mu\text{l}/\text{min}$ . Elution profiles were monitored at 280 nm with an ISCO (Lincoln, NE) type 9 optical unit, and samples were collected using an ISCO Retriever II fraction collector. Fractions were quantitatively analyzed for their IgG content using the sandwich ELISA described above.

#### Histology and immunohistochemistry

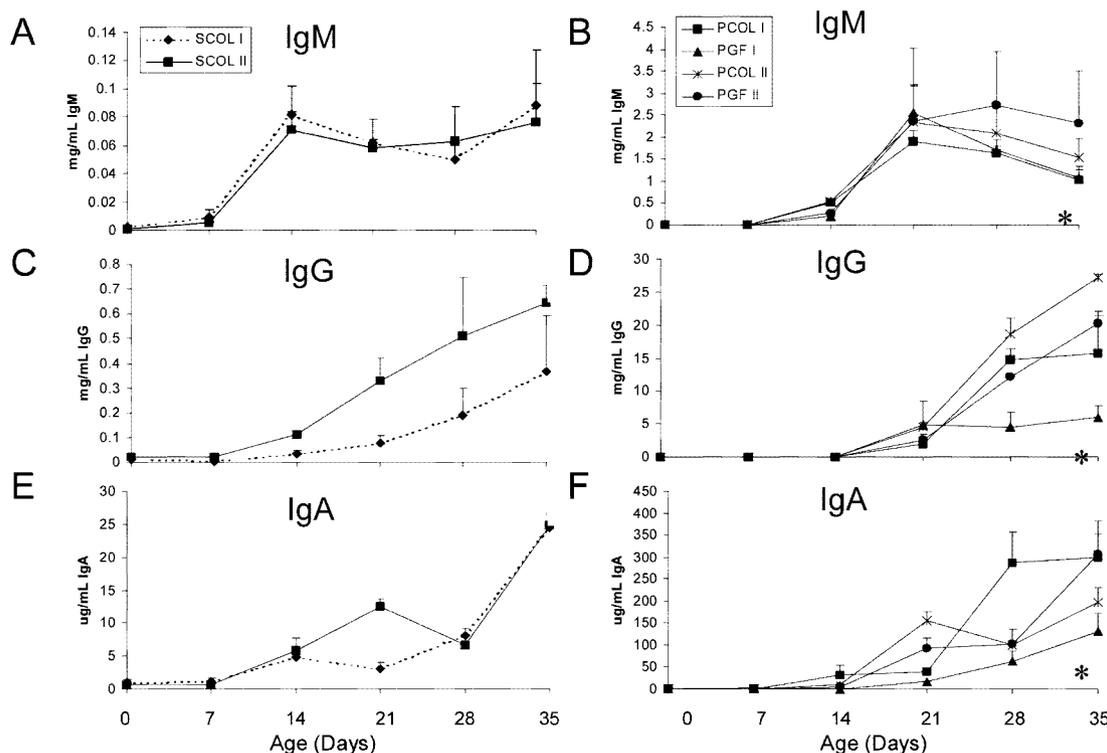
Paraffin-embedded kidney sections were stained with H&E. Enzyme and fluorescence immunohistochemistry were used to visualize IgG deposition in kidney sections. Immunofluorescence was also used to visualize PRRSV nucleocapsid Ag, IgM, and IgA in kidney sections. Eight- to 10- $\mu\text{m}$ -thick sections of kidney frozen in OCT were prepared using a Microm cryostat (Mikron Instruments, San Marcos, CA), adhered to Superfrost glass slides (Fisherbrand; Fisher Scientific, Pittsburgh, PA), and fixed in 10% cold

formalin. IgG was detected using a monoclonal mouse anti-swine IgG (M155; kindly provided by K. Nielsen (Animal Disease Research Institute, Nepean, Ontario, Canada)) followed by either a goat anti-mouse IgG conjugated to Alexa 488 or a goat anti-mouse IgG conjugated to HRP (DAKO, Carpinteria, CA). IgM and IgA were detected using mAbs M160 and 1459, respectively (also kindly provided by K. Nielsen). Nucleocapsid Ag was detected using a monoclonal mouse Ab, SDOW17 (kindly provided by E. Nelson (South Dakota State University, Brookings, SD)). These were followed by the same goat anti-mouse IgG conjugated to Alexa 488, as was used to detect IgG. Propidium iodide was used to counterstain nuclei for immunofluorescence. Diaminobenzidine was used as the HRP substrate, and methyl green counterstained nuclei. The sections were visualized by either fluorescence microscopy or light microscopy and photographed at  $\times 200$  or  $\times 160$ , respectively.

#### Detection of autoantibodies

Anti-nuclear and/or anti-cytoplasmic Abs of the IgG isotype were detected in piglet serum using the HEp-2 fluorescent anti-nuclear Ab (ANA) test system (Immunoconcepts, Sacramento, CA). The protocol was based on the manufacturer's instructions, with slight modifications to allow detection of swine IgG. Briefly, a 1/100 dilution of rabbit anti-swine IgG (Fc-specific) serum (18) followed by a 1/50 dilution of swine anti-rabbit conjugated to FITC (DAKO) was used for the detection system. Cells were visualized at  $\times 400$  magnification on a fluorescence microscope. Samples were deemed positive or negative for autoantibodies, and the staining patterns were determined according to the manufacturer's protocol.

IgG anti-dsDNA was detected using the QUANTA Lite dsDNA ELISA kit (INOVA Diagnostics, San Diego, CA). The protocol was based on the manufacturer's instructions with slight modifications to allow detection of swine IgG. Briefly, the detection system consisted of a 1/500 dilution of rabbit anti-swine IgG (Fc-specific) serum (18), followed by a 1/4000 dilution of goat anti-rabbit IgG conjugated to HRP (Pierce, Rockford, IL) and then tetramethylbenzidine as the substrate. The anti-dsDNA activity of the samples was calculated according to the manufacturer's instructions.



**FIGURE 1.** Serum Ig levels in SCOL, PCOL, and PGF isolator piglets. Data are expressed as the mean + SEM for IgM (A and B), IgG (C and D), and IgA (E and F). Piglets were colonized on day 3, inoculated on day 7 and again on day 28. Data from SCOL piglets (A, C, and E) are plotted separately from PCOL and PGF piglets (B, D, and F) due to the large differences in Ig levels. Note that the y-axis scale for B, D, and F, is >10-fold the scale for the y-axis in A, C, and E. Each point represents an average from two piglets, and each line represents data from two separate litters (I and II). The asterisks in B, D, and F, represent the highest value for SCOL piglets if they were shown on the same plots for PCOL and PGF piglets.

Table I. Serum IgG concentrations as determined by sandwich ELISA (SW) and single radial diffusion (SRD)<sup>a</sup>

Group	Pig	Day 28		Day 35	
		SW <sup>b</sup>	SRD <sup>c</sup>	SW	SRD
PCOL	2C	16.3 ± 0.2	26.2 ± 0	21.3 ± 0.3	21.7 ± 0.8
	2D	13.4 ± 0.4	21.7 ± 0.8	10.0 ± 0.4	16.6 ± 1.3
	4B	21.0 ± 0.5	29.0 ± 2.2	26.7 ± 0.9	32.8 ± 1.1
	4C	16.1 ± 0.7	26.2 ± 0	27.8 ± 0.6	34.1 ± 3.1
Average		16.7 ± 1.8	25.8 ± 1.5	21.5 ± 8.1	26.3 ± 8.5
PGF	3C	2.3 ± 0.1	5.1 ± 0	4.2 ± 0.2	7.8 ± 0.2
	3D	6.8 ± 0.2	12.0 ± 0.1	7.8 ± 0.4	11.5 ± 0.8
	6B	12.7 ± 0.3	26.7 ± 0.9	22.0 ± 0.7	39.3 ± 1.9
	6D	11.7 ± 0.3	17.1 ± 0.8	18.5 ± 0.5	26.2 ± 0
Average		8.4 ± 4.8	15.2 ± 9.1	13.1 ± 8.5	21.2 ± 14.5

<sup>a</sup> IgG levels in SCOL animals were below detection by SRD.

<sup>b</sup> Mean concentration (mg/ml) ± SD; samples were tested in triplicate.

<sup>c</sup> Mean concentration (mg/ml) ± SD; samples were tested in triplicate.

## Results

### *PRRSV-infected piglets develop remarkably elevated levels of serum Ig*

Weekly serum Ig levels were determined as described, and results are shown in Fig. 1. Two weeks after inoculation, on day 21 of life, significantly higher levels of serum IgM, IgG, and IgA were detected in infected piglets (Fig. 1, *right panels*) compared with controls (*left panels*). The dramatic increases were not dependent on bacterial colonization, although some PCOL piglets had higher levels. Serum IgM levels peaked 3 wk after inoculation (day 28) in PCOL and PGF animals and then began to decline, whereas serum IgG and IgA progressively increased until necropsy. Three to 4 wk after inoculation (days 28 and 35), levels of serum IgM, IgG, and IgA in sham-inoculated piglets were on average only 3–10% of levels seen in PRRSV-infected animals (Fig. 1, *left vs right panels*). In a pool of normal adult swine sera, the average concentrations of IgM, IgG, and IgA were 4, 14, and 0.4 mg/ml, respectively. Thus, PCOL piglets' serum IgM levels at day 35 were on average 32% of adult levels, IgG levels were 154%, and IgA levels

were 69%. At the same time point after infection, PGF piglets' serum levels of IgM were 42% of adult levels, IgG levels were 94%, and IgA levels were 61%. Because of the unexpectedly high concentration determined by sandwich ELISA, single radial diffusion was also used to determine serum IgG concentrations. The values obtained by single radial diffusion supported the observation from sandwich ELISA, indicating the high serum IgG levels were not an artifact of the initial quantitation method (Table I).

### *Detection of PRRSV and PRRSV-specific Ab*

PRRSV was not isolated from any of the SCOL piglets (Table II). At the time of necropsy (day 35), PRRSV was isolated from the sera of all PCOL and PGF pigs, and all of these pigs had developed PRRSV-specific Abs, with a mean group IDEXX ELISA S/P ratio of 1.21 and 1.70, respectively. Depletion of Ab specific for PRRSV by IDEXX plates in five different PRRSV-infected piglets indicated that <1% of the total IgG in the sera of PRRSV-infected piglets was specific for the PRRSV Ags that were immobilized on the IDEXX plates (data not shown).

Table II. Clinical and serological history<sup>a</sup>

Group	Pig	VI <sup>b</sup>	ELISA <sup>c</sup>	Renal Lesions <sup>d</sup>	Anorexic Days <sup>e</sup>	IgG Deposits in Kidneys <sup>f</sup>	IgM/IgA Deposits in Kidneys <sup>g</sup>
SCOL	1A	–	0.00	No	0	–	ND
	1B	–	0.00	No	0	–	–
	5C	–	0.00	No	0	ND	ND
	5D	–	0.00	No	0	–	–
PGF	3C	+	1.92	Yes	10	–	+
	3D	+	0.95	Yes	12	+	ND
	6B	+	1.39	Yes	3	+	+
	6D	+	2.53	Yes	7	+/-	ND
PCOL	2C	+	0.71	Yes	13	+	ND
	2D	+	1.38	Yes	13	+	+
	4B	+	0.85	Yes	7	ND	ND
	4C	+	1.88	Yes	7	+	+

<sup>a</sup> Data from piglets infected on day 7 of life.

<sup>b</sup> Virus isolation (VI) from serum reported as positive or negative.

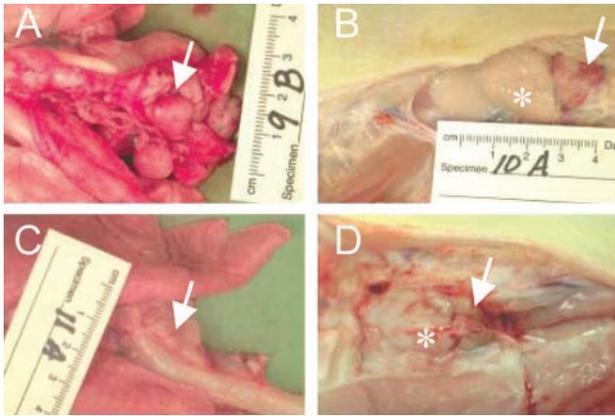
<sup>c</sup> ELISA S/P ratio detecting PRRSV-specific Ab using the IDEXX assay. Values >0.4 are considered positive.

<sup>d</sup> Lesions observed in the kidney were compatible with IC deposition.

<sup>e</sup> Number of days of anorexia observed for each pig. The time of onset for anorexia varied from 1 to 7 days postinfection in PGF and PCOL piglets.

<sup>f</sup> IgG deposits were visualized by immunohistochemistry and immunofluorescence and were localized to the glomeruli and renal blood vessels (Fig. 5) and reported as positive or negative.

<sup>g</sup> IgM and IgA deposits were visualized by immunofluorescence and were localized to glomeruli and renal blood vessels (Fig. 5) and reported as positive or negative.



**FIGURE 2.** PRRSV infection results in lymphoid hyperplasia in isolator piglets. *A* and *B*, Representative enlarged and hemorrhagic BLN and SMLN, respectively, in PRRSV-infected piglets. *C* and *D*, Representative normal BLN and SMLN, respectively, in SCOL piglets. Lymphoid tissue was examined at the time of sacrifice (day 35 of life). Arrows denote lymph nodes; asterisks denote parotid glands that were not enlarged.

#### Lymph nodes are hyperplastic in PRRSV-infected piglets

The unexpected increases in serum Ig in infected animals suggested hyperactivity of B cells. Thus, lymph nodes were examined at the time of sacrifice (day 35). Because PRRSV causes respiratory illness, the bronchial lymph nodes (BLN) were of interest since they would most likely drain the main site of infection. In all PRRSV-infected piglets, both PCOL and PGF, these lymph nodes were hemorrhagic and enlarged ~5- to 10-fold, compared with those of SCOL piglets (Fig. 2, *A* and *C*). The submandibular lymph nodes (SMLN) were also similarly affected in PCOL and PGF piglets (Fig. 2*B*). In contrast, mesenteric lymph nodes (MLN), which do not drain the main site of infection, were comparable in size and appearance between infected and uninfected animals (data not shown).

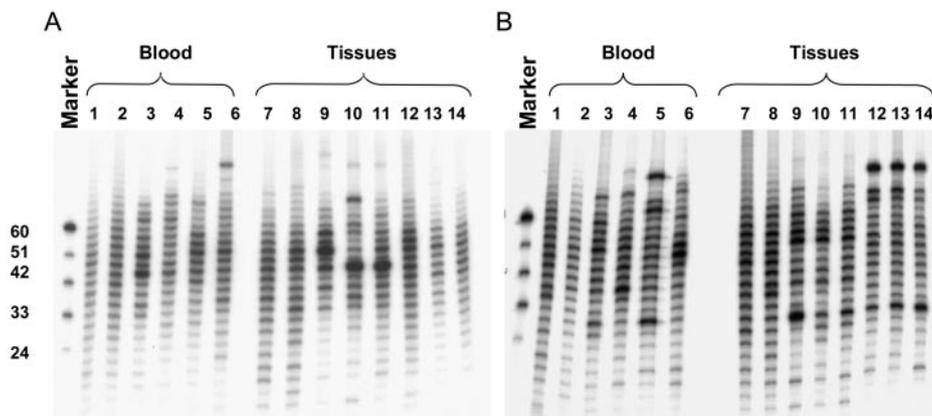
#### B cell repertoire clonality in SCOL, PCOL, and PGF piglets

It had been previously reported that both LDV and PRRSV infection lead to increased B cell numbers and serum Ig concentrations in mice (3, 4, 7, 8, 10, 11) and pigs (28), respectively. In addition, serum Ig from LDV-infected mice have been analyzed by two-

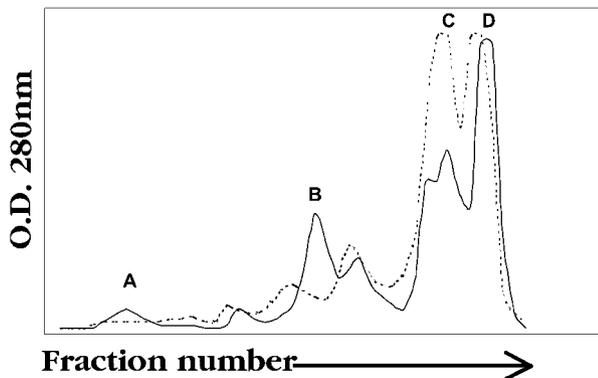
dimensional isoelectric focusing to better characterize B cell clonality (39). The authors concluded from this study that LDV infection induced polyclonal B cell activation. However, repertoire clonality of pigs infected with PRRSV has not been directly examined. Thus, to better characterize the repertoire of B cells in PRRSV-infected isolator piglets, CDR3 spectratyping was performed. Initially, we analyzed the clonality of PBLs in SCOL, PCOL, and PGF piglets. CDR3 spectratyping of PBLs isolated from SCOL piglets (Fig. 3, *A* and *B*, lanes 1 and 2) revealed a Gaussian distribution of CDR3 lengths characteristic of a polyclonal B cell population. PBLs isolated from PCOL and PGF piglets also appeared polyclonal, although CDR3s of certain lengths were favored over others, as indicated by the bands of greater intensity (Fig. 3, *A* and *B*, lanes 3–6). Because the peripheral blood B cell repertoire is composed mainly (~90%) of IgM-bearing naive B cells, we wanted to determine whether B cell clonality of various lymph nodes differs from that of peripheral blood. Results showed that B cells in the BLN and MLN of the control animals were generally polyclonal (Fig. 3, *A* and *B*, lanes 7 and 8). This was consistent for all uninfected animals. However, individual variation between lymphoid tissue B cell clonality of the PRRSV-infected animals was detected (Fig. 3, *A* and *B*, lanes 9–14) in that most of the animals showed several pronounced CDR3 lengths, whereas a few exhibited a distribution of CDR3 lengths more similar to uninfected animals. In no case was there evidence for expansion of a single B cell clone that characterizes virus-induced B cell tumors.

#### PRRSV infection is associated with high-molecular-mass IgG

Because higher levels of serum Ig were observed in PRRSV-infected piglets, we wanted to better characterize their molecular properties and distribution in serum. Analysis of sera by SE-FPLC yielded the representative protein profiles shown in Fig. 4. Monomeric IgG normally elutes in peak C, peak B contains higher molecular mass proteins, peak D is mainly albumin, and peak A contains proteins of >1000 kDa. A comparison of the serum protein profiles of a normal adult swine and a PRRSV-infected isolator piglet that had comparable serum IgG levels revealed that peak B in the infected piglet was much larger. Fractions were collected, and the IgG content in the peaks was measured by sandwich ELISA. Peak B from the PRRSV-infected piglet contained a higher proportion of IgG compared with normal adult sera (data



**FIGURE 3.** B cell clonality in blood and peripheral lymphoid tissue. Spectratyping analysis of CDR3 lengths in PBL (lanes 1–6 of *A* and *B*) and peripheral lymphoid tissue (lanes 7–14 of *A* and *B*) from SCOL, PCOL, and PGF piglets. *A*, Lanes 1–2, SCOL piglets 1A and 1B (refer to Table II); lanes 3–4, PCOL piglets 2C and 2D; lanes 5–6, PGF piglets 3C and 3D. Lanes 7–8, SCOL piglet 1B, MLN and BLN; lanes 9–11, PCOL piglet 2C, MLN, tonsil, and BLN, respectively; lanes 12–14, PGF piglet 3C, MLN, tonsil, and BLN, respectively. *B*, Lanes 1–2, SCOL piglets 5C and 5D; lanes 3–4, PCOL piglets 4B and 4C; lanes 5–6, PGF piglets 6B and 6D. Lanes 7–8, SCOL piglet 5C, MLN and BLN; lanes 9–11, PCOL piglet 4B, MLN, tonsil, and BLN, respectively; lanes 12–14, PGF piglet 6B, MLN, tonsil, and BLN, respectively. Marker indicates reference standard ladder of CDR3 lengths in base pairs.



**FIGURE 4.** Serum protein profile determined by SE-FPLC. Representative SE-FPLC protein profiles of normal adult swine serum (dotted line) and PRRSV-infected swine serum (solid line). Serum protein elution peaks are labeled A–D. Monomeric IgG normally elutes in peak C, whereas larger IC would elute in peak B.

not shown). This profile was observed particularly 3 wk after viral inoculation (day 28), but not 1 wk later (day 35). High-molecular-mass peaks were also seen in some sera samples from SCOL piglets (data not shown), although the IgG content in these peaks varied between individual piglets.

#### *Kidney lesions and IgG, IgM, and IgA deposition in PRRSV-infected piglets*

PRRSV-infected piglets (PCOL and PGF) had glomerular lesions in contrast to PRRSV-free piglets (SCOL), which did not have glomerular lesions (Table II). In PRRSV-infected pigs, ~50–80% of renal corpuscles had lesions characterized by enlarged, hypersegmented glomeruli that obliterated the urinary space (Fig. 5A). Affected glomeruli had expanded mesangial matrix and thickened basement membrane in peripheral capillary loops. In addition, there was hypertrophy of mesangial cells and endothelial cells. Some glomeruli contained a few polymorphonuclear leukocytes and a modest amount of nuclear debris. Also, there was a multifocal infiltrate of lymphocytes and plasma cells around Bowman's

capsule and scattered throughout the cortical interstitium. Deposition of IgG, IgA, and IgM was visualized in the kidneys from PRRSV-infected piglets by immunofluorescence and/or enzyme immunohistochemistry (Fig. 5, C–H; Table II). The staining was localized to the endothelium of renal blood vessels, to the basement membrane (especially IgM), and to the glomeruli. The latter gave the lumpy-bumpy pattern characteristic of complex deposition (Fig. 5, C and D, and F–H, arrows). SCOL piglets were negative for staining of IgG, IgM, and IgA in the kidneys (Fig. 5B and data not shown).

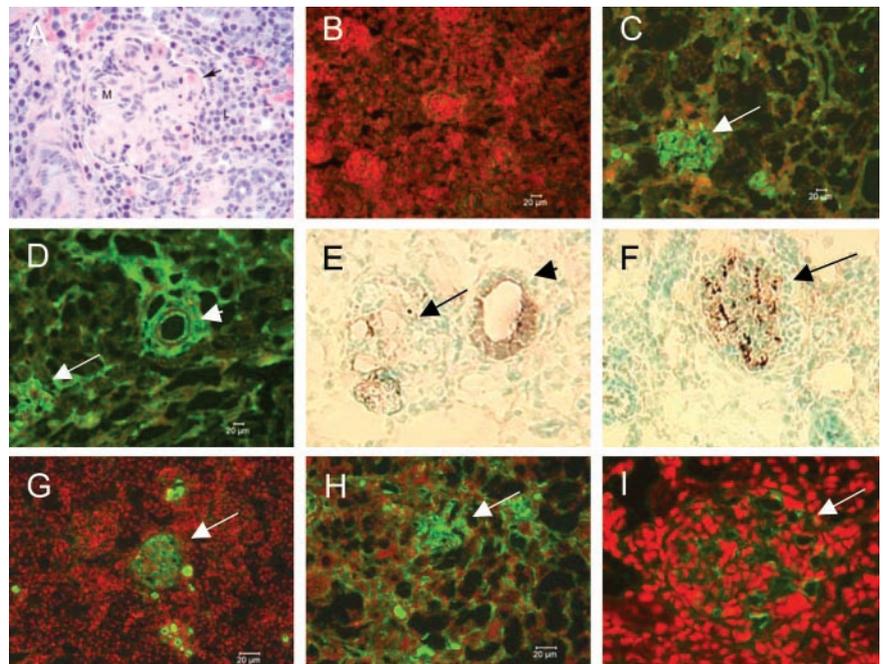
#### *Viral Ag is present in kidney glomeruli*

PRRSV nucleocapsid Ag was detected in kidney sections from PRRSV-infected animals using fluorescence immunohistochemistry. The staining was mainly localized to the glomeruli (Fig. 5I), with lesser staining in the renal blood vessels (data not shown). In contrast to the staining of IgG, IgM, and IgA in the renal blood vessels, the nucleocapsid staining pattern was not homogeneous and resembled IC deposition. SCOL animals were negative for nucleocapsid staining (data not shown).

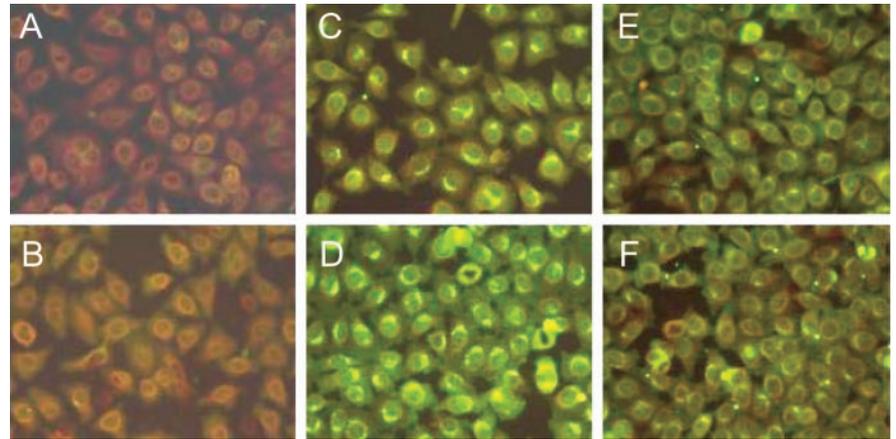
#### *Autoantibodies against multiple autoantigens are induced in PRRSV-infected piglets*

IgG anti-Golgi and anti-nuclear staining were obtained using sera from PRRSV-infected piglets (Fig. 6, C–F). The observed unilateral perinuclear staining is characteristic of anti-Golgi apparatus reactivity. The nuclear staining was seen as a speckled pattern, which is characteristic of autoantibodies directed against nuclear proteins and ribonuclear proteins (40). Despite variations in intensity of reactivity, all of the infected piglets (PCOL and PGF) tested positive by immunofluorescent ANA, whereas the sham-inoculated (SCOL) piglets were negative (Fig. 6, A and B). To rule out that positive staining was due to high IgG concentrations, PCOL and PGF sera samples were titrated and those from infected piglets remained positive, whereas no positive results were obtained with SCOL piglets, including those having IgG concentrations equal to infected piglets. Interestingly, a difference in the kinetics of autoantibody development between PCOL and PGF animals was observed. Positive immunofluorescent staining was first detected in

**FIGURE 5.** Kidney pathology associated with PRRSV infection. A, Typical renal corpuscle from a PRRSV-infected piglet. The glomerulus is enlarged and hypersegmented. The mesangial matrix (M) is expanded, and the basement membrane of the peripheral capillary loops is thickened (arrow). Periglomerular and interstitial infiltrate of lymphocytes and plasma cells are apparent (L). H&E staining,  $\times 400$ . IgG deposition in kidneys of sham (B) and infected (C and D) piglets was visualized by immunofluorescence. IgA (G), IgM (H), and PRRSV nucleocapsid protein (I; enlarged image of glomerulus) were also visualized in infected kidney sections by immunofluorescence. Nuclei were counterstained with propidium iodide (red), and Igs/Ag were detected with an Alexa-labeled Ab (green). IgG deposition was also visualized by enzyme immunohistochemistry (E and F) in the kidneys of infected piglets. Nuclei were counterstained with methyl green. Igs/Ag are localized to the renal blood vessels (arrowhead) and the glomeruli (arrow). B–D and G and H,  $\times 200$ . E and F,  $\times 160$ . Alexa fluorescence (green) was visualized with a constant exposure time.



**FIGURE 6.** Anti-nuclear and anti-Golgi apparatus IgG detected by immunofluorescent ANA. *A* and *B*, SCOL sera tested on Hep-2 cells. *C* and *D*, PCOL sera tested on Hep-2 cells. *E* and *F*, PGF sera tested on Hep-2 cells. Data were obtained from sera collected 3 wk after inoculation (day 28 of life).



PCOL animals 2 wk after inoculation (day 21), but in PGF animals 1 wk later (day 28). Autoantibodies reactive against nuclear and Golgi Ags were transient in both groups and began to diminish within 2 wk of their appearance.

Because molecular mimicry has been suggested to explain anti-Golgi activity, sera samples were also tested for the presence of autoantibodies specific for dsDNA. By ELISA, all PRRSV-infected piglets' sera at days 28 and 35 had detectable IgG anti-dsDNA (Table III). None of the uninfected piglets had detectable Abs against dsDNA.

## Discussion

The extraordinary increases in serum Ig we observed (Fig. 1) were accompanied by lymphoid hyperplasia that was especially apparent in the BLN and SMLN. Serum Ig data would suggest that B cells are responsible. In addition to lymph node pathology, infected piglets exhibited kidney abnormalities, including IgG, IgA, and IgM in glomeruli, which are characteristic of IC deposition. Because viral Ag was also deposited in the glomeruli (Fig. 5*I*), IC could merely be those formed with virus. However, the vascular endothelia and basement membrane stained brightly for all three isotypes, suggesting autoantibodies. The latter is consistent with our detection of autoantibodies specific for cytoplasmic and nuclear autoantigens in the sera of infected piglets. Collectively,

these data indicate a serious immune dysregulation following PRRSV infection.

Although higher IgG levels were detected by single radial diffusion than by sandwich ELISA, both methods show that the IgG levels were elevated 30- to 50-fold 3–4 wk after PRRSV inoculation compared with uninfected control piglets. Because there are six to eight IgG subclasses in swine (41), differences in specificity bias between the capture Ab used for sandwich and that used for single radial diffusion may explain the disparity apparent in Table I. Although there was a tendency for PCOL piglets to have higher IgG levels than PGF piglets (Table I), it was not universal because IgG levels in some PGF piglets' sera were equivalent to or higher than those of PCOL piglets.

Unlike previous data on PRRSV-induced elevation of Ig levels, which was believed to be polyclonal (28), we directly demonstrate this by spectratypic analysis. Our data rule out virus-induced lymphoma as a cause of the unexpectedly high Ig levels. That B cell clonality in the examined lymphoid tissues reflected that of blood is not surprising considering the dependence of lymphocytes on blood vessels to enter and exit the inverted lymph node of the pig (42). Because CDR3s could only be amplified from the tonsils of PRRSV-infected animals, it suggests that this organ appears to act as a secondary lymphoid tissue equivalent to a lymph node. Nevertheless, the spectratype patterns of PBLs and lymphoid tissues revealed the expansion of certain clones (Fig. 3, *A* and *B*, lanes 3–6 and 9–14). This pattern was seen in both colonized and non-colonized animals infected with PRRSV (PCOL and PGF), suggesting that the stimulation of certain B cell clones was not dependent on bacterial Ags. These clones could therefore be those specific for viral Ags. However, we found that a very small proportion of the serum IgG in PRRSV-infected animals could be accounted for as virus specific. This agrees with the polyclonal nature of the spectratype and data recently presented for LCMV (13). One might wonder why the serological IDEXX is positive, whereas only trace amounts of IgG were depleted. However, 1% is still 100–200  $\mu$ g of Ab, and ELISA-based assays detect picogram to nanogram Ab levels. Furthermore, the Ag source used (IDEXX plates) is proprietary and may capture only 10% of the total PRRSV-specific Ab in serum. Assuming this scenario is correct, it still means that <10% of the total serum IgG in PRRSV-infected piglets is virus specific.

Although the effect of PRRSV on specific leukocyte populations remains unclear, our data indicate that B cells ultimately become a target. Viral superantigens interacting with T or B cells could potentially cause the lymphoid hyperactivation we observed (43, 44), but PRRSV has not been shown to express any T or B cell

Table III. Detection of IgG anti-dsDNA<sup>a</sup>

Group	Pig	IU/ml <sup>b</sup>	
		28	35
SCOL	1A	190	69
	1B	26	37
	5C	59	73
	5D	58	48
PCOL	2C	409	457
	2D	312	685
	4B	510	216
	4C	417	422
PGF	3C	391	387
	3D	590	556
	6B	323	307
	6D	424	318

<sup>a</sup> Sera from days 28 and days 35 were assayed using the INOVA QUANTA Lite dsDNA ELISA system.

<sup>b</sup> Samples were run in duplicate, and the average OD at 405/620 nm was used to calculate values. Values of 0–200 IU/ml were considered negative; 201–300, equivocal; 301–800, moderately positive; and  $\geq$ 801, strongly positive.

superantigens. Furthermore, activation by most T and B cell superantigens is characterized by clonal anergy or a short burst of clonal proliferation followed by deletion. Whether this occurred in the PRRSV-infected piglets was not determined. Although T cell superantigens are common in various viral infections, B cell superantigens have been less well characterized. However, staphylococcal protein A targets the framework region of all B cell receptors encoded by the  $V_H3$  family in mice, humans, and swine (Ref. 44; G. J. Silverman, unpublished observations), and all porcine B cell receptors are encoded by  $V_H3$  (45). Thus a direct viral-B cell interaction resulting in B cell hyperplasia cannot be ruled out. Alternatively, the polyclonal B cell activation seen during PRRSV infection may result from costimulation by macrophages, T cells, or cytokines produced by these leukocytes.

Circulating IC at a low frequency can be effectively cleared by erythrocytes via binding to complement receptors and ultimately removed from circulation by phagocytic cells in the liver and spleen (46). However, during certain disease courses, circulating IC are produced in higher amounts, which overwhelms the innate clearing mechanism and results in their deposition in tissues and organs (14, 46). The kidney can be especially affected by increased IgG deposition and subsequent inflammatory cell infiltration. The SE-FPLC data were suggestive of circulating IC, which was consistent with IgG, IgA, and IgM deposition in the glomeruli of PRRSV-infected piglets (Fig. 5; Table II). The detection of PRRSV nucleocapsid protein in the glomeruli (Fig. 5I) suggests that some of these circulating IC may contain viral Ags. However, IgG, IgA, and IgM were also readily detected in the renal blood vessel endothelia and on the basement membrane in an evenly distributed pattern. This is characteristic of uniform autoantibody activity, not IC deposition. It is known that a small percentage of autoreactive B cells make it into the periphery despite negative selection in the bone marrow (47). However, these do not normally proliferate and differentiate in healthy individuals, but could do so under the influence of factors that promote indiscriminate B cell activation. Therefore, the autoantibodies to Golgi, dsDNA, and apparently basement membrane and vascular endothelium are most likely the result of this indiscriminate B cell activation. Previously, a correlation between PRRSV infection and a necrotizing vasculitis/nephropathy syndrome was reported in 12 conventional pigs (15). Unfortunately, the conventional nature of the animals examined by these investigators cannot exclude other environmental factors as the cause of this syndrome. In any case, the investigators found that blood vessels in the upper dermis of affected piglets stained positively for Igs, particularly IgM, which they concluded might be involved in the development of vasculitis. Similarly, we observed intense uniform IgM staining around renal blood vessels. If the vasculitis/nephropathy observed previously was a result of PRRSV infection, it supports the view that our results are not artifacts of the isolator model, and that kidney pathology is a direct result of virus-induced dysregulation.

Molecular mimicry between viral and cellular Ags was previously implicated in the production of autoantibodies during arboviral infections of mice, especially those specific for the Golgi apparatus (12). However, when considering the number of RNA, DNA, and retroviruses that induce autoantibodies, it seems unlikely that they would all express Ags that mimic identical cellular Ags. Recently, it was demonstrated that PRRSV-infected cells *in vitro* undergo an unusual cell death that exhibits characteristics of both apoptosis and necrosis (48). Interestingly, it has been shown that when cells undergo cell death, cleavage of Golgi complex proteins (golgins) occurs, which leads to the production of specific antigenic fragments (49). It was hypothesized that during injury or infection, cells would be undergoing unregulated cell death that

could lead to the release of immunostimulatory Golgi autoantigens into the system. Thus, the anti-Golgi Abs we report need not be a consequence of molecular mimicry but rather the activation of autoreactive T and/or B cells by excessive release of self-Ag. In addition, if the anti-Golgi Abs result only from molecular mimicry during PRRSV infection, then Abs to other self-Ag should not be present. In this study, we also demonstrated autoantibodies to dsDNA and apparently to basement membrane and vascular endothelia in infected piglets (Table III; Fig. 5). Thus, it is unlikely that the anti-Golgi activity (Fig. 6) results exclusively from molecular mimicry. The exposure of various intracellular autoantigens to the immune system in conjunction with viral danger signals that activate cells via Toll-like receptors (50–55) could be responsible for the resultant autoantibody formation. Recently, it was reported that Toll-like receptor 9 ligands were able to deliver signal 2 to autoreactive B cells receiving signal 1 via their cognate Ag (56). Thus, it was proposed that other Toll-like receptors might also be capable of inducing autoimmunity by similar means (57).

The choice of the isolator piglet model for studies on immunological development was discussed in the introduction. However, traditional veterinary immunologists/virologists have questioned its application to PRRSV research because conventional piglets are colonized by commensal bacteria and receive maternal Ig via colostrum. The former may stimulate development of the neonatal immune system (18) and perhaps permit some protective immunity to PRRSV, whereas the latter, on average, raises serum IgM, IgG, and IgA levels to 3, 30, and 15 mg/ml, respectively. If able to recognize PRRSV, they could reduce the striking effect we observed. Because maternal Ig can also suppress *de novo* Ig synthesis (20), the PRRSV effect we describe here could be masked in conventional animals. However, there are at least five arguments against viewing our results as either 1) system artifacts or 2) irrelevant to natural PRRSV infections. First, colonized piglets in our study (PCOL) had the same level of immune pathology as noncolonized (PGF) piglets. Second, conventional animals are exposed to numerous commensal and pathogenic microorganisms, so it would be difficult to pinpoint the immune pathology as exclusively a result of infection with PRRSV. Third, the elevated Ig levels we report could have been overlooked in studies on conventional animals in which large increases could be masked by passive maternal Ig, which in extreme cases can result in 5, 40, and 25 mg/ml IgM, IgG, and IgA, respectively (20). In fact, a previous report showed extremely high IgG levels in PRRSV-infected piglets, although the authors did not comment on this result (58). Fourth, PRRSV-associated lymphoproliferation and lymphoid hyperplasia (29, 59) and even kidney disease and IC (15) have been reported in less well-controlled studies in both specific pathogen-free and conventional piglets. Fifth, we have preliminary data that demonstrates that conventionally reared piglets infected with PRRSV also exhibit hypergammaglobulinemia and autoantibody production (60). Therefore, we do not believe that our isolator piglet data represent artifacts or are irrelevant to the situation in conventional animals. Rather, we take the position that, by reducing the number of variables and controlling the bacterial and viral exposure of isolator piglets, our results can more accurately measure the direct effect of PRRSV (or any other virus that targets swine) on the piglet's immune system.

Many human and murine viruses induce polyclonal B cell activation, and subsequent IC and autoantibody formation during the course of their infections (1–13). We have demonstrated that similar phenomena accompany PRRSV infection in isolator piglets and argue that these are not artifacts of the model. Because mice infected with LDV exhibit a similar pathology, our data suggest

that such a response pattern is characteristic of arteriviral infections in their natural hosts. Thus, PRRSV can be added to a growing list of viruses and other infectious agents that induce immune dysregulation and can be used as a model to identify the cellular and molecular basis of virus-induced immune dysregulation.

## Acknowledgments

We thank Lisa Horning and David Bohlken from the University of Iowa Hospitals and Clinics Immunopathology Laboratory for their assistance with the autoantibody assays, as well as H. Stuart Henrichs from Immunocore and Brett Heil from INOVA for providing the HEp-2 fluorescent ANA test system and QUANTA Lite dsDNA ELISA kits, respectively. We especially acknowledge the National Pork Board and the National Science Foundation for their support of this basic research on the development of the porcine immune system and agents that may alter normal development.

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