Antigen-Specific B-Cell Responses to Porcine Reproductive and Respiratory Syndrome Virus Infection

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Porcine reproductive and respiratory syndrome virus (PRRSV) causes an acute, viremic infection of 4 to 6 weeks, followed by a persistent infection lasting for several months. We characterized antibody and B-cell responses to viral proteins in acute and persistent infection to better understand the immunological basis of the prolonged infection. The humoral immune response to PRRSV was robust overall and varied among individual viral proteins, with the important exception of a delayed and relatively weak response to envelope glycoprotein 5 (GP5). Memory B cells were in secondary lymphoid organs, not in bone marrow or Peyer’s patches, in contrast to the case for many mammalian species. Potent anti-PRRSV memory responses were elicited to recall antigen in vitro, even though a second infection did not increase the B-cell response in vivo, suggesting that productive reinfection does not occur in vivo. Antibody titers to several viral proteins decline over time, even though abundant antigen is known to be present in lymphoid tissues, possibly indicating ineffective antigen presentation. The appearance of antibodies to GP5 is delayed relative to the resolution of viremia, suggesting that anti-GP5 antibodies are not crucial for resolving viremia. Lastly, viral infection had no immunosuppressive effect on the humoral response to a second, unrelated antigen. Taking these data together, the active effector and memory B-cell responses to PRRSV are robust, and over time the humoral immune response to PRRSV is effective. However, the delayed response against GP5 early in infection may contribute to the prolonged acute infection and the establishment of persistence.
MATERIALS AND METHODS

Animal procedures and housing. Conventional cross-bred commercial pigs of various ages that were negative for PRRSV by IDEXX ELISA were used as follows.

Twenty-two weaned pigs, 2 to 3 weeks of age, were maintained at the Livestock Infectious Disease Isolation Facility, Iowa State University, Ames, IA. Animals were assigned to four treatment groups at random and observed daily. Rectal temperatures and clinical scores were recorded every other day. After 1 week of acclimatization, four pigs were inoculated intramuscularly with 2 ml of 10^5 TCID_50 tissue culture infectious doses (TCID_50) of the VR2332 isolate of PRRSV, four pigs were inoculated intramuscularly with keyhole limpet hemocyanin (KLH) emulsion, 12 pigs were inoculated with both PRRSV and KLH, and two pigs were mock inoculated with 2 ml of saline diluent (PRRSV control) and incomplete Freund's adjuvant (KLH control). KLH emulsions were prepared by mixing equal volumes of KLH (2 mg in 1 ml saline; Sigma Chemical Co., St. Louis, MO) and Freund's incomplete adjuvant (Sigma Chemical Co.) in Micromate inter-changeable syringes (Popper and Sons Inc., New Hyde Park, NY). Animals were immunized intramuscularly in the ham and boosted on day 14 with 50 µg of KLH emulsion.

The design was replicated one time for a total of 44 animals. Whole blood was collected in EDTA tubes and serum samples in clot tubes at weekly intervals. At 27 and 120 days after infection, animals were sacrificed and samples of spleen, tonsil, inguinal lymph node (ILN), sternal lymph node (SLN), and femur were collected. Isolation Facility, Iowa State University, Ames, IA. Animals were assigned to treatment groups at random and, after 1 week, were inoculated intramuscularly with 2 ml of 10^5 TCID_50 per ml of PRRSV strain VR2332. Both PRRSV-infected and uninfected animals were sacrificed at postinoculation days 98, 112, 147, 175, and 205 for the isolation of mononuclear cells (MNCs) from various lymphoid tissues.

To further analyze B-cell responses in lymphoid tissues, 6- to 8-week-old PRRSV naive pigs were housed at the Swine Disease Eradication Center (University of Minnesota) research farm. Animals were assigned to treatment and control groups at random. Animals were inoculated intranasally with 2 ml of cell culture fluid containing 3 x 10^5 TCID_50 of PRRSV (MN-30100 field isolate) alone, with strain MN-30100 at day 0 and a boost of 2 ml of Ingelvac PRRS MLV vaccine (Boehringer Ingelheim Vetmedica) on day 7, or with 2 ml of saline diluent only. Animals were sacrificed to isolate MNCs from various lymphoid tissues at 37 days after the first infection.

Pigs at 4 weeks and 2 years of age were maintained at Vet Resources, Ames, IA. After acclimatization, animals were infected with the virulent PRRSV strain JAI42 or were given diluent alone. Pigs were sacrificed 63 days after infection, and hemagglutination inhibition (HAI) and SLN and SLN tissues were harvested for fluorescence-activated cell sorter analysis.

All of the experiments were performed under IACUC-approved animal protocols at the University of Minnesota or Iowa State University.

Viruses and viral quantitation. Strains of PRRSV used, doses, and routes of inoculation used in each experiment are described separately in Results. Virulent PRRSV isolates VR2332, MN-30100, and JAI42 and the vaccine Ingelvac PRRS MLV were used in this study. A litered stock of PRRSV strain MN-30100 (5 x 10^5 TCID_50) was used to generate a quantitative one-step real-time reverse transcriptase PCR (qRT-PCR) standard curve for use as a positive control. RNA was extracted from 200 µl of serum using a Nucleospin II RNA isolation kit (BD Biosciences, San Jose, CA), eluted in 50 µl of RNase-free water, and stored at −80°C. For qRT-PCR assays, samples were dried in a Savant SpeedVac (GMI Inc., Ramsey, MN) and resuspended in 5 µl of RNase-free water. Primers and a dual-labeled 5-carboxyfluorescein-6-carboxytetramethylrhodamine probe were designed from the orf 7-3 untranslated region of PRRSV (58). PRRSV RNA reverse transcription and PCR were performed on infected samples, a water-only control, and primers and probe were expressed and purified using a modification of the Qiagen Ni-nitrilotriacetic acid agarose affinity column purification method for denatured protein isolation. Denatured proteins were dialyzed overnight in 0.1 M Tris-HCl, pH 8.0, 6 M guanidine HCl, 2 mM EDTA at 4°C and were concentrated to a concentration of 3 mg/ml. Dithiothreitol was added to 300 mM, and the solution was filtered through a 0.45-µm membrane. Reduced protein was added into refolding buffer, filtered (0.22 µm), and stirred overnight at 4°C. The purified protein was concentrated by tangential flow filtration (Pellicon XL UltraceL PLC 5 KD; Millipore) and dialyzed against 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. Protein concentrations were analyzed by Bradford assays (Bio-Rad, Hercules, CA). Purified protein solutions were stored at −80°C. For studying memory B-cell responses, a cocktail containing equal amounts of nsp2, N, and GP3 was pre-fractionated overnight and stored at −80°C.

ELISPOT assay for antigen-specific ASC. ELISPOT assays were performed on the day of MNC isolation to enumerate antigen-specific plasma cells actively secreting antibodies in vivo. Briefly, nitrocellulose-based 96-well microtiter plates (Millipore, Bedford, MA) were prewetted with 70% ethanol for 60 s and were washed three times with sterile water. The wells were coated with 500 ng/well of recombinant KLH, nsp2, N, or GP3 polypeptide separately to measure antigen-specific B-cell responses. After overnight incubation at 4°C, wells were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) for 1 h at 37°C. Plates were washed three times with PBS, and MNCs were plated at three fivefold dilutions in duplicate wells, starting with 5 x 10^5 cells/well. After incubation for 12 to 16 h at 37°C in 5% CO2 incubator, ELISPOT plates were washed three times with PBS and four times with PBS containing 0.05% Tween 20 (20 Fisher Scientific, Pittsburg, PA). To each well we added 100 µl of horseradish peroxidase-labeled affinity-purified goat anti-pig antibody specific for immunoglobulin G (IgG) (KPL, Gaithersburg, MD, and Bethyl Laboratories, Montgomery, TX), IgM, or IgA (both from Bethyl Laboratories) diluted 1:200 in PBS-Tween and containing 1% BSA for 2 h at 37°C. Plates were washed three times in PBS and developed with 3-amino-9-ethylcarbazole (Sigma Chemical Co.). Spot patterns were analyzed using the immunospot image analyzer (Cellular Technology, Cleveland, OH) in the laboratory of Bernhard Herling, Department of Surgery, University of Minnesota. Data are presented as the mean numbers of SPFC per 10^5 MNCs from duplicate wells of each sample. The assay was performed as described here for all samples, including analysis at three fivefold dilutions of the cell number so that at least one dilution was within the counting range of the image analyzer.

Isolation of MNCs from blood and lymphoid tissues. Animals were sacrificed by intravenous administration of Beuthanasia-D solution (Schering Plough Animal Health, Union, NJ). All of the tissue and cell culture reagents were obtained from Mediatech except as noted. Blood samples were collected in EDTA tubes (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ). Spleen, tonsil, ILN, SLN, and femur samples were collected in RPMI 1640 containing 10 µg/ml of gentamicin (GIBCO BRL, Grand Island, NY). MNCs from spleen, tonsil, ILN, and SLN samples were isolated by mechanical disruption using 80-mesh screens in a cell strainer (Ceciliter, St. Petersburg, FL). Bone marrow cells were isolated by being flushed with sterile phosphate-buffered saline (PBS). Peripheral blood MNCs (PBMC) were isolated by density gradient centrifugation of blood diluted 1:2 with sterile PBS on lymphocyte separation medium (ICN Biomedicals, Aurora, OH). PBMC and isolated cells from other lymphoid tissues were filtered through 70-µm cell strainers (BD Falcon, Bedford, MA), washed with PBS, and freed of red blood cells by hypotonic water lysis. Cells were resuspended in RPMI complete medium containing 5% fetal bovine serum (Sigma Chemical Co.), 2 mM l-glutamine, 0.02 mM nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 U), streptomycin (100 µg/ml) (GIBCO BRL), 50 mM HEPES, pH 7.2, and gentamicin (20 µg/ml) (GIBCO BRL). Cell viability was confirmed by trypan blue exclusion, and cells were counted using a hemocytometer.

Antigens used for ELISA and enzyme-linked immunosorbent (ELISPOT) assays. Recombinant PRRSV nucleocapsid (N), envelope glycoprotein 5 ectodomain (GP5 5′ total), envelope glycoprotein 5 endodomain (GP5 3′), and nonstructural protein 2 (nsp2) proteins and polypeptides were prepared by PCR amplification and cloning from cDNA of PRRSV strain VR2332 (4, 31). The GP5 ectodomain containing amino acids 1-396 was expressed and purified as a recombinant nsp2, N, or GP3 was pre-fractionated overnight and stored at −80°C. For studying memory B-cell responses, a cocktail containing equal amounts of nsp2, N, and GP3 was pre-fractionated overnight and stored at −80°C.

ELISPOT assay for antigen-specific ASC. ELISPOT assays were performed on the day of MNC isolation to enumerate antigen-specific plasma cells actively secreting antibodies in vivo. Briefly, nitrocellulose-based 96-well microtiter plates (Millipore, Bedford, MA) were prewetted with 70% ethanol for 60 s and were washed three times with sterile water. The wells were coated with 500 ng/well of recombinant KLH, nsp2, N, or GP3 polypeptide separately to measure antigen-specific B-cell responses. After overnight incubation at 4°C, wells were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) for 1 h at 37°C. Plates were washed three times with PBS, and MNCs were plated at three fivefold dilutions in duplicate wells, starting with 5 x 10^5 cells/well. After incubation for 12 to 16 h at 37°C in a 5% CO2 incubator, ELISPOT plates were washed three times with PBS and four times with PBS containing 0.05% Tween 20 (20 Fisher Scientific, Pittsburg, PA). To each well we added 100 µl of horseradish peroxidase-labeled affinity-purified goat anti-pig antibody specific for immunoglobulin G (IgG) (KPL, Gaithersburg, MD, and Bethyl Laboratories, Montgomery, TX), IgM, or IgA (both from Bethyl Laboratories) diluted 1:200 in PBS-Tween and containing 1% BSA for 2 h at 37°C. Plates were washed three times in PBS and developed with 3-amino-9-ethylcarbazole (Sigma Chemical Co.). Spot patterns were analyzed using the immunospot image analyzer (Cellular Technology, Cleveland, OH) in the laboratory of Bernhard Herling, Department of Surgery, University of Minnesota.
RESULTS

Characterization of the humoral antibody response to PRRSV infection. We characterized the humoral response to PRRSV by determining specific IgM and IgG antibody responses in serum to individual structural and nonstructural proteins of PRRSV. We compared these results to the antibody response to KLH, a common protein antigen, administered in the presence or absence of viral infection. The IgM response to KLH was present at day 3 (the earliest time point examined), peaked at day 7, and declined thereafter (Fig. 1A). The pattern of response to KLH was the same irrespective of concurrent PRRSV infection. A similar IgM response was observed to PRRSV nsp2 and N; both showed a peak at day 7 and a decline to low levels by day 21 (Fig. 1B and C). In striking contrast, the IgM response to GP5 endodomain epitopes (GP5 3') was inapparent; IgM antibodies were not significantly increased at any time point up to 56 days (Fig. 1D). To determine if an IgM response to another region of GP5 was present, an ELISA was performed with GP5 ectodomains (GP5 5') total epitopes was delayed by 2 weeks, first being detected at 21 days after infection and peaking at day 35 (Fig. 1I and J). Anti-GP5 levels were maintained over time on average, but in the case of the ectodomain epitopes (GP5 5' total) there was marked variation among animals, as indicated by the large error bars.

The delayed IgG response to PRRSV proteins was not due to low-level or delayed production of viral antigens. A high level of viremia was present 3 days after infection and remained high for about 2 weeks (Fig. 2). Individual animals resolved the acute, viremic infection by 21 to 28 days of infection. Thus, viral antigens were actively produced for an extended period of 3 to 4 weeks, during which the onset of antiviral IgM responses varied from 3 days (nsp2) to no apparent response (GP5 3').

Overall, PRRSV infection had no substantial effect on the antibody response to an unrelated antigen, KLH, and the presence of KLH antigen had no effect on anti-PRRSV responses. In all cases, only infected or immunized animals responded, demonstrating that all of the humoral responses were antigen specific. Although PRRSV is reported to induce polyclonal B-cell activation and hypergammaglobulinemia (39, 41, 54), we observed no significant difference in anti-KLH antibody levels in the presence or absence of PRRSV infection (Fig. 1F).

Distribution of PRRSV- and KLH-specific plasma cells in acute and persistent infection. The persistent infection of lymphoid tissues by PRRSV suggests that antigenic stimulation of B-cell differentiation to actively secreting plasma cells is occurring for an extended period of time. However, anti-PRRSV antibody levels consistently declined over time, whereas anti-KLH antibody levels continued to increase for more than 60 days after the boost (Fig. 1G to J). Since serum antibody levels are determined by actively secreting plasma cells, we analyzed the distribution of plasma cells in various lymphoid tissues near the end of acute infection (27 days) and in persistent infection (120 days) (Fig. 3). In uninfected or unimmunized animals, antigen-specific plasma cells were not observed at any time (data not shown). Because the frequencies of antigen-specific IgM and IgA isotypes were low or undetectable following infection, further studies examined only IgG-secreting cells.

At the end of acute infection (27 days), the frequency of IgG plasma cell responses to all PRRSV antigens was highest in SLN, in the range of 100 to 200 ASC per million MNCs, for nsp2 and N (Fig. 3A). Slightly lower frequencies were observed in spleen, followed by tonsil and superficial ILN. ASC for the GP5 3' endodomain were present at substantially lower levels in all tissues, consistent with the lower levels of anti-GP5 3' endodomain antibody titers in serum at 27 days after infection. The presence of KLH did not affect the anti-PRRSV antigen response. IgG ASC specific for KLH were predominantly localized to spleen (~200 IgG ASC/10^6 cells) and tonsil (~150 to 200 IgG ASC/10^6 cells), followed by superficial levels in ILN and SLN. The presence of PRRSV infection had no effect on the plasma cell response to KLH. Interestingly, few plasma cells specific for either PRRSV proteins or KLH were detected in bone marrow under any treatment condition.

At 120 days, far into the persistent phase of infection, the frequencies of plasma cells specific for PRRSV antigens were low, in the range of 10 to 40 ASC/10^6 cells, and without differences in tissue distribution (Fig. 3B). At this time, the num-

Memory B-cell response assay. Memory B-cell responses were assessed by in vitro stimulation of MNCs with recombinant PRRSV antigens and mitogens for 4 days in cell culture, followed by measuring antigen-specific, actively secreting B cells. The duration of antigen stimulation and the dose of antigen were optimized as previously described and were based on the culture conditions that yielded the greatest number of ASC in the ELISPOT assay (19). MNCs were plated in 24-well culture plates at 2 x 10^6 cells/well in 2 ml of RPMI complete medium supplemented with phytohemagglutinin (2.5 μg/ml Sigma Chemical Co.), pokeweed mitogen (2.5 μg/ml; Sigma Chemical Co.), and 1.67 μg/ml each of nsp2, N, and GP5 3'. Cells from each lymphoid tissue from PRRSV-infected and uninfected pigs were cultured in 12- or 24-well plates at 37°C for 4 days in a 5% CO2 incubator. On the second, third, and fourth days of incubation, 0.5 ml of RPMI complete medium was added to each well. On the fifth day, cells were collected, washed three times with PBS, resuspended in RPMI complete medium, counted, and plated in antigen-coated ELISPOT plates using three fivefold dilutions in duplicate wells, starting with 5 x 10^9 cells/well as described above. Memory B-cell responses were quantified based on the number of spots obtained in response to restimulation.

Protein-specific ELISA. Microwell plates were coated overnight with 100 ng/well of KLH, nsp2, or N or 200 ng/well of either GP5 5' total or GP5 3' in carbonate buffer (pH 9.6). Plates were washed and blocked with 5% nonfat dry milk (pH 9.6) in PBS containing 0.05% Tween 20 for 1 h. Serum samples were diluted 1:1,000, and 100 μl of each sample was applied to duplicate wells on ELISA plates (Corning, Acton, MA) for 1.5 h. Plates were washed and incubated for 1 h with horseradish peroxidase-conjugated goat-anti-pig antibody, either anti-IgG at 1:1,500 dilution (Bethyl Laboratories), anti-IgG at 1:5,000 (KPL), or anti-IgM at 1:1,500 dilution (Bethyl Laboratories). Plates were washed, and color development was performed with 100 μl of tetramethylbenzidine substrate (KPL). Reactions were stopped with 100 μl of 1 M phosphoric acid, and plates were read at 450 nm on a Molecular Devices ThermoMax plate reader (Sunnyvale, CA).

Statistical analysis. Statistical analysis was performed using nonparametric one-way analysis of variance in GraphPad Prism software (San Diego, CA). Multiple pair-wise comparisons were performed using MacAnova (http://www.stat.unm.edu/macanova/).
The numbers of plasma cells secreting anti-GP5 3' endodomain were equal to the numbers of cells secreting anti-nsp2 and anti-N. The numbers of anti-KLH plasma cells also were low, though they still were more frequently observed in spleen and tonsil than in other lymphoid tissues. Bone marrow showed no plasma cells specific for either PRRSV proteins or KLH at 120 days. Altogether, antigen-specific plasma cells exhibited different patterns of tissue distribution in KLH immunization and
PRRSV infection. PRRSV-specific cells were predominantly localized to SLN draining the lungs, which is the primary site of acute infection. KLH-secreting cells were predominantly localized to spleen, which filters systemic circulation, and tonsil, a B-cell-rich tissue that samples oral antigens.

To further characterize antibody and B-cell responses over time, results from several experiments involving the challenge of pigs with various PRRSV strains were pooled. Pooling was justified because the immune response of pigs to individual viral proteins is equivalent and not substantially affected by the strain of virus, even between attenuated and virulent strains (31, 32). We were able to determine the frequencies of PRRSV-specific plasma cells in lymphoid tissues from near the end of acute infection (27 days) and at days 37, 67, 98, 112, 120, 147, 175, and 203 after infection. Three animals infected with the VR2332 isolate of PRRSV were sacrificed at 27 days, six animals infected with MN-30100 were sacrificed at each of 37 and 67 days, and three animals infected with VR2332 were sacrificed at each time point from 98 days and thereafter. A peak abundance of plasma cells specific for all three viral proteins was observed at 37 days after infection and was predominantly localized in SLN and spleen (Fig. 4A). The response in tonsil consistently peaked at the 67-day time point at a level equivalent to the response in SLN at the same time. The strongest response was to nsp2, followed by that to N and then the GP5 3’ endodomain. At the time of peak response, about 0.1% of total mononuclear leukocytes were actively secreting antibodies to these three viral proteins. These data also con-

FIG. 2. Viremia levels in pigs infected with VR2332 with (n = 12) or without (n = 4) KLH immunization and boosting at 14 days. Viral levels were determined by qRT-PCR as described in Materials and Methods. Data from the two groups were pooled after no significant differences were found and are presented as box-and-whisker plots, with the horizontal bar representing the mean, boxes at the 75th percentile, and the whiskers extended to the minimum and maximum values.

FIG. 3. Effect of PRRSV infection and KLH immunization on antigen-specific plasma cell responses. Two-week-old pigs were mock inoculated or were inoculated intramuscularly with 10^5 TCID_{50} of VR2332, 2 mg KLH, or both, as indicated in the treatment legend. Antigen-specific plasma cell responses to KLH, nsp2, N, and GP5 3’ at (A) 27 days postinfection and (B) 120 days postinfection were determined by ELISPOT assay. BM, bone marrow.
firm that bone marrow is not a site of plasma cell residence (<20/10^6 MNCs).

Characterization of memory B-cell responses. In vitro recall responses were measured in MNCs obtained from different lymphoid tissues at different times after PRRSV infection to assess antigen-specific memory B-cell responses. Anti-PRRSV memory B-cell responses already were high when first measured at 37 days after infection, remained high in the case of anti-nsp2- and anti-N-secreting cells for about 100 days, and declined but remained readily detectable at 200 days (Fig. 4B). Anti-GP5 3’ endodomain memory cells were present at about one-quarter to one-third of the peak level of anti-nsp2- and anti-N-specific memory cells but did not decline; they were maintained at the same frequencies for 200 days. All of the lymphoid tissues contained anti-PRRSV memory B cells at substantially higher (threefold or greater) levels than plasma cells at all time points up to 200 days.

At 37 days of PRRSV infection, spleen and tonsil had equivalent frequencies of anti-PRRSV memory B cells. At later time points the frequencies were higher in tonsil and lower in spleen, particularly for anti-nsp2 and anti-N memory B cells (Fig. 4B). As we noted previously, bone marrow was not a site of residence for memory B-cell differentiation. Multiple pairwise comparisons of kinetics of tissue distribution of memory B-cell responses revealed no difference between SLN and tonsil at the peak of antibody response at 37 days, whereas memory B cells were predominantly localized to tonsil in persistent PRRSV infection (Table 1). Except as indicated, tonsil samples had a significantly higher number of memory B cells than other lymphoid tissues in persistent PRRSV infection (P < 0.05). This indicates that, unlike actively secreting plasma cells, memory B cells were predominantly localized to tonsil in persistent PRRSV infection, perhaps because of the higher viral loads in tonsil tissue in persistent PRRSV infection (9, 17, 40, 56, 64, 67).

A second exposure to PRRSV does not alter plasma cell or memory B-cell responses. Reexposure of an animal to an antigen, i.e., boosting, typically induces a large increase in antibody response. To better understand B-cell and anti-PRRSV-specific plasma cell responses of pigs upon reexposure to PRRSV, we determined the effect of inoculation with PRRSV MN-30100 alone or MN-30100 plus, 7 days later, Ingelvac MLV vaccine. Responses were assessed at 37 days after the first exposure. In animals that received MN-30100 alone, the strongest antigen-specific IgG-secreting plasma cell response was to nsp2, with approximately twice as many cells than responded to N and three to four times more cells than responded to GP5 3’ (Fig. 5A). A second exposure to PRRSV Ingelvac MLV had no effect on antigen-specific plasma cell numbers in any of the tissues examined. Plasma cells specific for each of the three viral proteins were significantly more abundant in SLN than in other cells and tissues, including spleen (P < 0.05). The frequencies in spleen, in turn, were significantly higher than those in PBMC, tonsil, ILN, and bone marrow (P < 0.05). Thus, spleen and lymph nodes draining the lung appear to be the major sites of anti-PRRSV antibody production, with circulating lymphocytes, tonsil, and ILN playing minor roles and bone marrow playing no role at all. These results were antigen specific, since uninfected controls showed no antigen-specific actively secreting plasma cell response (data not shown).

Memory B-cell responses to recall antigen were two to three times higher than plasma cell responses, with the nsp2 response being the strongest, followed by N and GP5 3’ endodomain responses (Fig. 5B). A second exposure to PRRSV had no effect in any of the tissues examined (Fig. 5B). Tonsil was equivalent to SLN and spleen in anti-PRRSV memory B-cell responses, in contrast to its minor role in actively secreting plasma cell production. Memory B cells were uncommon in blood and were at extremely low levels in bone marrow. Recall

FIG. 4. Kinetics of antigen-specific B-cell responses in various lymphoid tissues in PRRSV infection. An ELISPOT assay was used to determine the kinetics of (A) antigen-specific plasma cell responses and (B) memory B-cell responses to nsp2, N, and GP5 3’. For plasma cell assays, three animals infected with the VR2332 isolate of PRRSV were sacrificed at day 27, six animals infected with MN-30100 were sacrificed at each of days 37 and 67, and three animals infected with VR2332 were sacrificed at each time point from day 98 on. Memory cell responses were performed on days 37, 98, and at each time point thereafter. BM, bone marrow.
The decline in the number of antiviral plasma cells was secondary to a generalized reduction in B-cell frequencies in lymphoid tissues as pigs age, total IgG-secreting cells were absent or present at very low frequencies in bone marrow of young pigs. Cells could not be isolated from bone marrow of adult pigs.

Antigen-specific and nonspecific B-cell responses in Peyer’s patches. Since B-cell responses in bone marrow, the site of long-term plasma and memory B-cell production in some mammalian species (34, 59), were nil, and Peyer’s patches are reported to be the principal site of B-cell differentiation in the pig (3, 14, 15), we examined anti-PRRSV and total primary and memory B-cell responses in both the jejunal and ileal Peyer’s patches. Five-week-old conventional pigs were inoculated with PRRSV MN-30100 on day 0 and were reinoculated with Ingelvac MLV on days 7, 37, and 67. Animals were sacrificed 97 days after the initial infection, and Peyer’s patch lymphoid tissues were present in both jejunal and ileal Peyer’s patches.

Since tissue differences in PRRSV-specific B-cell frequencies might be due to a global effect of viral infection on B-cell differentiation or survival, we enumerated the frequencies of IgM+ cells in spleen, tonsil, SLN, superficial ILN, and bone marrow of pigs that were infected at 4 weeks or 2 years of age with PRRSV strain JA142. As shown in Fig. 8, the frequencies of IgM+ cells was not affected by PRRSV infection in tissue of young or adult pigs. B-cell frequencies in spleen showed substantial individual variation among animals regardless of age; there was a tendency for lower frequencies in young pigs infected with PRRSV, but there was no such tendency in adult pigs. Young pigs also demonstrated more variation in B-cell frequencies in other tissues compared to those in tissues of adult pigs, with the variation being skewed to higher frequencies in young pigs.

Table 1 shows the analysis of tissue distribution of memory B-cell responses in PRRSV infection. Mean values that are not significantly different from those for tonsil samples in persistent infection are in boldface.

**Table 1. Analysis of tissue distribution of memory B-cell responses in PRRSV infection**

<table>
<thead>
<tr>
<th>Protein and day</th>
<th>PBMC</th>
<th>Spleen</th>
<th>Tonsil</th>
<th>Superficial ILN</th>
<th>SLN</th>
<th>Bone marrow</th>
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<td>nsp2 37</td>
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<sup>a</sup>Means in the same horizontal row and not sharing the same superscript are significantly different from each other. Multiple pairwise comparisons were performed using MacAnova. The mean numbers of memory B cells/10<sup>6</sup> MNC from three animals at each time point in each tissue were computed. Superscripts indicating significance (P < 0.05) were ranked in order (A > B > C > D). Mean values that are not significantly different from those for tonsil samples in persistent infection are in boldface.

DISCUSSION

In an effective mammalian immune response, the B-cell response to antigen involves the specific activation and proliferation of naïve B cells in the draining lymph nodes and spleen, resulting in the generation of plasma cells and memory B cells in a matter of days (20). Plasma cells and memory B cells were enumerated in PBMC, spleen, SLN, superficial ILN, tonsil, and bone marrow of pigs at 7, 12, and 20 weeks of age. As shown in Fig. 7, the proportion of in vivo ASC increased steadily over time in all tissues examined, with the highest numbers of responding cells in spleen and tonsil and the lowest numbers in bone marrow and blood (PBMC).

Since tissue differences in PRRSV-specific B-cell frequencies might be due to a global effect of viral infection on B-cell differentiation or survival, we enumerated the frequencies of IgM+ cells in spleen, tonsil, SLN, superficial ILN, and bone marrow of pigs that were infected at 4 weeks or 2 years of age with PRRSV strain JA142. As shown in Fig. 8, the frequencies of IgM+ cells was not affected by PRRSV infection in tissue of young or adult pigs. B-cell frequencies in spleen showed substantial individual variation among animals regardless of age; there was a tendency for lower frequencies in young pigs infected with PRRSV, but there was no such tendency in adult pigs. Young pigs also demonstrated more variation in B-cell frequencies in other tissues compared to those in tissues of adult pigs, with the variation being skewed to higher frequencies in all tissues of young pigs. IgM+ cells were absent or present at very low frequencies in bone marrow of young pigs. Cells could not be isolated from bone marrow of adult pigs.
migrate to the sites of infection and to bone marrow, which is the major site of sustained antibody production and memory B-cell residence (44, 60). Antigen-specific plasma cells secrete IgM or IgG into the circulation or release dimeric IgA adjacent to the mucosal epithelium to generate antiviral responses (13, 49). The porcine B-cell response conforms to this model with regard to viral infections caused by influenza and foot-and-mouth disease viruses (1, 12). Specifically, active infection with peak viremia occurring within 24 to 72 h is followed by rapid diminution of viral load in the circulation and clearance of

![Graph A](image1.png)

**FIG. 5.** Effect of a second dose of PRRSV in vivo on (A) antigen-specific plasma cell responses and (B) memory B cell responses in various lymphoid tissues at 37 days after initial infection. Six- to 8-week-old PRRSV-naive pigs were inoculated intranasally with 2 ml of 3 × 10⁴ TCID₅₀/ml of PRRSV MN-30100 (white circles). Another group of pigs was infected with MN-30100 on day 0 and boosted with 2 ml of Ingelvac PRRS MLV on day 7 (black circles). An ELISPOT assay was used to measure antigen-specific responses to nsp2, N, and GP5 3’. Each circle represents the response of an individual animal. The horizontal bar is the mean value of all data points within a group. BM, bone marrow.

![Graph B](image2.png)

**FIG. 6.** Anti-PRRSV and total B-cell responses in Peyer’s patches. Pigs were inoculated with PRRSV strain MN-30100 and reinoculated with Ingelvac MLV on days 7, 37, and 67 after primary infection. Animals were sacrificed 97 days after primary infection. Jejunal (circles) and ileal (triangles) Peyer’s patches from three animals were dissected. MNCs were isolated and cultured to determine IgG-ASC, IgM-ASC, and IgA-ASC for PRRSV nsp2, N, and GP5 3’, respectively, as well as total IgG-ASC and IgA-ASC. Each symbol is the mean response from one animal. Cultures of ileal Peyer’s patch cells from one animal became contaminated and were discarded.
virus by 4 to 7 days. The reduction and resolution of viral infection is associated with the appearance of antiviral antibodies (1, 12, 22). These previous studies demonstrated that swine possess the full repertoire of innate and adaptive immune responses to rapidly respond to and resist infections by naturally occurring viral pathogens. However, the porcine response to PRRSV deviates from this model in its dynamics, lymphoid tissue distribution, dissociation from viral clearance in blood, and absence of a boosting response.

Delayed response to PRRSV protein GP5. The IgM response to the viral proteins nsp2 and N following infection with PRRSV occurs rapidly, transiently, and before any appearance of IgG. However, no IgM response to the endodomain fragment of GP5 (GP5 3′/H11032) was detected, and the response to the GP5 ectodomain (GP5 5′/H11032 total) peaked at 28 to 35 days, when viremia essentially was resolved. The IgG response to both domains also was delayed relative to that of other viral proteins. Since serum samples were tested three times under a variety of conditions for anti-GP5 3′ endodomain IgM, it appears that either transcription or translation is inefficient, secretion of the antibodies is at a very low level, or that anti-GP5 3′ endodomain IgM is unusually sensitive to degradation or elimination from the blood. GP5 3′ also might be poorly im-

FIG. 7. Effect of age on IgG-secreting plasma cell numbers in blood and lymphoid tissues. MNCs from different lymphoid tissues from conventional healthy pigs were isolated and assayed with an ELISPOT assay to measure total IgG B-cell responses. BM, bone marrow.

FIG. 8. Effect of PRRSV infection on IgM-positive B-cell frequencies in lymphoid tissues. Pigs at 4 weeks of age (young) and 2 years of age (adult) were infected with PRRSV strain JA142. Lymphoid tissue samples were obtained at sacrifice on day 63 of infection. Isolated cells were analyzed by a fluorescence-activated cell sorter for IgM-positive B-cell frequencies from three to five tissues from five pigs per group. MNCs could not be isolated from adult bone marrow. The gray line is the isotype-matched control. BM, bone marrow.
munogenic, though antigenicity and hydrophilicity plots suggest the region is antigenic (data not shown). Masking epitopes in the GP5 5’ total ectodomain, which have been hypothesized to account for a delayed appearance of neutralizing antibodies to PRRSV, do not explain our results, since a neutralizing function was not required for the detection of anti-GP5 antibody responses (51). Neutralizing antibodies are believed to be directed to conserved sequences in the GP5 ectodomain (24, 25, 27, 28, 43, 52, 53, 55, 56, 61, 62, 65, 66). Our results indicate that anti-GP5 antibodies do not play a significant role in the resolution of viremia and suggest that their delayed appearance contributes to the establishment of persistent infection.

The atypical response to GP5 is particularly interesting, since viral infection did not alter the response to an unrelated antigen, KLH, or (apparently) to other viral proteins, as evidenced by N and nsp2. The more rapid kinetics of the IgG response to KLH may reflect the immediate presence of a high antigen load rather than a gradual accumulation of viral antigen through the spread of acute infection. However, viral load as measured by viremia was maximal at 2 to 7 days after infection, so other factors must be involved in the lag to peak antibody production, which occurs at about 4 weeks after infection with PRRSV and even longer after infection in the case of the anti-GP5 response.

Declining humoral response despite antigen persistence. A hallmark of PRRSV infection is the persistence of infectious virus and viral antigens in lymphoid tissues, particularly the tonsil, for 150 or more days (5, 7, 11, 17, 30, 56, 63). Nevertheless, the cell-mediated immune response to PRRSV declines over time (67). In this study, the plasma cell response to nsp2, N, and GP5 also decreases, and at 120 days it was lower than the plasma cell response to KLH. PRRSV cytolytic infection destroys macrophage and dendritic cells, but Xiao et al. (67) and Samsom et al. (57) showed that macrophage cell numbers as a whole in lung and lymphoid tissues are not affected by PRRSV infection. Infection might suppress macrophage or dendritic cell presentation of antigen. While little evidence supports this hypothesis, more expression studies of the molecules involved in antigen presentation processes may address the question.

The decline of anti-PRRSV antibody levels and plasma cell frequencies indicates a nonsignificant level of stimulation rather than a lack of responsive cells, as demonstrated by the presence of a large reservoir of B cells able to respond to recall antigen in lymphoid tissues. Figure 9, redrawn from data depicted in Fig. 5, shows that memory cells are abundantly distributed in lymphoid tissues at 37 days after infection (the MN-30100 plus in vitro restimulation treatment group), whereas plasma cell frequencies are 3- to 10-fold lower and are not increased by a booster inoculation with a second viral strain 7 days after primary infection (MN-30100 group + MLV in vivo). Thus, in sum, neither PRRSV persistence nor a second infection at 7 days, prior to the establishment of specific immunity, facilitates a robust humoral antibody response. The decline in plasma cell numbers after the peak at 27 days also suggests that viral replication resulting in cytolytic infection is low during this period. Lysis of infected cells would release nonstructural and structural proteins into extracellular space for recognition by PRRSV-antigen-receptor-bearing B cells and presumably activate renewed antibody production, as was demonstrated in vitro.

In vitro recall response without enhanced response to second infection in vivo. An interesting feature of PRRSV humoral immunity is the absence of an anamnestic response (29), even though recall responses in vitro to PRRSV antigens are present from early times of infection and are more than twofold higher than plasma cell responses. The lack of an in vivo anamnestic response may be due to the depletion of permissive macrophages that renders the host resistant to reinfection (although evidence of macrophage resistance to infection is lacking [67]) or to an absence of antigen that occurs when re-exposure fails to result in reinfection (29). In this study, reexposure to virus on day 7, before antigen-specific immunity is observed, did not enhance ASC numbers in vivo, whereas the memory response to recall antigen in vitro was quite strong in tonsil, spleen, SLN, and ILN (Fig. 9). Thus, other mechanisms of immune resistance appear to prevent antigen production following reinfection so that more PRRSV-specific plasma cells are not elicited.

Previous attempts to assess an immunosuppressive effect of PRRSV infection on porcine disease resistance have not demonstrated convincing proof that PRRSV exacerbates other disease conditions, nor have they provided a mechanistic model of immunosuppression (reviewed in references 26 and 47). Here, we show that PRRSV infection had no effect on the humoral antibody and B-cell response to a second, specific, independent antigen. In addition, viral infection did not reduce B-cell frequencies in lymphoid tissues.

Regionalization of plasma and memory cell responses. In acute PRRSV infection, the viral load is high in a variety of lymphoid tissues (56, 67), but antigen-specific plasma cells are predominantly localized to SLN and spleen at the peak of the antibody response (Fig. 5). A prominent role for the SLN is
expected because it drains the lungs, the primary site of acute infection. The minor role of ILN was surprising, since gross enlargement of the superficial ILN, often discernible to the naked eye, is a hallmark pathological sign of PRRS. PRRSV-specific B cells also were abundant in spleen, which may be the most important source of plasma and memory cells due to its large size and intimate contact with the circulation. Tonsil was not significant as a PRRSV-specific plasma cell reservoir but was equivalent to SLN and spleen for memory B cells specific for all PRRSV proteins. Overall, these findings indicate that spleen and SLN are the major effector and surveillance sites containing actively secreting plasma cells and memory cells. In contrast, tonsil is a site for surveillance but not effector function, since it contains memory cells but not plasma cells. The ILN shows B-cell characteristics similar to those of tonsil but is not a principal site of persistent infection.

In persistent PRRSV infection, both plasma cells and memory B cells exhibited a different pattern of anatomical localization than was present at the peak of antibody responses. Antigen-specific plasma cell responses declined dramatically in all of the lymphoid tissues, but memory B cells showed no significant change. The decline in plasma cells might be attributed to the migration from SLN and spleen to sites of infection or to disappearance through apoptosis. Unlike plasma cell responses, memory B cells remained at high levels up to 200 days after infection. Interestingly, memory B cells were predominantly localized to tonsil in persistent PRRSV infection, consistent with higher viral loads (67).

Because bone marrow is regarded as the major site of long-lived memory B cell production and residence (44, 60), the paucity of PRRSV-specific plasma cells and memory B cells in bone marrow was particularly interesting. In rotavirus infection, bone marrow also was not the primary site of antibody production or the site of residence of memory B cells until 83 days after infection (68). The failure of ASC and memory B cells to migrate to the bone marrow could be due to a species-specific lack of expression of homing molecules on the responding B cells. Alternatively, the ileal Peyer’s patches, not bone marrow, may be the primary site of B-cell differentiation in swine (10, 15). We observed that ileal and jejunal Peyer’s patches have high frequencies of ASC, as shown in Fig. 8, but the frequencies of PRRSV-specific B cells was unremarkable. Therefore, it appears that PRRSV-specific long-lived plasma cell and memory B-cell maturation occurs not in generalized sites of B-cell differentiation but in secondary lymphoid tissues that drain sites of PRRSV infection, especially SLN draining the lung (23, 67), and tonsil, the primary site of persistent PRRSV infection (17, 40, 63, 67). Consistent with this interpretation is the observation that the tissue distribution of PRRSV-specific ASC from SLN and spleen in acute infection to tonsil and spleen in persistent infection.

In conclusion, PRRSV infection induces a significant and specific antibody and B-cell response to a variety of PRRSV proteins without affecting humoral antibody or B-cell responses to an irrelevant protein antigen, KLH. Thus, viral infection did not suppress the immune response to a second immunological challenge. The predominant localization of IgG plasma cells to SLN and spleen suggests that they are the primary sites of effector antibody production in acute infection. In persistent infection, memory B cells were predominantly localized to tonsil, the primary site of viral persistence. Since viral antigen is widespread in lymphoid tissues (67), the striking localization of anti-PRRSV memory B cells in tonsils indicates that mechanisms of specific B-cell differentiation and growth are different here than in other lymphoid tissues. The IgM response to GP5 is total, the putative neutralization determinant, was notably deficient, which might contribute to viral persistence. Memory B-cell responses generated in vitro were more than twofold higher than plasma cell responses and were maintained for 200 days. Durable recall responses are consistent with prolonged periods of protective immunity beyond 600 days (36). A lack of anamnestic responses in the presence of resistance to reinfection suggests that viral antigens do not encounter memory B cells in sufficient amounts to trigger their activation and proliferation.

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