Cytokine Profiles in Peripheral Blood Mononuclear Cells and Lymph Node Cells from Piglets Infected In Utero with Porcine Reproductive and Respiratory Syndrome Virus

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The aim of the present study was to investigate at 2, 4, and 6 weeks after birth cytokine expression by peripheral blood mononuclear cells and bronchial lymph node cells from piglets infected in utero with porcine reproductive and respiratory syndrome virus (PRRSV). Technically, by flow cytometry we were able to measure gamma interferon (γ-IFN), tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), and IL-8 levels. In general, we found increases in the percentages of IL-4-, γ-IFN-, and TNF-α-producing lymphocytes in the infected piglets compared to the percentages in the uninfected control animals, while there was a decrease in the percentage of IL-8-producing monocytes. We believe that these findings reflect a general lymphocyte activation stage that is created due to the infection and that occurs in combination with impairment of the monocyte function, possibly due to the ongoing viral replication in these cells. Single-cell bronchial lymph node preparations exhibited very much the same cytokine profiles as peripheral blood mononuclear cells except for a lack of IL-8 production. When the levels of the individual cytokines in the three groups of PRRSV-infected piglets were compared, the levels of cytokine expression at 4 weeks diverged from those at 2 and 6 weeks, in that there was a significant decrease in the numbers of lymphocytes producing γ-IFN and TNF-α. This tendency was also observed among blood monocytes and lymph node macrophages. Possible reasons for this temporary immunosuppression in the piglets at 4 weeks are discussed.

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the family *Arteriviridae*, a family of positive-stranded RNA viruses (17). Like the other members of the arteriviruses, PRRSV replicates preferentially in cells of the monocyte/macrophage lineage (30). Since macrophages are known to be important in initiating immune responses, ongoing viral replication in these cells may cause immune dysfunctions (11, 18, 22), as observed at several levels. Infected macrophages themselves are compromised in their phagocytosis capacity and in their capacity to generate oxidative metabolites (10, 20, 26) and produce reduced amounts of tumor necrosis factor alpha (TNF-α) in vitro (10, 16, 27, 28). In vivo cytokine studies with lung lavage fluid from PRRSV-infected pigs did find some interleukin-1 (IL-1) but only small amounts of alpha interferon (α-IFN) and no TNF-α (29). PRRSV-infected animals may show a transient diminished T-cell immunity (2) as well as an increase in the rate of secondary infections (12, 31). Certain infected animals may show late or no viral clearance (2, 3, 6).

The aim of this study was to investigate single-cell cytokine production in leukocytes from piglets infected with PRRSV in utero. Since the flow cytometric technique requires the use of monoclonal antibodies (MAbs) reacting with porcine cytokines, in recent years we have been defining useful antibodies (21). That technique allows us to measure porcine IL-4, IL-8, γ-IFN, and TNF-α levels, which is the sole reason for limiting our study to these cytokines.

MATERIALS AND METHODS

Animals. Four healthy, pregnant sows were transferred to the animal isolation units at the Danish Veterinary Institute. On day 90 of gestation, two of the sows (sows 97 and 208) were challenged intranasally with 1 ml of the sixth cell culture passage of a Danish PRRSV isolate at a titer of 10^7 50% tissue culture infective doses/ml (7, 19). The two sows gave birth to 23 piglets. Examination of precolostral serum samples from the piglets for PRRSV was carried out with porcine pulmonary alveolar macrophages as described previously (7), and viremia was observed in all 23 piglets. Eight piglets from sow 97 still tested PRRSV positive 2 weeks after birth, at which time they were euthanized. The 14 piglets from sow 208 were divided into two groups. Eight piglets were euthanized 4 weeks after birth. Five of these were found to be positive for PRRSV and three were negative. The remaining six piglets from sow 208 were euthanized 6 weeks after birth, and none of these were found to contain PRRSV in their sera.

The other two sows (sows 199 and 303) were sham inoculated intranasally with sterile tissue culture medium. Sow 199 gave birth to 12 piglets, and sow 303 gave birth to 13 piglets. Precolostral serum samples from all 25 piglets were found to be negative for PRRSV, and all animals stayed negative throughout the experiment. Seven piglets from sow 303 were euthanized 2 weeks after birth. Four piglets from sow 199 and six piglets from sow 303 were euthanized 4 weeks after birth, and the remaining eight piglets from sow 199 were euthanized 6 weeks after birth. The noninfected piglets were used as negative control animals in the study.

Chemicals. All chemicals used in this study, including cell culture media, were of the best quality and to our knowledge nonpyrogenic.

Preparation of PBMCs. Heparinized blood samples from the 2-, 4-, and 6-week old piglets were diluted twice in phosphate-buffered saline (PBS; pH 7.3) and centrifuged through a Ficoll-Paque density gradient (specific gravity, 1.077; Pharmacia Biotech, Uppsala, Sweden) at $1,200 \times g$ for 15 min at room temperature. The interphase cells (peripheral blood mononuclear cells [PBMCs]) were washed twice in PBS (5 min at $925 \times g$) and were immediately counted and cultured.

Preparation of bronchial lymph node cells. Bronchial lymph nodes were surgically removed immediately after the animals were euthanized and kept in PBS supplemented with 10 mM EDTA. Single-cell suspensions were made by dicing the tissue and straining the specimens through a stainless steel mesh. Clumps and cellular aggregates were removed by filtering the specimens through a 100-μm-pore-size filter (Filtron N 100; Dako, Glostrup, Denmark).

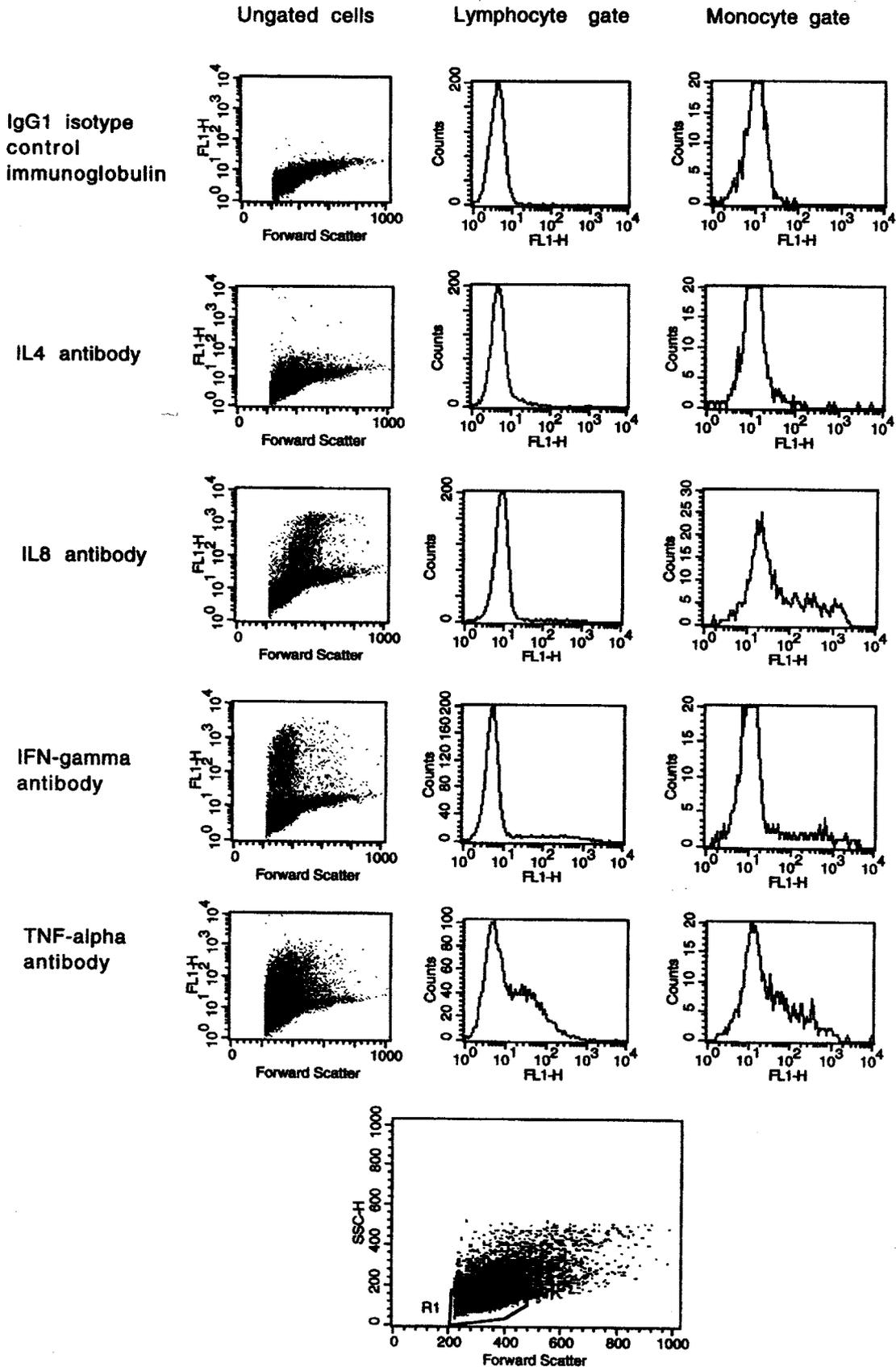* Corresponding author. Mailing address: Laboratory of Virology and Immunology, Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Stigbojlen 7, DK-1870 Frederiksberg C, Copenhagen, Denmark. Phone: 45 35282727. Fax: 45 35282742. E-mail: bas@kvl.dk.

FIG. 1. IL-4, IL-8, γ-IFN, and TNF-α antibody staining profiles obtained by flow cytometry with Ficoll-purified PBMCs from a PRRSV-infected piglet. The staining profile for a nonreactive IgG1 isotype control MAb is also included.
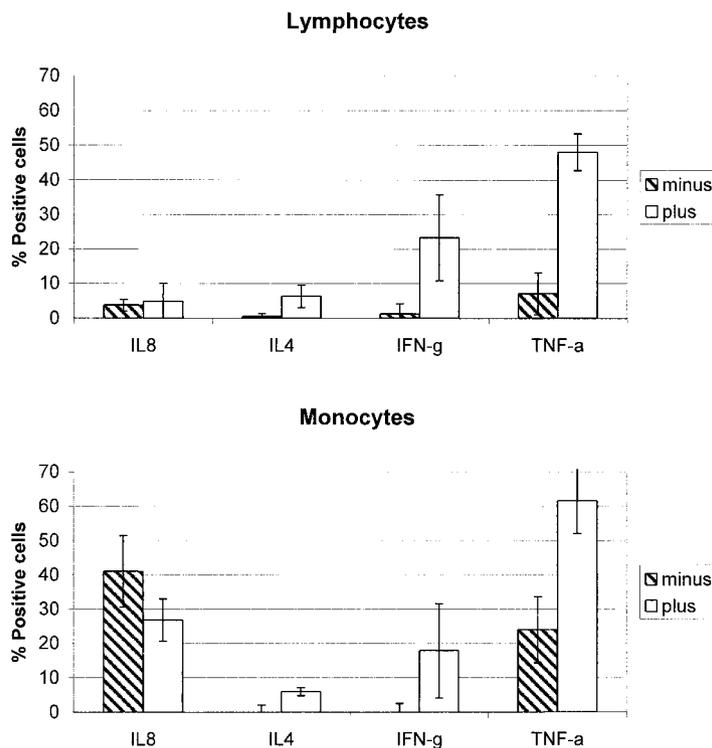
FIG. 2. Percentages of IL-8-, IL-4-, γ-IFN-, and TNF-α-positive lymphocytes and monocytes in 4-h cell cultures to which PMA and ionomycin were (□) or were not (▧) added.

Cell culture. PBMCs and lymph node cells were cultured (2×10^6/ml) at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 medium (Gibco-BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Gibco-BRL), 1 mM sodium pyruvate, 200 IU of penicillin per ml, and 200 μg of streptomycin (Gibco-BRL) per ml in the presence of 10 μg of brefeldin A (Sigma, St. Louis, Mo.) per ml, 1 μg of ionomycin (Sigma) per ml, and 20 ng of phorbol-12-myristate-13-acetate (PMA; Sigma) per ml. After 4 h, the cells were washed twice in PBS (5 min at 925 $\times$ g) and the cell pellet was resuspended in 5 ml of freshly prepared 4% (wt/vol in PBS) paraformaldehyde for 5 min, followed by addition of 2 ml of 1% bovine serum albumin (BSA; fraction V; Sigma) in PBS and centrifugation at 2,000 $\times$ g for 10 min. The cells were then suspended in the buffer of 1% BSA in PBS mentioned above and kept at 4°C for up to 3 days.

Cytokine detection by flow cytometry. On the day of testing the cells were washed twice (5 min at 925 $\times$ g) with PBS and 0.1% (wt/vol) saponin (Sigma) (PBS-S) supplemented with 1% plasma from the animal under investigation. These fixed and saponin-treated cells were then transferred to U-bottom microtiter plates (0.2×10^6 to 1×10^6 cells in a volume of 50 μl), and the plates were incubated for 1 h at room temperature with 500 ng of MAbs. We used the following four MAbs (21): a cross-reactive anti-ovine IL-8-specific MAb (Serotec MCA 1660; immunoglobulin G2a [IgG2a] isotype), a cross-reactive anti-bovine IL-4-specific MAb (Serotec MCA 1820; IgG2a isotype), a cross-reactive anti-bovine γ-IFN-specific MAb (Serotec MCA 1783; IgG1 isotype), and an anti-porcine TNF-α-specific MAb (Endogen MP-390; IgG1 isotype). Nonreactive MAbs of the IgG1 isotype (DAK-G01; Dako) and the IgG2a isotype (DAK-G05; Dako) were used to stain the isotype controls. The cells were then washed twice with PBS-S and incubated with 500 ng of a fluorescein isothiocyanate-conjugated F(ab')$_2$ fragment of a rabbit anti-mouse immunoglobulin (F313; Dako). The cells were then washed once in PBS-S and once in FACS sheath fluid (J. T. Baker, Deventer, The Netherlands) and analyzed with a Calibur flow cytometer (Becton Dickinson, San Jose, Calif.). The results presented in this study were based on lymphocyte gating on a forward scatter-versus-side scatter diagram (see region 1 [R1] in Fig. 1). All cells outside the lymphocyte gate (region 1) are named and regarded as monocytes (named macrophages in the lymph node preparations). Fluorescent histogram markers were defined and quantitated positively from negatively stained cells. The percentage of cytokine-positive cells was calculated after subtraction of eventual positive signals from the isotype control immunoglobulin preparations. The values for all animals in a group were plotted and

were found to be normally distributed. Student's t test was used for statistical evaluations.

RESULTS

Examples of single-cell cytokine staining profiles. Figure 1 illustrates the antibody staining profiles obtained by flow cytometry for IL-4, IL-8, γ-IFN, and TNF-α with Ficoll-purified PBMCs isolated from a PRRSV-infected piglet. Figure 1 also shows the staining profile for a nonreactive IgG1 isotype control MAb. Identical (negative) staining with nonreactive IgG2a (the isotype control) was found (data not shown). It is evident from Fig. 1 that the IL-4 signals are considerably weaker than the signals found for the other cytokines that were stained. The few IL-4-positive cells primarily fall in the lymphocyte gate. It should also be noted that the IL-8-positive cells almost exclusively belong to the monocyte cell population, while cells positive for γ-IFN and TNF-α fall in both gated cell populations.

Cytokine production with and without addition of PMA and ionomycin to cell cultures. In order to investigate the effects of the addition of cellular stimulants (PMA and ionomycin) on the number of cytokine-producing cells, blood was drawn from 4-week-old healthy piglets (four animals) and the PBMCs were isolated. Four-hour cell cultures were set up with and without the addition of PMA and ionomycin. The cellular protein export pathway was blocked with the drug brefeldin A in all cell cultures. After fixation and membrane disruption, the cells were stained for the cytokines IL-4, IL-8, γ-IFN, and TNF-α, as mentioned above for Fig. 1. Figure 2 presents the percentage of cytokine-positive lymphocytes and monocytes. Signifi-
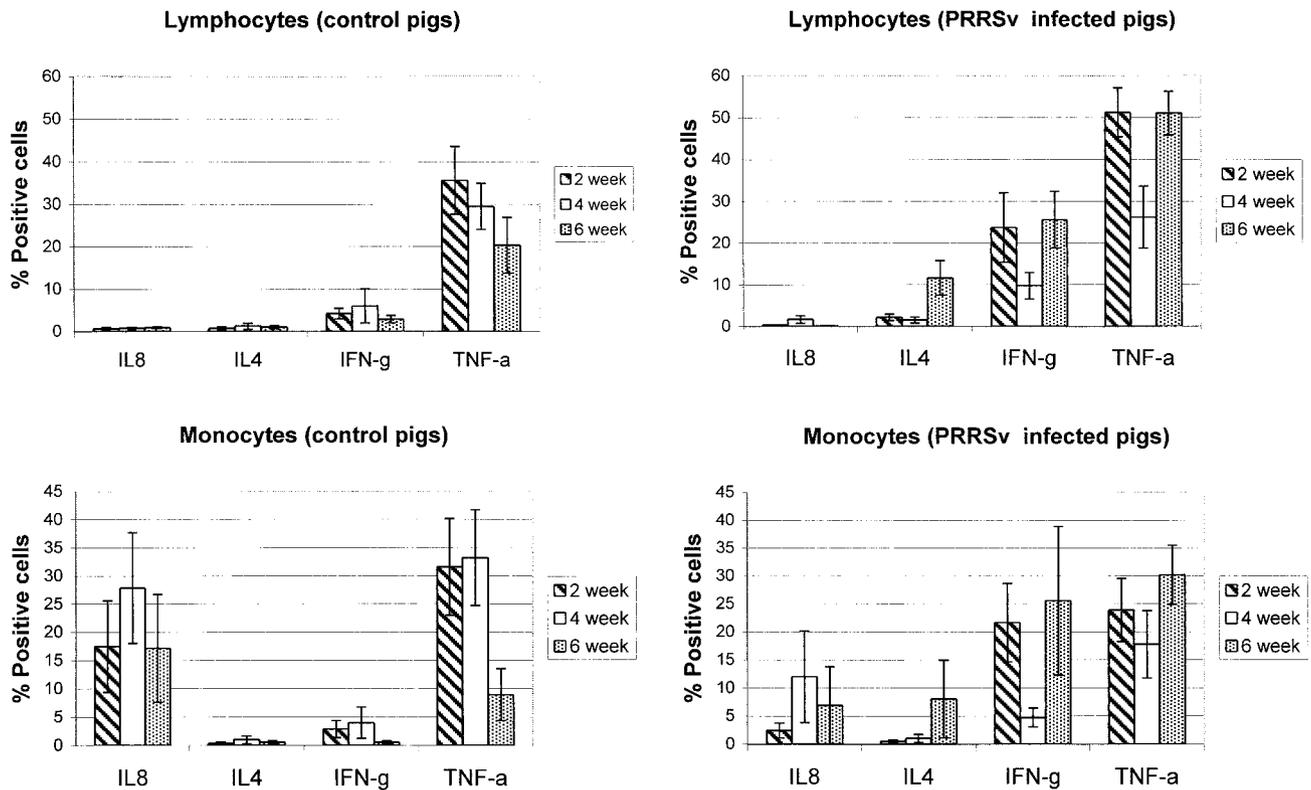
FIG. 3. Cytokine profiles for PBMCs from piglets infected with PRRSV in utero and control piglets. The percentages of IL-8-, IL-4-, γ-IFN-, and TNF-α-positive lymphocytes and monocytes in control animals and PRRSV-infected piglets are shown.

cantly enhanced IL-4, γ-IFN, and TNF-α levels (defined as $P <$ 0.05 by Student's t test) were found for the PMA- and ionomycin-treated lymphocytes and monocytes compared to the corresponding levels for nontreated cells. This effect was quite drastic with regard to γ-IFN and TNF-α. The PMA and ionomycin treatment had a slight suppressive effect on IL-8 production by monocytes. As a consequence, we decided to include PMA and ionomycin in the cell culture medium in this study.

Cytokine production by PBMCs and lymph node cells from control piglets and piglets infected with PRRSV with in utero. The experimental infection model is described in Materials and Methods. Two groups of piglets (one control group consisting of seven animals and one infected group consisting of eight animals) were euthanized 2 weeks after birth. Another two groups of piglets (10 control piglets and 8 infected piglets) were euthanized 4 weeks after birth. The rest of the piglets (eight control piglets and six infected piglets) were euthanized 6 weeks after birth. Purified PBMCs and single-cell lymph node cell preparations were cultured for 4 h in the presence of the strong cellular stimulants PMA and ionomycin. The cellular protein export pathway was blocked with the drug brefeldin A. After 4 h of culture, the gated lymphocytes and monocytes/macrophages were fixed and stained for IL-4, IL-8, γ-IFN, and TNF-α (Fig. 1).

Figure 3 presents the percentage of IL-4-, IL-8-, γ-IFN-, and TNF-α-positive lymphocytes in control animals and PRRSV-infected piglets.

The main significant findings ($P < 0.05$ by Student's t test)

were as follows. We found an increase in the proportion of IL-4-producing lymphocytes at 6 weeks and increases in the proportions of γ-IFN- and TNF-α-producing lymphocytes at 2 and 6 weeks. A similar pattern was found for monocytes (Fig. 3). In addition, we found a decrease in the number of IL-8-producing monocytes in all groups of infected piglets. There were also reduced numbers of TNF-α-producing monocytes at 2 and 4 weeks but, interestingly, increased numbers of TNF-α-producing monocytes at 6 weeks.

When the cytokine levels at 4 weeks were compared with those at 2 and 6 weeks, there was a significant reduction in γ-IFN and TNF-α levels combined with enhanced monocyte production of IL-8. Since such a pattern was not evident from the control group, it is likely that this partial reduction in lymphocyte cytokine levels at 4 weeks indicates a temporary immunosuppressive stage.

Flow cytometric staining identical to that described above for PBMCs was performed with single-cell preparations of bronchial lymph node cells (Fig. 4). Surprisingly, findings almost identical to those described above and presented in Fig. 3 were found for lymph node cells, except for a lack of IL-8 production by the large leukocytes in the lymph nodes (in this study we identify them as macrophages).

DISCUSSION

We performed this study in order to gain insight at the single-cell level of cytokine production during a phase of PRRSV infection in which immune suppression is suspected

Lymphocytes (control pigs)

Lymphocytes (PRRSv infected pigs)

Macrophages (control pigs)

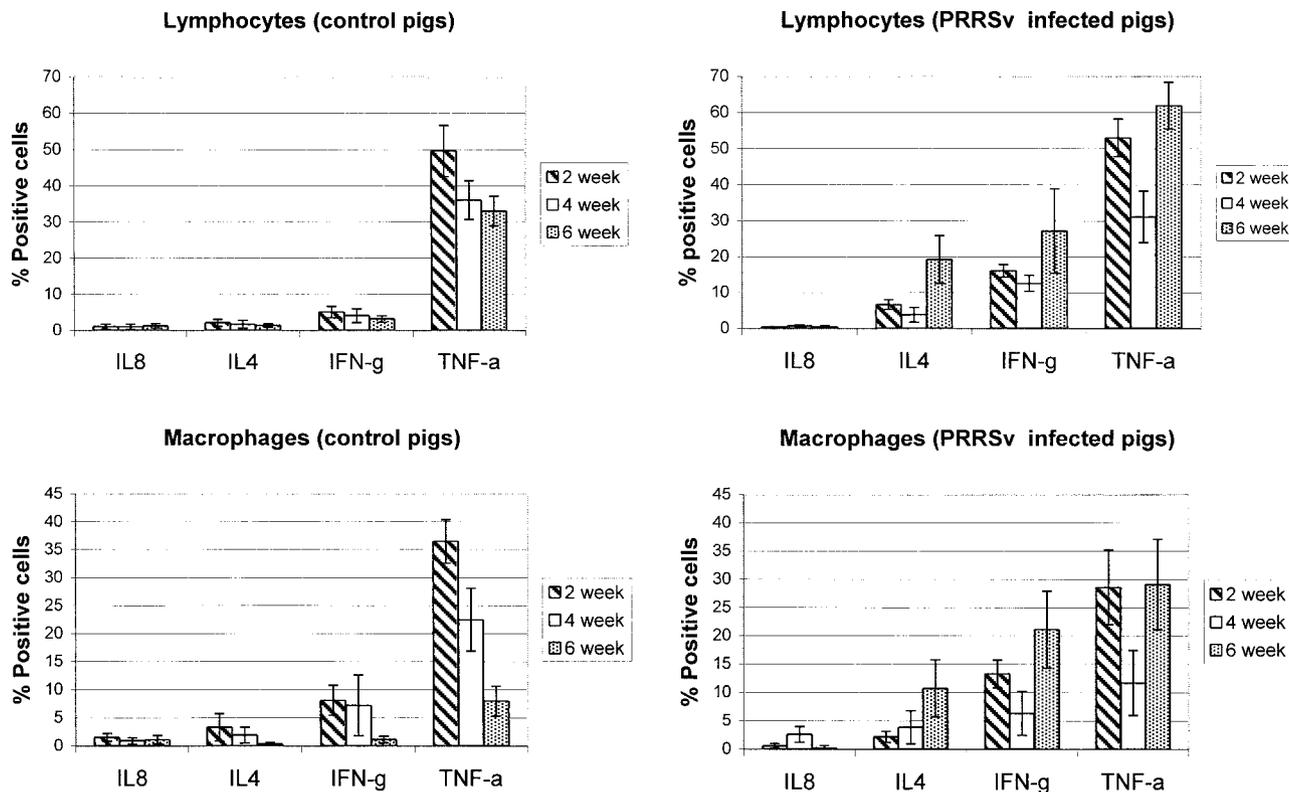**Macrophages (PRRSv infected pigs)**

FIG. 4. Cytokine profiles in single-cell preparations of bronchial lymph nodes from piglets infected with PRRSV in utero and control piglets. The percentages of IL-8-, IL-4-, γ-IFN-, and TNF-α-positive lymphocytes and large leukocytes (most likely representing macrophages) in control animals and PRRSV-infected piglets are shown.

(10, 16). We focused on 2-, 4-, and 6-week-old piglets and used the transplacental infection route because in our opinion this route of infection is very important under normal farming conditions. We compared the levels of IL-4-, IL-8-, γ-IFN-, and TNF-α-producing leukocytes in infected piglets to the ones found in noninfected piglets.

In order to obtain sufficient sensitivity in the measurement of cytokines at the single-cell level by flow cytometry, we found that it was necessary to cultivate purified PBMCs in the presence of the strong cellular stimulants PMA and ionomycin and combined that treatment with brefeldin A blockage of the Golgi complex. We found that the addition of these drugs significantly enhanced IL-4, γ-IFN, and TNF-α levels compared to the levels found in cells treated only with brefeldin A (Fig. 1). Although the cell culture method can be said to be highly artificial, it is our belief that the relative comparisons of the percentage of cytokine-producing cells in infected versus noninfected animals made in this study do reflect the in vivo situation. The sole purpose of culturing PBMCs is to create sufficient sensitivity by flow cytometry to allow statistical analysis of the data.

Several findings were apparent from the study. At 6 weeks after birth we found increases in the number of IL-4-producing lymphocytes from piglets infected in utero as well as increases in the numbers of γ-IFN- and TNF-α-producing cells at 2 and 6 weeks. Identical increases were found for monocytes. In addition to these increases, decreases in the numbers of IL-8-producing monocytes were noted. This was observed in all

groups of infected piglets. There are at least two explanations for the reduced levels of IL-8-producing monocytes. The first one is that the stimulated monocytes may have left the blood circulation and therefore would not be present in the blood samples. The other explanation is that the reduced level of IL-8 production could be due to an impairment of the monocyte (macrophage) functions due to ongoing viral replication. The latter explanation might also explain the reduced levels of TNF-α production found in this study as well as in other studies (10, 16, 27).

We take the increase in the number of IL-4-, γ-IFN-, and TNF-α-producing cells, which was most pronounced at 6 weeks after birth, as a sign of activation of the immune system. This rather late activation correlates quite well with information presented in other reports from studies with animals in the field, including reports on cell-mediated immunity and the generation of CD8⁺ cells (2, 4, 13, 15, 24, 25). This is the period when viral clearance most frequently occurs in piglets (6, 14), as was also found in the present study, since none of the six piglets infected with PRRSV in utero had viremia at this time. An important function of both type 1 and type 2 interferons is inhibition of viral replication (5, 23). We believe that the increased numbers of γ-IFN-producing lymphocytes and monocytes/macrophages found in this study most likely play a role in the clearance of PRRSV. In our mind, the increases in the levels of TNF-α- and IL-4-producing lymphocytes found at 6 weeks illustrate enhanced immune stages as well.

Enhanced levels of γ-IFN and TNF-α were already identi-

fied at 2 weeks after birth in the PRRSV-infected piglets compared to the levels found in noninfected piglets. This illustrates that there is an early immune recognition of infection. Surprisingly, this phase was followed by a phase at 4 weeks after birth in the PRRSV-infected piglets in which there was a reduction in the level of cytokine-producing cells. We found significant reductions in the levels of γ-IFN- and TNF-α-producing cells, including both lymphocytes and monocyte/macrophages (Fig. 3 and 4). The reason for this reduction in the levels of cytokine-producing cells is not apparent from our studies, but one likely explanation could be overproduction of the immunosuppressive IL-10 (Koefoed Johnsen, personal communication). It should been kept in mind that PRRSV-induced immunosuppression, as measured by decreased IFN-α levels, has been described previously (1, 9).

In this study we not only focused on cytokine expression by PBMCs, but bronchial lymph nodes were also collected from piglets that were euthanized during the study. Interestingly, the flow cytometric staining profiles of cytokines from single cells prepared from these lymph nodes (Fig. 4) were almost identical to the profiles of cytokines from PBMCs (Fig. 3), except for a lack of production of IL-8 by the large cell population, most likely representing macrophages. This finding was surprising, since we would have expected more intense cytokine production by these lymph nodes at the infection site.

Our data do not justify a closer analysis of possible Th1 and Th2 profiles for the PRRSV-infected piglets. Whether or not a dichotomy in Th1 and Th2 profiles of veterinary relevance exists at all in animals is under debate (8), and the model would most likely be an oversimplification.

ACKNOWLEDGMENT

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