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Characterization of Interaction Between Porcine Reproductive and Respiratory Syndrome Virus and Porcine Dendritic Cells

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The porcine reproductive and respiratory syndrome Virus (PRRSV) is an infectious disease that causes abortions and respiratory disorders in swine. In this study, the interaction between PRRSV and porcine dendritic cells generated from CD14$^+$ monocytes in the presence of GM-CSF and IL-4 was examined. As a result, it was shown that immature and mature dendritic cells can be productively infected with PRRSV. When the expression of surface MHC molecules on infected dendritic cells was determined, MHC classes I and II were found to be downregulated when compared with uninfected dendritic cells. With the exception of the IL-4 and IFN-γ cytokines, the induction of the IL-10, IL-12, and TNF-α cytokines all increased in dendritic cells infected with PRRSV. A mixed lymphocyte reaction showed that peripheral blood mononuclear cells cocultured with PRRSV-infected dendritic cells were less stimulated than peripheral blood mononuclear cells cocultured with dendritic cells treated with PBS, LPS, or UV-inactivated PRRSV. Therefore, these results suggest that PRRSV would appear to modulate the immune stimulatory function of porcine dendritic cells.

Keywords: Porcine reproductive and respiratory syndrome virus (PRRSV), monocyte-derived dendritic cells, major histocompatibility complex (MHC) classes I and II, cytokines

The porcine reproductive and respiratory syndrome virus (PRRSV) is the etiological agent of a disease characterized by reproductive loss in sows and respiratory disorders in piglets. PRRSV emerged in the late 1980s in the United States [13] and in the early 1990s in Europe [29]. The Lelystad and VR-2332 strains are considered as the reference strains for the European and American genotypes, respectively, and only share a 55–70% nucleotide identity in their genes [9, 23].

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PRRSV belongs to the *Arteriviridae* viral family in the genus *Arterivirus* and order *Nidovirales*, along with the lactate dehydrogenase elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian haremorrhagic fever virus (SHFV) [6]. PRRSV is a small-enveloped virus containing a positive-sense, single-stranded RNA genome with a poly(A) tail at its 3'end composed of nine open reading frames [20, 31]. PRRSV has a tropism for monocyte/macrophage lineages, particularly for porcine alveolar macrophages (PAM), both *in vivo* and *in vitro* [8]. It has also been reported that porcine testicular germ cells show a limited susceptibility to PRRSV infection [27]. The only non-porcine cells known to support PRRSV replication are African green monkey kidney cells, MA-104 and their derivatives MARC-145 and CL-2621 [8, 14].

Dendritic cells (DC) are recognized as having the ability to initiate primary immune responses in naive animals [2] and playing a role in inducing a protective immunity against viral infection. For example, DC are required for the *in vivo* response to prime B cells, cytotoxic T lymphocytes (CTL), and helper T cells [1]. The infection of DC by viruses has a marked effect on the cells for the generation of subsequent immune responses. Following viral infection, DC migrate from peripheral tissues to lymph nodes and activate CD4$^+$ and CD8$^+$T lymphocytes [1, 3, 5]. The stimulatory capacity for T lymphocytes can be mediated by increasing the expression of the major histocompatibility complex (MHC) classes I and II, costimulatory molecules of B7-1 and B7-2, and the secretion of many different cytokines in DC [1, 17]. A number of viruses have been known to infect DC and modulate immune responsiveness [7, 10–12, 15, 16, 18, 19, 24]. The human immunodeficiency virus, measles virus (MV), cytomegalovirus, and herpes simplex virus type 1 can all infect DC and induce immune suppression [16].

Accordingly, this study investigated whether PRRSV can efficiently infect porcine DC and modulate the immune stimulatory markers in DC, and which cytokines are induced in porcine DC infected with PRRSV.

Materials and Methods

Virus

PRRSV strain CNV-3 isolated from Korean pigs was used in this study. The virus was propagated in MARC-145 cells in MEM (GIBCO BRL, Gaithersburg, MD, U.S.A.) supplemented with 5% fetal bovine serum (FBS; GIBCO BRL), penicillin, and streptomycin (Sigma, MO, U.S.A.).

Generation of Porcine Monocyte-Derived Dendritic Cells

Peripheral blood mononuclear cells (PBMC) from pigs that were negative for PRRSV were isolated from the buffy coat fraction of blood using density centrifugation at 1,500 rpm for 30 min over Histopaque (1.077 g/l) (Sigma, MO, U.S.A.). Monocytes expressing $CD14^+$ on their surfaces were purified using magnetic beads coated with a $CD14^+$ monoclonal antibody in a Miltenyi magnetic-activated cell sorting (MACS) separation system with LD columns (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). To establish DC, the purified monocytes were cultured for 6 days in a RPMI medium supplemented with recombinant porcine granulocyte-macrophage colony stimulating factor (rpGM-CSF) (50 ng/ml) (R&D Systems, Minneapolis, U.S.A.) and recombinant porcine interleukin-4 (rpIL-4) (50 ng/ml) (R&D Systems, Minneapolis, U.S.A.). To obtain mature DC, the immature DC obtained after culturing for 6 days were cultured for an additional 2 days in a RPMI medium supplemented with recombinant porcine tumor necrosis factor-alpha (rpTNF-α) (50 ng/ml) (Biosource, Camarillo, CA, U.S.A.) and recombinant porcine prostaglandin (Pg) E2 (50 ng/ml) (Sigma, MO, U.S.A).

Inactivation of PRRSV by Ultraviolet (UV) Rays

The PRRSV propagated in the MARC-145 cells was purified using a sucrose gradient and irradiated using a UV cross-linker (Vilber Lourmat, Marne-la-Vallée Cedex 1, France) for 1 h at an optimal cross-linking value (0.120 J/cm^2). The inactivation of the PRRSV was then determined in MARC-145 cells by performing an immunofluorescent assay using a mouse PRRSV anti-nucleoprotein antibody.

Preparation of Porcine Alveolar Macrophages

Alveolar macrophages were obtained based on bronchoalveolar lavages of lungs from 6-week-old pigs that were negative for PRRSV. The cells were obtained by infusing 500 ml of cold PBS (pH 7.4) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 20 U/ml nystatin (Life Technologies), and by gently massaging the lungs to distribute the fluid through the lung parenchyma. The cells were washed twice in Hank's balanced salt solution (HBSS) (Life Technologies) without calcium chloride and magnesium chloride, by centrifugation for 10 min at 1,500 rpm 25°C, and the cell pellet was resuspended in a RPMI medium.

Infectivity of Porcine Dendritic Cells by PRRSV

The porcine DC (2×10^5 cells per well) and alveolar macrophages (2×10^5 cells per well) in 96-well plates (costar, Cambridge, MA, U.S.A.) were infected with PRRSV at a multiplicity of infection (MOI) of 1 for 2 h (37°C, 5% CO_2), and then the cells were washed five times with PBS (pH 7.4) and added to a RPMI medium. After incubating the infected cells for 3 days, the virus was collected by freezing and thawing the plates three times. The supernatants were collected by centrifugation at 1,500 rpm for 10 min and the viral titers in the supernatants determined by the tissue culture infectious dose 50 ($TCID_{50}$)/ml in MARC-145 cells. The presence of the virus was determined by the appearance of cytopathic effects (CPE) in the MARC-145 cells.

Determination of Phenotype Markers of Dendritic Cells by Flow Cytometric Analysis

After culturing the porcine monocytes for six days in a RPMI medium supplemented with the recombinant porcine granulocyte-macrophage colony stimulating factor (GM-CSF) (50 ng/ml) (R&D Systems, Minneapolis, U.S.A.) and recombinant porcine interleukin-4 (IL-4) (50 ng/ml) (R&D Systems, Minneapolis, U.S.A.), the nonadherent cells were harvested and labeled with the following monoclonal antibodies: anti-major histocompatibility complex classes I (MHC-I) (MCA2261; Serotec, Oxford, U.K.) and II (MHC-II) (MCA1335; Serotec), anti-human B7-1 (CD80)-phycoerythrin (PE) (R&D System), and anti-human B7-2 (CD86)-fluorescein isothiocyanate (FITC) (R&D Systems). The cells were also further stained with FITC or PE-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, CA, U.S.A.), and then washed three times with PBS (pH 7.4) containing 1% fetal bovine serum (FBS) and 0.1% sodium azide (Sigma) before being analyzed using an FACScan instrument operated with CELLQuest software (Becton Dickinson).

To determine the modulation of the surface markers on DC treated with PRRSV, the porcine DC (1×10^6 cells) in RPMI media were placed in 24-well plates and infected with PRRSV (MOI, 1) or treated with UV-inactivated PRRSV (15 μg) in a humidified incubator (37°C, 5% CO_2). After 36 h of infection, the porcine DC were recovered, incubated on ice for 30 min, and the surface markers on the DC were determined by a flow cytometric analysis using specific monoclonal antibodies for MHC class I and MHC class II.

Detection of Antigens in Porcine Dendritic Cells by Indirect Immunofluorescence Assay

Porcine DC (2×10^5 cells per well) and alveolar macrophages (2×10^5 cells per well) on a Lab-Tek II Chamber slide (Nalge Nunc international, IL, U.S.A.) were infected with 1 MOI of the PRRSV CNV-3 strain in a humidified incubator (37°C, 5% CO_2) for 20 h, and then the cells were fixed with 70% acetone in water for 20 minutes on ice. The fixed cells were then blocked with PBS (pH 7.4) containing 10% bovine serum albumin (BSA) (GIBCO BRL) for 1 h at room temperature and stained with a mouse anti-PRRSV nucleoprotein monoclonal antibody (2D6) (VMRD, Inc. Pullman, U.S.A.) and FITC or PE-conjugated goat anti-mouse IgG secondary antibody for 40 min at room temperature. The stained cells were observed under a confocal microscope (Olympus, Tokyo, Japan).

Detection of Cytokines Induced in Porcine Dendritic Cells by PRRSV

Porcine DC (5×10^6) in 6-well plates were infected/treated with PRRSV (MOI, 1), UV-inactivated PRRSV (15 μg), or lipopolysaccharide (LPS) (1 μg) (Sigma), and then the secretion of cytokines was assayed using enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions. The TNF-α, IL-4, and interferon (IFN)-γ ELISA kits were purchased from Biosource International, and the IL-10 and IL-12p70 ELISA kits were purchased from R&D Systems. The amount of cytokines was calculated based on the standard curve created by each cytokine standard.

Mixed Leukocyte Reaction (MLR) in Porcine Dendritic Cells

Allogeneic PBMCs isolated from outbred pigs by Histopaque (Sigma) were further depleted of $CD14^+$ monocytes using magnetic beads

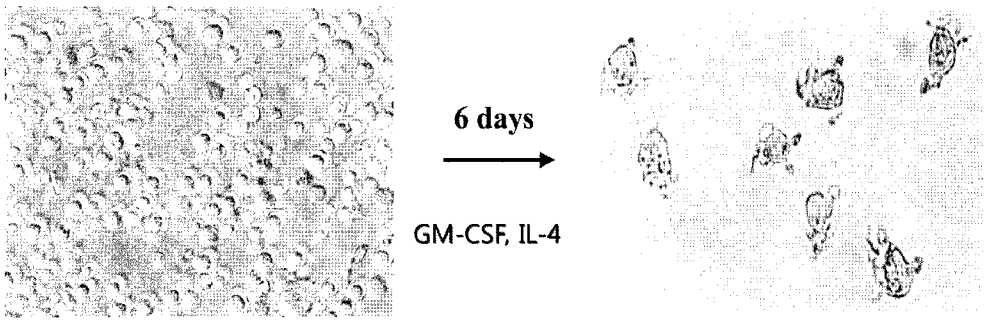

Fig. 1. Generation of immature denderitic cells from porcine CD14$^+$ monocytes.
Monocytes isolated by CD14$^+$ magnetic beads were cultured in a RPMI 1640 medium supplemented with GM-CSF and IL-4 for 6 days. The cell morphology was observed using an inverted microscope (×40).

coated with a CD14$^+$ monoclonal antibody (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) and labeled with CFSE (5- and 6-carboxy-fluorescein diacetate succinimidyl ester) (Molecular Probes, Eugene, OR, U.S.A.) in PBS (pH 7.4) for 15 min at 37°C according to the manufacturer's instructions. Meanwhile, porcine DC (1×10^5) were infected/treated with PRRSV (MOI, 1), UV-inactivated PRRSV (15 μg), or LPS (1 μg) for 24 h in 96-well U-bottom microtiter plates (Costar, Cambridge, MA, U.S.A.), and then the CFSE-labeled PBMCs (3×10^5) were added and the mixed cells incubated in a humidified incubator (37°C, 5% CO_2) for 5 days. Finally, the cells were harvested and the fluorescence intensity of the CFSE was determined by a flow cytometric analysis.

Statistical Analysis

The statistical analysis was performed using the Statistical Product and Services Solutions (SPSS) package, version 10.0. Student's *t* test was used for the group comparisons, and a *P* value of less than 0.05 was considered statistically significant.

Results

Generation of Dendritic Cells Using Porcine Monocytes and Determination of Cell Phenotypes

Porcine monocytes that were purified using CD14$^+$ magnetic beads were cultured for 6 days using RPMI 1640 media with recombinant porcine GM-CSF and IL-4. The resulting nonadherent cells showed a typical DC-like morphology, including a significant size increase, irregular shapes, presenting either as single cells or in clusters with veils, and pseudopodia on the cell surface (Fig. 1).

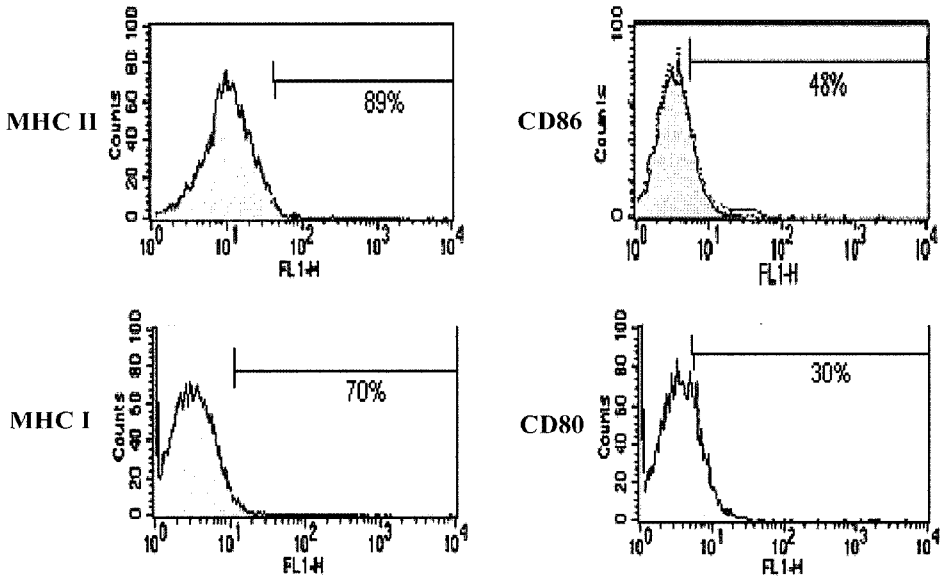

Fig. 2. Staining of cell surface molecules on dendritic cells using flow cytometric analysis.
Porcine CD14$^+$ monocytes were cultured for 6 days in the presence of GM-CSF and IL-4. The nonadherent cells were then harvested and analyzed for their expression of cell surface molecules, MHC class I/II and CD80/86, using a flow cytometeric analysis. Gray-filled histograms represent staining with an isotype-matched control antibody.

In addition, a phenotypic evaluation of the collected cells was carried out using a flow cytometric analysis, and when we investigated the expression of the costimulatory molecules CD80/86 and MHC I/II on the generated dendritic cells, the surface expression of MHC I and II was 70% and 89%, respectively, whereas the surface expression of CD80 and CD86 was 30% and 48%, respectively (Fig. 2), indicating that the generated cells were DC.

Infectivity of Porcine Dendritic Cells with PRRSV

It is already known that PRRSV has a tropism for monocyte/macrophage lineages, and in particular porcine alveolar macrophages (PAMs) [8]. Thus, to determine whether porcine DC were permissive to PRRSV infection, the cells were infected with PRRSV (MOI, 1). At 20 h post infection (p.i.), expression of the viral nucleocapsid (NP) protein was detected by an indirect immunofluorescence assay (Fig. 3). Therefore, similar to porcine alveolar macrophages, the immature and mature DC (pulsed with rpTNF-α and rpPGE2 for 2 days) were both permissive to PRRSV infection.

When we determined the viral titers in the immature and mature porcine DC infected with PRRSV (Fig. 4), the porcine alveolar macrophages and porcine DC were both shown to be productively infected, where the virus titers peaked at 24 h p.i. and then declined until 72 h p.i. At 24 h p.i., the viral titers in the infected immature DC, infected porcine alveolar macrophages, and infected mature DC were 3.3, 3.4, and 2.7 $\log_{10}TCID_{50}$/100 μl, respectively. Meanwhile, at 72 h p.i., the viral titers in the infected immature DC, infected porcine alveolar macrophages, and infected mature DC were 2.4, 1.3, and 1.6 $\log_{10}TCID_{50}$/100 μl, respectively. Thus, it seemed that the PRRSV viability declined rapidly 24 h after infection.

Modulation of MHC Class I/II and Costimulating Molecule Expression on PRRSV-infected Dendritic Cells

MHC class I and MHC class II are very important for inducing immune responses to viral infections. Therefore, to determine whether PRRSV infection would modulate MHC class I or MHC class II on porcine DC, porcine DC were infected or treated with PRRSV (MOI, 1), PBS, or UV-inactivated PRRSV (15 μg/ml), and then stained with antibodies for MHC class I and MHC class II (Fig. 5). The expressions of MHC class I and MHC class II on the DC infected with PRRSV was

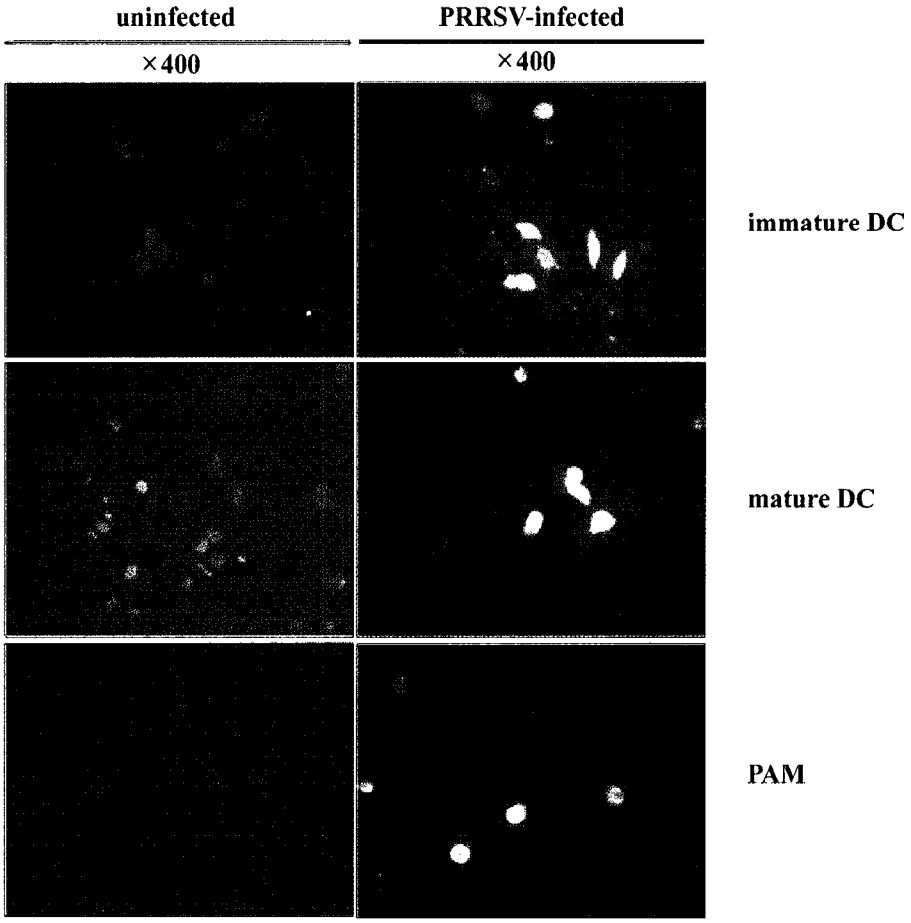

Fig. 3. Viral protein expression in immature or mature dendritic cells, or porcine alveolar macrophages.
Cells were infected with PRRSV (MOI, 1), and then 20 h later the infected cells were stained with a nucleocapsid-specific monoclonal antibody and PE- or FITC-labeled secondary antibody. The stained cells were observed under a confocal microscope (×200). DC, dendritic cells; PAM, porcine alveolar macrophages.

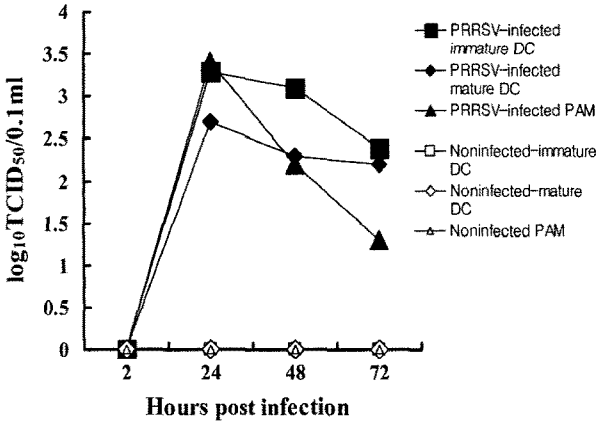

Fig. 4. Infectivity of PRRSV in immature or mature dendritic cells, or porcine alveolar macrophages.
Cells were infected with PRRSV (MOI, 1) for 3 days, and then the supernatants were collected based on three cycles of freeze-thawing at each time point. The viral titers in the supernatants were determined in the permissive cell line, MARC-145, based on $\log_{10}TCID_{50}$/100 μl. Each point represents the mean ± SD in triplicate.

downregulated compared with that on the PBS mock-infected DC, whereas the expressions of MHC class I and MHC class II on the DC treated with UV-inactivated PRRSV increased compared with that on the PBS mock-infected DC. When measuring the costimulating molecules CD80 and CD86 on the infected DC (Fig. 5), the CD80 expression (51.3%) was similar to that on the uninfected DC (52%), whereas the CD86 expression (51.6%) on the infected DC was upregulated compared with that on the uninfected DC (32.9%).

Therefore, the results indicate that PRRSV infection downregulates the expression of MHC class I and MHC class II on porcine DC.

Modulation of Stimulatory Capacity of Dendritic Cells for Leukocytes by PRRSV

Since PRRSV infection was found to modulate the expressions of MHC class I and MCH class II on porcine DC, the capacity of PRRSV to affect the ability of porcine

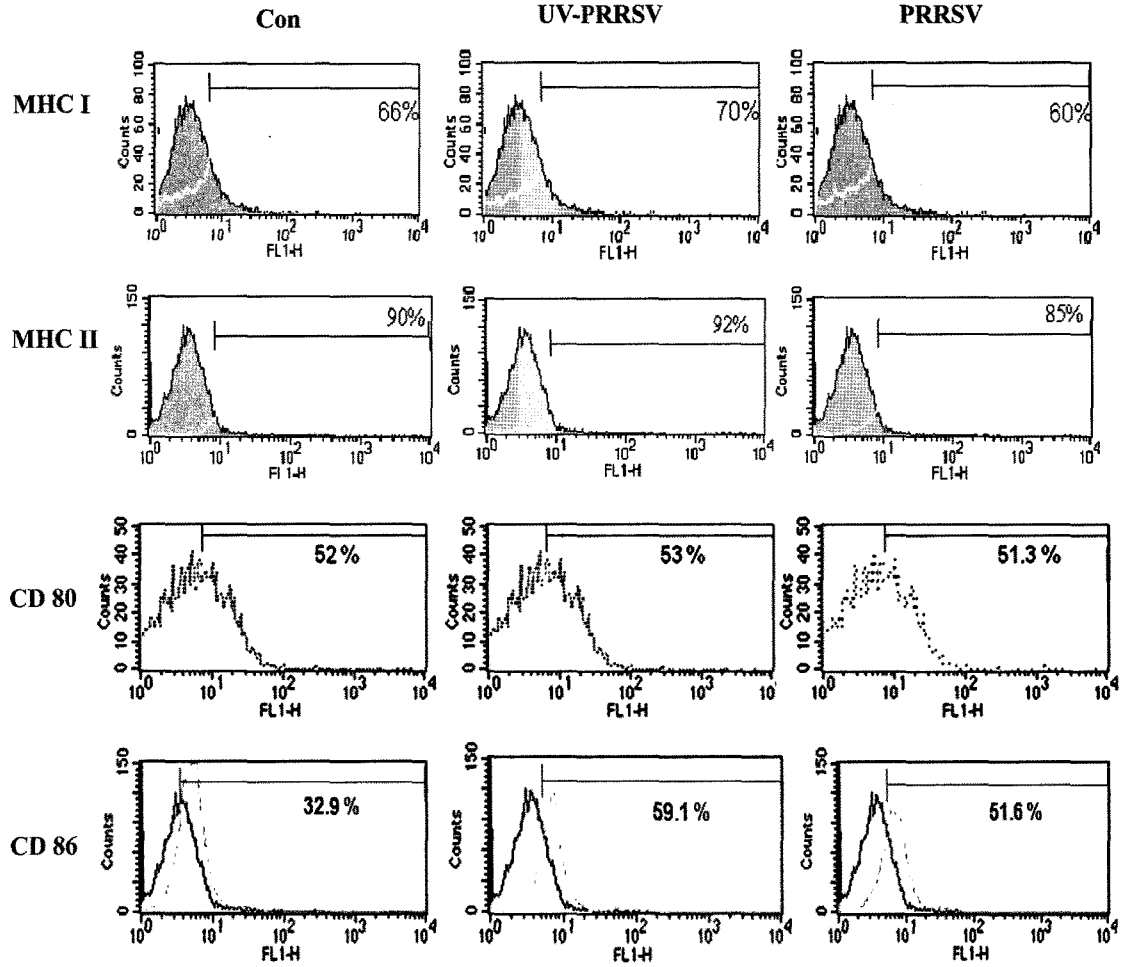

Fig. 5. Analysis of expression of MHC I and II, CD80, and CD86 on PRRSV-infected dendritic cells.
Immature DC were infected with PRRSV or UV-inactivated PRRSV (1.5 μg/ml) for 36 h. The cells were collected, and the expression of MHC I and II, CD80, and CD86 on the surface measured using MHC I and II, CD80, and CD86 antibodies based on a flow cytometric analysis. Gray-filled histograms represent staining with isotype-matched antibody. Data are representative of one of three independent experiments. Con, PBS mock-infected immature dendritic cells; UV-PRRSV, UV-inactivated PRRSV.

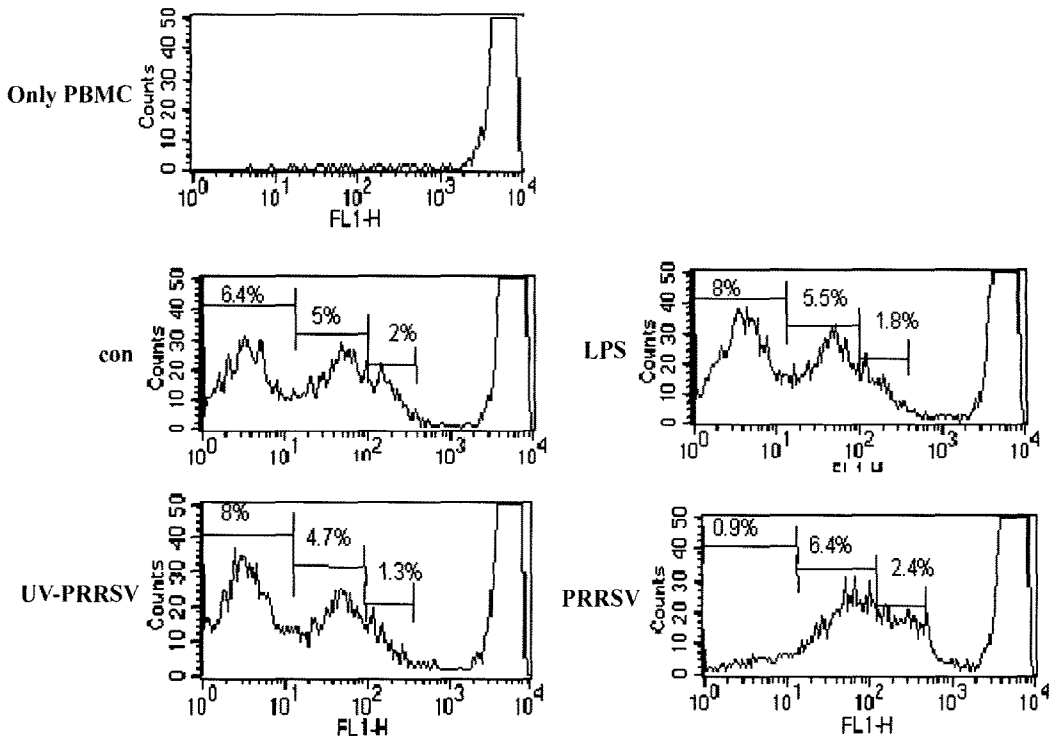

Fig. 6. Mixed leukocyte reaction of PRRSV-infected dendritic cells with PBMC.
Freshly prepared $CD14^+$ monocyte-depleted allogenec PBMC were incubated with the vital dye CFSE, and cocultured with DC treated/infected with PBS, LPS (1 μg/ml), UV-inactivated PRRSV (1.5 μg/ml), or PRRSV (MOI, 1) at a ratio of 1:3. After 5 days of coculturing, the cells were harvested and the fluorescence intensity of CFSE determined by a flow cytometric analysis. Only PBMC, not cocultured with immature DC; Con, PBMC cocultured with immature DC mock infected with PBS; LPS, PBMC cocultured with immature DC treated with LPS; UV-PRRSV, PBMC cocultured with immature DC treated with UV-inactivated PRRSV; PRRSV, PBMC cocultured with immature DC infected with PRRSV.

DC to stimulate leukocytes was also tested based on a mixed leukocyte reaction. Thus, $CD14^+$ monocyte-depleted, MHC mismatched allogeneic PBMC were labeled with the vital dye CFSE and cocultured with porcine DC treated/infected with PBS, LPS (1 μg/ml), UV-PRRSV (1.5 μg/ml), or PRRSV. After 5 days of coculturing, the amount of divided cells that contained CFSE was measured using a flow cytometric analysis. The PBMC cocultured with the porcine DC treated with PBS, LPS, or UV-PRRSV divided three times over the 5-day incubation period, whereas the PBMC cocultured with the PRRSV-infected porcine DC divided almost two times (Fig. 6). The PBMC not cocultured with any porcine DC did not divide. The percentage of daughter PBMC cocultured with the PRRSV-infected porcine DC was lower than that with the porcine DC treated with PBS, LPS, or UV-inactivated PRRSV. Therefore, the results suggest that PRRSV infection may reduce the stimulatory capacity of porcine DC for immune responses.

Induction of Cytokines in Porcine Dendritic Cells by PRRSV

As it is already known that cytokines are important for stimulating DC for antigen presentation, the cytokine induction in porcine DC infected with PRRSV was investigated. To determine the cytokines secreted in PRRSV-infected porcine DC, supernatants were collected from porcine DC treated/infected with PBS, LPS (1 μg/ml), UV-irradiated PRRSV (1.5 μg/ml), or PRRSV (MOI, 1) at 24 h and 48 h p.i., and the induced amounts of IL-4, IL-10, IL-12, TNF-α, and IFN-γ was determined (Fig. 7). When measuring the TNF-α, which is known to be involved in stimulating the recruitment of neutrophils and monocytes to an infection site, the PRRSV-infected DC produced a higher amount of TNF-α compared with the UV-inactivated PRRSV-treated DC. At 48 h p.i., the amount of TNF-α in the PRRSV-infected DC was 44,106.7 pg/ml, whereas the amount in the UV-inactivated PRRSV-treated DC was only 19,386.3 pg/ml (P=0.001) (Fig. 7A). Meanwhile, the induced amount of IL-12, which is known to be involved in T cell clonal expansion after antigen recognition, was higher in the PRRSV-infected DC than in the UV-inactivated PRRSV-treated DC. At 48 h p.i., the amount of IL-12 in the PRRSV-infected DC was 1,845.3 pg/ml, whereas the amount in the UV-inactivated PRRSV-treated DC was only 691.7 pg/ml (Fig. 7B). When we measured the immune-suppressive cytokine IL-10 at 48 h p.i., the amount of IL-10 in the PRRSV-infected DC was 226.7 pg/ml, whereas the amount in the UV-inactivated PRRSV-

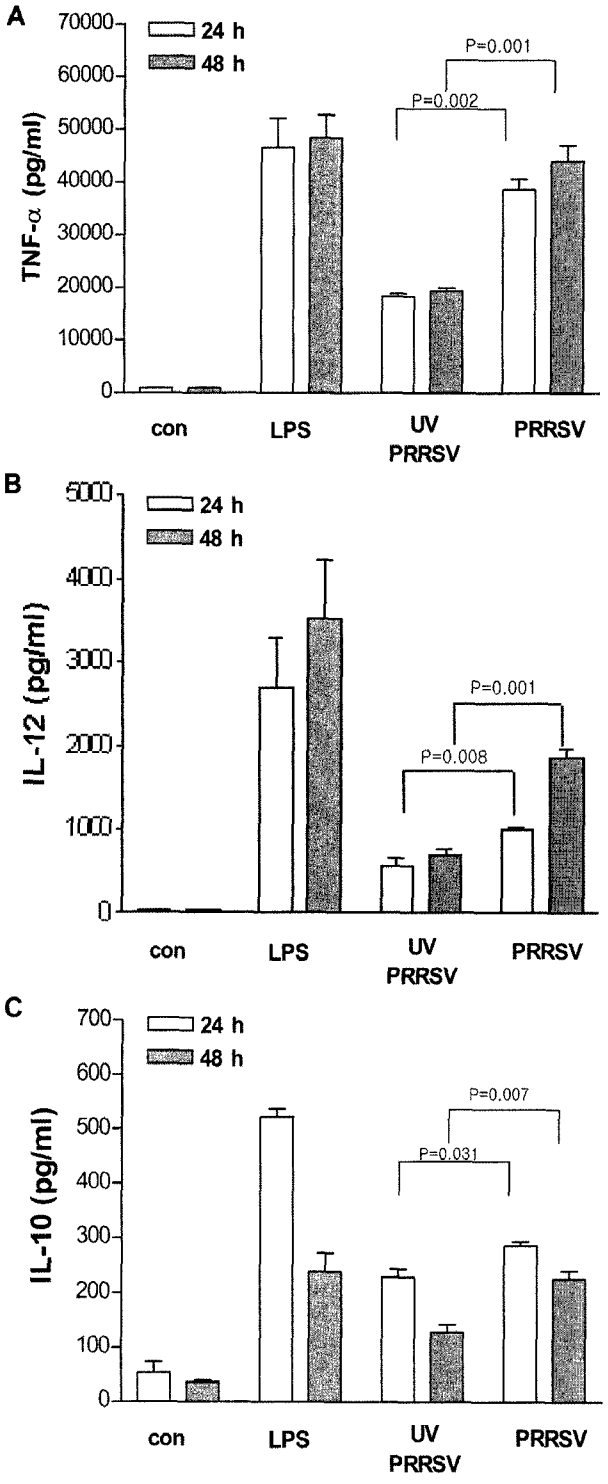

Fig. 7. Detection of cytokines in PRRSV-infected dendritic cells. Immature DC were treated or infected with LPS (1 μg/ml), UV-inactivated PRRSV (1.5 μg/ml), or PRRSV (MOI, 1) for 48 h. The induced amount of cytokines was determined using an ELISA kit specific for each cytokine. The data are representative of the mean±SD in triplicate. **A**, TNF-α; **B**, IL-12; **C**, IL-10.

treated DC was only 128.0 pg/ml (Fig. 7C). In contrast, the induction of the IL-4 and IFN-γ cytokines in the PRRSV-infected DC and UV-inactivated PRRSV-treated DC was similar to that in the untreated DC (data not shown).

DISCUSSION

PRRSV is an infectious disease that causes abortions and respiratory disorders in swine [4, 23, 26]. This study examined whether PRRSV can infect DC generated from $CD14^+$ monocytes in the presence of GM-CSF and IL-4 [25]. As a result, it was shown that immature and mature DC could be productively infected with PRRSV. When we determined the surface expression of MHC I and II molecules on the infected DC, MHC classes I and II were downregulated when compared with the uninfected DC. Furthermore, a mixed leukocyte reaction showed that PBMC cocultured with immature DC infected with PRRSV was less stimulated than PBMC cocultured with DC treated with PBS, LPS, or UV-inactivated PRRSV. Therefore, these results suggest that PRRSV infection may interfere with the functions of DC for stimulating immune responses in pigs.

The downregulation of MHC classes I and II on the PRRSV-infected DC may be responsible for previous observations of a poor immune response to PRRSV in infected pigs. The present study also showed that MHC classes I and II were downregulated by 6% and 5%, respectively, on the PRRSV-infected DC and upregulated by 4% and 2%, respectively, on the UV-inactivated PRRSV-infected DC when compared with the uninfected DC. Therefore, this result indicates that only live viruses, and not killed viruses, were able to downregulate the expression of MHC classes I and II on DC. However, the exact effect of the downregulation of MHC classes I and II on the immune responses to PRRSV requires further study. Several previous studies have suggested that PRRSV may negatively modulate the host immune system [21, 28]. Following infection, PRRSV persists in infected pigs for up to 12 weeks and infectious viruses can be shed during this stage [30], suggesting that the immune response is not able to completely eliminate viruses from the infected host.

Although PRRSV is highly contagious, virus replication appears to be mainly limited to phagocytic cell populations, including macrophages and activated monocytes [21]. In addition, the present study showed that PRRSV can productively infect DC, and this may be responsible for the weak immune responses of pigs to PRRSV based on interference with the DC functions of antigen presentation. A previous study also showed that poor innate immune responses were related to a delayed and ineffective adaptive immunity to PRRSV in pigs [22, 32].

Finally, the present data showed that PRRSV infection induced a considerable amount of immune-stimulatory cytokines, such as TNF-α and IL-12, suggesting that the poor immune responses to PRRSV in pigs may be not due to the impaired production of stimulatory cytokines for DC, but rather due to the downregulation of MHC classes I and II on the surface of DC.

In conclusion, the results of this study suggest that the productive infection of DC with PRRSV may be responsible for the poor immune responses in infected pigs.

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