

Porcine Reproductive and Respiratory Syndrome Virus Infects Mature Porcine Dendritic Cells and Up-Regulates Interleukin-10 Production[∇]

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Porcine reproductive and respiratory syndrome virus (PRRSV) infects mature dendritic cells (mDCs) derived from porcine monocytes and matured with lipopolysaccharide. The infection of mDCs induced apoptosis, reduced the expression of CD80/86 and major histocompatibility complex class II molecules, and increased the expression of interleukin-10, thus suggesting that such mDC modulation results in the impairment of T-cell activation.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus that belongs to the *Arteriviridae* family (15). PRRSV is responsible for significant economic losses and is the cause of the most important infectious disease affecting swine production worldwide. PRRSV infects pigs of different ages, and cells supporting PRRSV replication are located in different organs and tissues, with alveolar macrophages being the principal cell type that supports replication. Apoptotic cells have been associated with PRRSV infection, and they are distributed in different infected tissues, including lung, testes, and lymph node (21, 22). Protective immunity against PRRSV is not clearly understood. A delay in the appearance of the cellular immune response suggests that PRRSV infection involves a mechanism of immunosuppression or immunomodulation (18). Recently, it was reported that PRRSV infects and interferes with some functions of immature monocyte-derived dendritic cells (DCs) (3, 11, 27), as evidenced by the down-regulation of alpha interferon (IFN- α) mRNA and the up-regulation of IFN- β and tumor necrosis factor alpha (TNF- α) mRNA without changes in the production of interleukin-10 (IL-10), IL-12, and IFN- γ (27). No report exists, however, on the effects of PRRSV on mature DCs (mDCs). In this paper, we report the effects of PRRSV on mature porcine monocyte-derived DCs. Our results show that PRRSV replicated in mDCs, induced apoptosis, down-regulated the expression of CD80/86 costimulatory and major histocompatibility complex class II (MHC-II) molecules, reduced the allogeneic stimulation of T cells, and up-regulated the expression of IL-10 (mRNA and protein).

The PRRSV strain NVSL 97-7895 (GenBank accession no. AY545985) was propagated in MARC-145 cells, and the supernatant was collected, titrated, and stored at -70°C . Porcine alveolar macrophages (PAM) were collected and maintained as described previously (14). The following monoclonal antibodies were used: for MHC-II, MSA3; for CD172a, SWC3

(74-22-15); and for CD14, CAM36A; all were from VMRD (Pullman, WA). Other key reagents included mouse immunoglobulin G (IgG) Fc-human cytotoxic T-lymphocyte-associated antigen 4 fusion protein (501-820; Ancell, Bayport, MN), goat anti-mouse IgG-fluorescein isothiocyanate (IgG-FITC) (Southern Biotech), and recombinant porcine IL-4 (PSC0045) and recombinant porcine granulocyte-macrophage colony-stimulating factor (PSC2015) from Biosource International (Camarillo, CA). *Escherichia coli* O55:B5 lipopolysaccharide (LPS) and PCR primers and probe were from Sigma (St. Louis, MO). Blood samples were taken from 4- to 8-week-old White/Yorkshire hybrid pigs, and peripheral blood mononuclear cells (PBMCs) were isolated in gradients of Ficoll-Hypaque as previously described (8) and resuspended in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 50 mM 2-mercaptoethanol, 100 IU penicillin/ml, and 100 μg streptomycin/ml. PBMCs were placed in 75-cm² tissue culture flasks, and monocytes were allowed to adhere through overnight incubation at 37°C in 5% CO₂. Nonadherent cells were removed by being washed with DMEM, and the adherent cells were cultured in complete DMEM containing 20 ng/ml of each recombinant porcine granulocyte-macrophage colony-stimulating factor and IL-4 at 37°C and 5% CO₂ in a humidified incubator. The cultures were incubated for 5 days, and half of the culture medium was changed on days 2 and 5. On day 5, LPS was added (2 $\mu\text{g}/\text{ml}$), and the cells were cultured for two more days. On day 7, nonadherent cells (mDCs) were harvested from the supernatant by being washed twice with DMEM, resuspended in complete DMEM, and infected with PRRSV.

The mDCs were infected at a multiplicity of infection of 0.1 for 1 h. To eliminate unabsorbed virus, cells were washed three times by centrifugation at $200 \times g$ at 4°C , resuspended in fresh medium, and cultured for 72 h. Samples of PRRSV-infected mDCs were taken every 24 h and were stored at -70°C until the infectivity assay was performed. The expression of CD14, CD172a, MHC-II, CD80/86, and PRRSV N protein (determined by intracellular staining) was evaluated 24 h postinfection (hpi) by flow cytometry as previously described (8). At 24

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TABLE 1. Primer and probe sequences^a

Gene	Primer sequence (5'→3')		Probe sequence ^b (5'→3')
	Forward	Reverse	
IFN- α	TCAGCTGCAATGCCATCTG	AGGGAGAGATTCTCCTCATTGTG	TET-TGACCTGCCTCAGACCCACAG CC-BHQ1
IL-10	TGAGAACAGCTGCATCCACTTC	TCTGGTCCTTCGTTTGAAAGAAA	TET-CAACCAGCCTGCCCCACATGC- BHQ1
PPIA ^c	GCCATGGAGCGCTTTGG	TTATTAGATTTGTCCACAGTCAG CAAT	TET-TGATCTTCTTGCTGGTCTTGCC ATTCTT-BHQ1
ORF5-outer	CCTGAGACCATGAGGTGGG	GCGGTCACTACTATATACGG	
ORF5-nest	ATGTTGGGGAAATGCTTGA CCGC	CTGCAGCTAAGGACGACCCCATGT TCCG	

^a Sequences for cytokines and peptidylprolyl isomerase A are from the Porcine Immunology and Nutrition database (<http://www.ars.usda.gov/Services/docs.htm?docid=6065>).

^b TET, 6-carboxy-2',4,7,7'-tetrachlorofluorescein; BHQ1, black hole quencher.

^c PPIA, peptidylprolyl isomerase A.

hpi, RNA was extracted from infected and noninfected mDCs using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was done using Superscript II reverse transcriptase (Invitrogen) in a total volume of 20 μ l. Real-time PCR was performed with a Brilliant quantitative PCR core reagent kit (Stratagene, La Jolla, CA)

and a SmartCycler system (Cepheid, Sunnyvale, CA). The amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers and probes are listed in Table 1. For the quantification of mRNA, differences in threshold cycle (C_T) values between infected and noninfected DCs were evaluated with

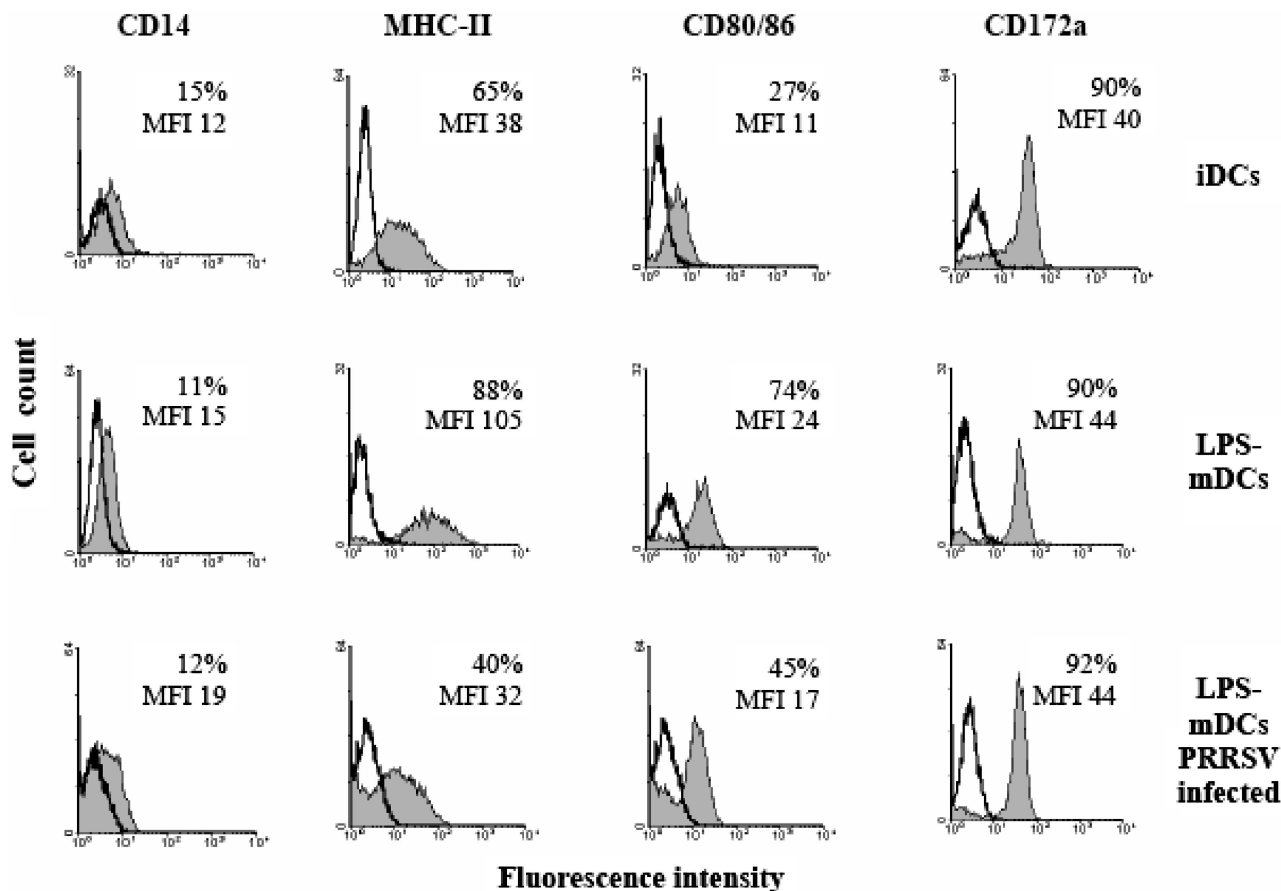


FIG. 1. PRRSV induces phenotypic modulation of DCs. Five-day-old immature DCs (iDCs) were generated as described in Materials and Methods and were treated with LPS for 48 h to induce maturation. mDCs treated with LPS (LPS-mDCs) were infected with PRRSV (LPS-mDCs PRRSV infected). Twenty-four hours later, the cells were collected, and the expression of cell surface molecules was analyzed by flow cytometry. Open histograms represent the background staining of secondary antibody. Values indicate the percentage of positive cells and the mean fluorescence intensity (MFI) for each sample. The results from one representative experiment of a total of four experiments are shown.

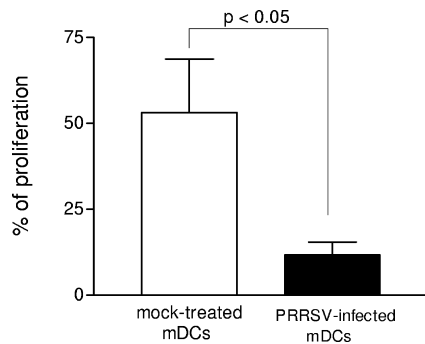


FIG. 2. Allogeneic proliferative response. T cells were labeled with carboxyfluorescein succinimidyl ester and were cultured in the presence of allogeneic infected or mock-treated DCs for 5 days, and then they were analyzed by flow cytometry. Results represent the means \pm standard deviations from three pigs. Data were analyzed by paired *t* tests by using the NCCS 2001 package.

the $2^{-\Delta\Delta CT}$ formula. The C_{7s} from infected and noninfected cells were normalized against an endogenous control (peptidylprolyl isomerase A).

To detect PRRSV RNA, ORF5 gene-specific nested reverse transcription-PCR was used as previously described (12). Apoptosis was analyzed by annexin V-FITC and propidium iodide (PI) staining and by flow cytometry following the manufacturer's instructions (Zymed Laboratories, San Francisco, CA). IL-10 was quantified using an enzyme-linked immunosorbent assay kit (Biosource, Camarillo, CA), and an allogeneic assay was performed as previously described (7). Data were analyzed as follows. A Mann-Whitney U test was used to assess differences between infected and noninfected DCs, the allogeneic proliferative response and IL-10 production in the culture supernatant were analyzed by a paired *t* test, and cytokine

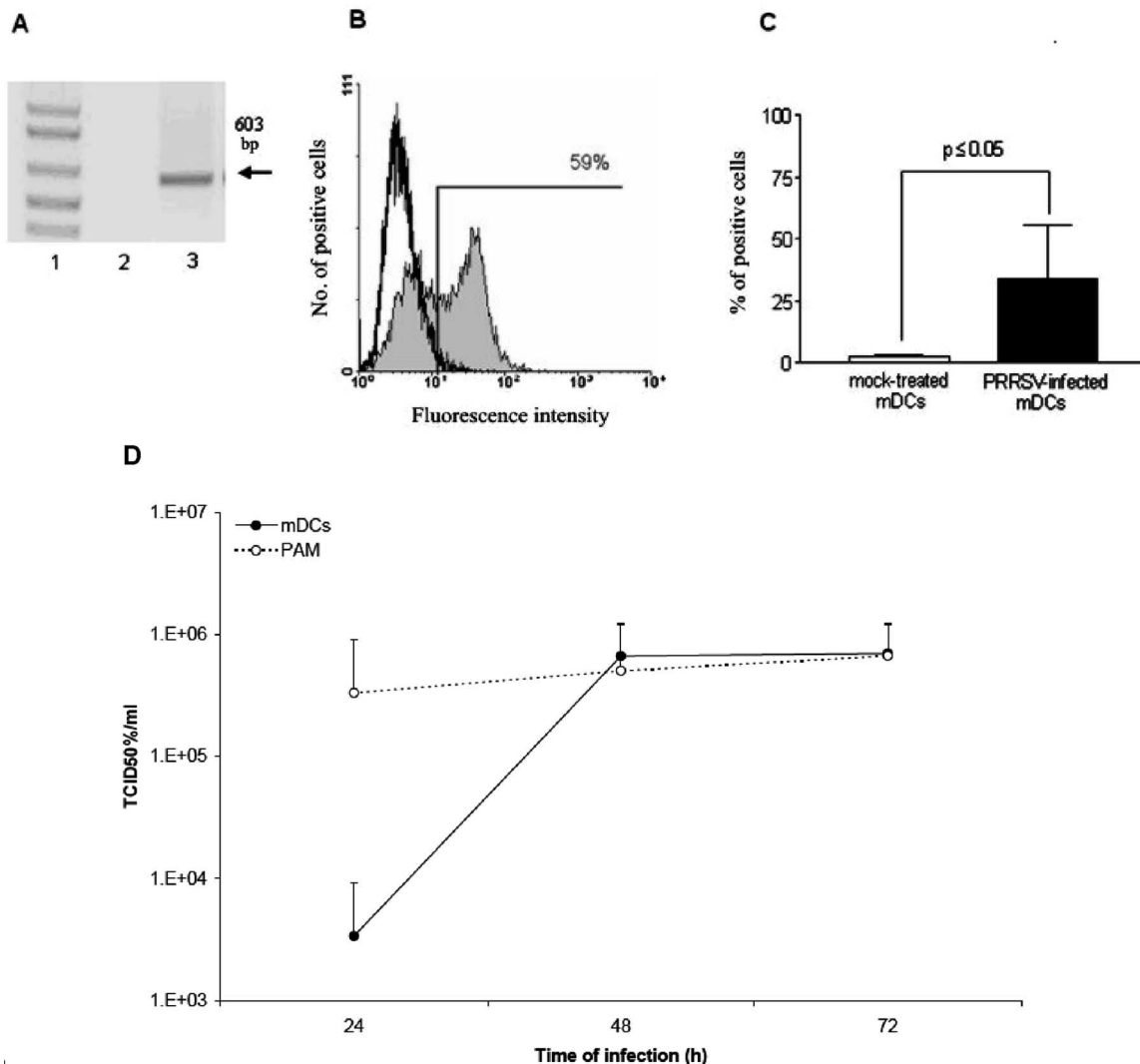


FIG. 3. PRRSV infects mDCs. (A) mDCs were infected with PRRSV, and after 24 h total RNA was extracted, and the expression of the ORF5 gene was evaluated by nested reverse transcription-PCR (lane 1, size marker; lane 2, mock-treated mDCs; and lane 3, PRRSV-infected mDCs). (B) mDCs were infected with PRRSV, and after 24 h the mDCs were fixed and permeabilized to detect the expression of intracellular N protein. The open histogram represents the background staining of secondary antibody, and the number of N protein-expressing cells is shown for one representative experiment. (C) The results are expressed as percentages of positive cells \pm standard deviations from four experiments (significance was tested by the Mann-Whitney U test). (D) The infectious virus yields in the culture supernatants of PAM and mDCs were quantified on MARC-145 cells ($n = 3$). TCID₅₀/ml, 50% tissue culture infective dose in ml.

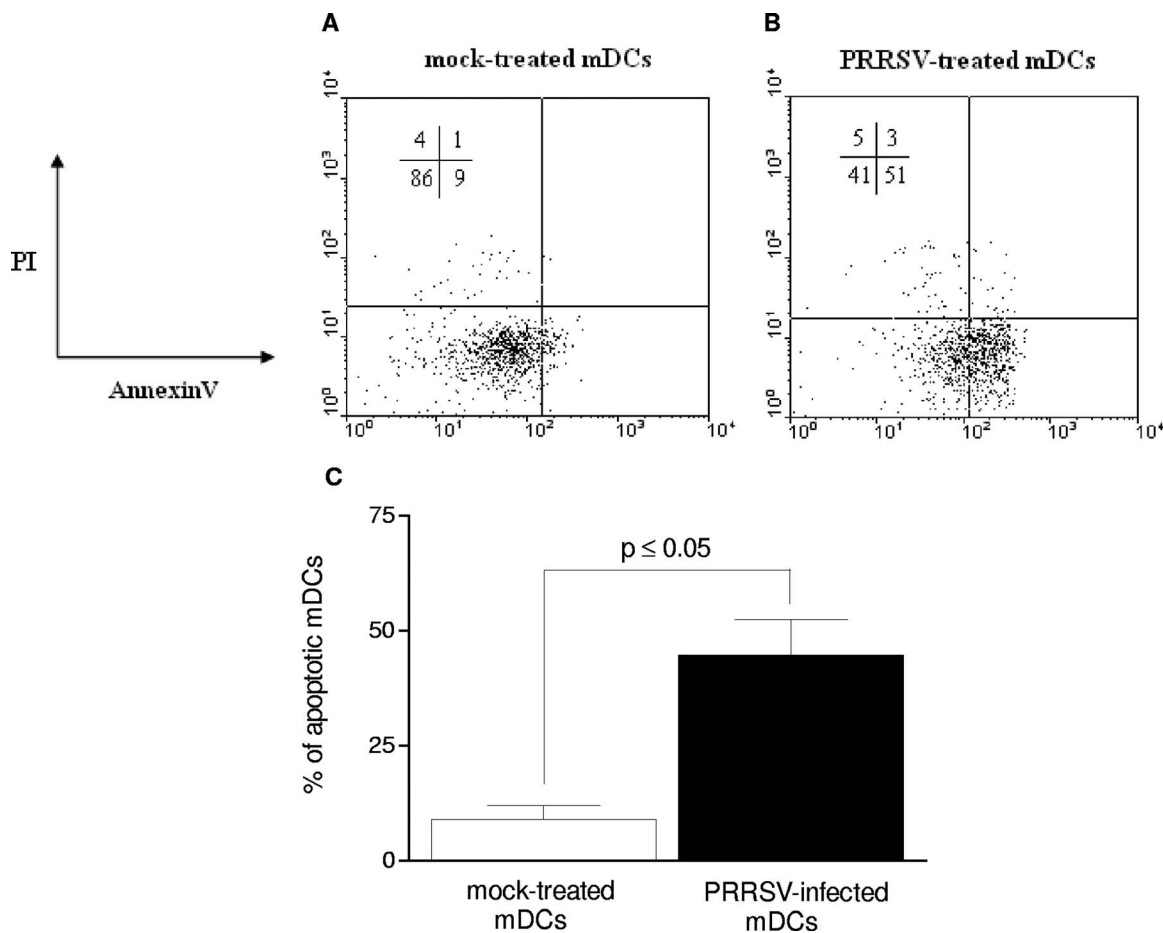


FIG. 4. PRRSV-infected DCs undergo apoptosis. FITC-conjugated annexin V and PI staining of PRRSV-infected DCs. The percentages of annexin V-positive, PI-negative cells representing early apoptosis stages and the percentages of annexin V-positive, PI-positive labeling representing end-stage apoptosis are shown. (A) Representative experiment of mock-treated mDCs. (B) Representative experiment of PRRSV-infected mDCs. For panel C, the means \pm standard deviations from three independent experiments are shown. Significance was tested by the Mann-Whitney U test.

mRNA expression was analyzed using one-way analysis of variance followed by Tukey's multiple-comparison test. All of the statistical analyses were carried out using the NCCS 2001 package.

Flow cytometric analysis revealed that, after 5 days of culture, the predominant cell phenotype was CD14^{low}, MHC-II⁺, CD80/86⁺, and CD172a⁺ ($n = 4$) (Fig. 1). LPS stimulation increased the expression of MHC-II and CD80/86 molecules but did not affect the expression of CD14 or CD172a (Fig. 1). The exposure of mDCs to PRRSV consistently induced the same phenotypic changes throughout four independent experiments. A reduction in the expression of CD80/86 costimulatory molecules and MHC-II molecules was observed with a phenotype similar to that of immature DCs, although no significant changes in CD14 or CD172a antigens were noticed (Fig. 1). An allogeneic assay was conducted to quantify the effects of the PRRSV-induced down-regulation of costimulatory and MHC-II molecules. The results showed that PRRSV-infected mDCs induced a 4.5-fold lower level of proliferation than mock-treated mDCs (Fig. 2).

At 24 hpi, GP5 glycoprotein RNA was expressed in infected mDCs ($n = 3$) (Fig. 3A). N protein expression occurred in

about 30% (range, 20 to 59%; $n = 3$) of mDCs (Fig. 3B and C), and offspring virus could be detected in the supernatant of the infected cells after 24, 48, and 72 hpi (Fig. 3D). Importantly, no differences were observed between the levels of PRRSV replication in PAM and mDCs at 48 and 72 h, but significant differences were observed at 24 h (Fig. 3D). These results suggest that mDCs are fully permissive to PRRSV replication, leading to the release of infectious viral progeny. Annexin V-FITC and PI staining of PRRSV-infected mDCs showed that the number of early apoptotic cells increased by 44% at 24 hpi (range, 36 to 51%; $n = 3$; $P < 0.05$) (Fig. 4). No significant changes were observed in the frequency of late apoptotic or necrotic cells between infected and noninfected mock-treated mDCs.

The expression of cytokine mRNA was quantified by real-time PCR, and the results are expressed as relative increments of IL-10 and IFN- α in infected versus noninfected mDCs (Fig. 5A). Our results showed that the infection of mDCs by PRRSV increases the expression of IL-10 mRNA by 6.20-fold ($P < 0.05$), but only a 1.04-fold increase in IFN- α mRNA was seen ($P > 0.05$). These data also suggest that a significant increase in IL-10 release takes place compared to the release

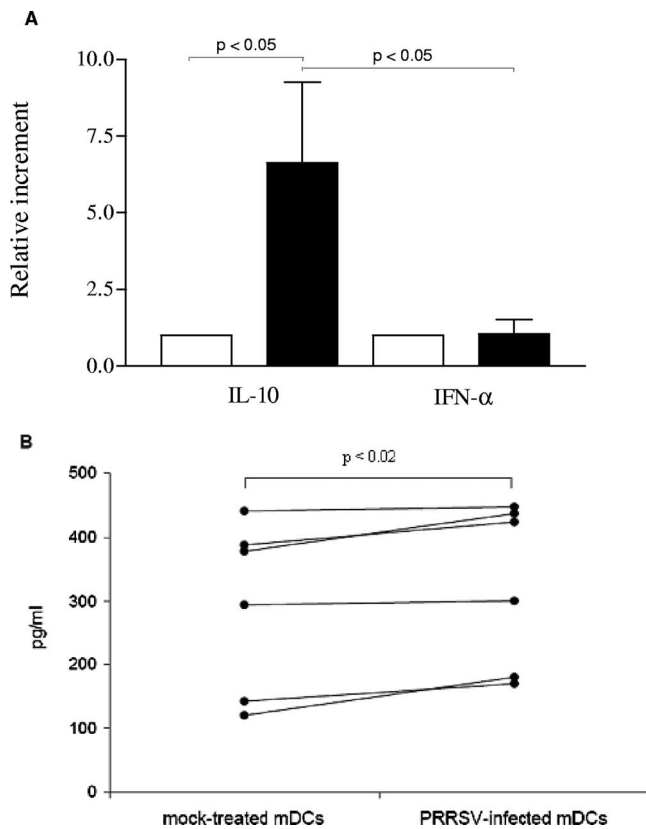


FIG. 5. PRRSV modulates cytokine expression in infected DCs. (A) Total RNA was extracted, and the expression of IL-10 and IFN- α mRNA was quantified by real-time PCR. For quantification, differences in C_T s between infected and noninfected DCs were evaluated with the formula $2^{-\Delta\Delta C_T}$. The C_T s from infected and noninfected cells were normalized against the peptidylprolyl isomerase A gene as an endogenous control, and the results (means \pm standard deviations from six pigs) are reported as the (n -fold) change in expression between uninfected DCs (clear bars) and infected DCs (black bars). Statistical analyses were performed using one-way analysis of variance followed by Tukey's multiple-comparison test. (B) The production of IL-10 was evaluated by enzyme-linked immunosorbent assay in the supernatant of infected or noninfected (mock-treated) mDCs. Data represent pairs from the same preparations of six pigs and were analyzed by the paired t test by using the NCCS 2001 package.

of IFN- α ($P < 0.05$). The increase in IL-10 mRNA expression was in accordance with the production of IL-10 in the culture of infected mDCs. Consistent increases in IL-10 production were observed in PRRSV-infected DCs compared to the production level in mock-treated DCs ($P < 0.02$) (Fig. 5B).

The mDCs generated in this study showed a morphology and phenotype consistent with that of previous reports (2, 19). We induced the maturation process by stimulating immature DCs with LPS, and we observed increments in the percentage and mean fluorescence intensity of CD80/86 and MHC-II (Fig. 2), which demonstrate the mature status of the cells. No changes were observed in CD172a⁺ and CD14^{low} expression. At 24 hpi, the PRRSV GP5 and N proteins were expressed, and PRRSV could be detected in infected mDCs, suggesting that mDCs are permissive to infection and that such permissiveness is similar in mDCs and in PAM. This outcome confirms and extends previous reports that PRRSV can produc-

tively infect immature DCs (3, 11, 27) but, apparently, not lung DCs (11). The differences in the lineage of the cells could explain such discrepancies (11), but further experiments are needed to test these differences.

By evaluating changes in PRRSV-infected mDCs, we found that PRRSV down-regulates the cell surface expression of MHC-II and CD80/D86, as has been reported for other viruses, such as varicella-zoster virus (17). Similar observations have been reported for PRRSV infection of immature DCs (27). A logical consequence of this down-regulation would be the reduced allogeneic proliferation that we observed upon the infection of mDCs with PRRSV. However, the infection of porcine lung DCs with PRRSV induces a down-regulation of MHC-II, but not CD80/86, expression or the ability to stimulate allogeneic T-cell proliferation. The addition of IFN- α reverses the MHC-II down-regulation without affecting T-cell proliferation (11).

Upon the infection of mDCs by PRRSV, we observed low levels of IFN- α mRNA expression but high levels of IL-10 mRNA expression and production in the cell supernatant. Based on these results, we can assume that the reduced proliferation of T cells incubated with the infected DCs is caused by the low level of expression of the CD80/86 and MHC-II molecules and by the production of antiinflammatory IL-10. These results are in agreement with those of Loving et al. (11), who detected no production of IFN- α , but differ from those of Wang et al. (27), who described unaltered IL-10 production after the infection of immature DCs. One possibility for this discrepancy resides in the state of differentiation of the DCs. Immature DCs have a minimal ability to produce IL-10, but after stimulation with bacteria, soluble CD40 ligand, or LPS, mDCs produce high levels of IL-10 (5). IL-10 production down-regulates the production of inflammatory cytokines, and the neutralization of IL-10 increases the production of IL-12 and TNF- α (5). Interestingly, Wang et al. (27) found a significant increase in the levels of TNF- α but no changes in IL-10 secretion. Therefore, it is possible that the differential IL-10 production is a property of some DC subsets, as described for human epidermal Langerhans cells and mouse spleen DCs, which do not express IL-10 mRNA (9, 25). Another possibility is that PRRSV up-regulates IL-10 production by mDC but not by immature DCs by a yet-to-be-defined mechanism.

IL-10 levels are enhanced in pigs infected with PRRSV (23, 24), and the production in PBMC from vaccinated pigs is dependent on the virus strain involved (6). IL-10 plays a role in immune suppression and can be inhibitory in a variety of ways. The secretion of IL-10 down-regulates MHC expression, as do costimulatory and other surface molecules that induce depressed antigen presentation and a suppressed immune response. IL-10 also has down-regulatory properties for a number of proinflammatory cytokines, including IL-12. IL-10 is essential in limiting the immune response to numerous pathogens and subsequent immune pathologies (16).

Previous studies demonstrated that PRRSV encodes two separate immune evasion strategies, specifically by (i) down-regulating the inflammatory cytokine response and inducing the up-regulation of IL-10 expression during PRRSV infection (4, 18, 24, 26) or vaccination against PRRSV (20), and (ii) delaying the appearance of the protective humoral response and the initial clonal amplification of PRRSV-specific T lym-

phocytes while transiently enhancing the ability of PRRSV to replicate in lung, lymphoid, and reproductive tract sites (1, 10, 13, 17). According to our present results and those of others (3, 11, 27), a third possible immune evasion mechanism for PRRSV is productive DC infection and the modulation of cytokine production. Such an outcome could impair the ability of DCs to function properly.

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