Role of porcine reproductive and respiratory syndrome virus nucleocapsid protein in induction of interleukin-10 and regulatory T-lymphocytes (T\textsubscript{reg})

Piya Wongyanin,\textsuperscript{1,2} Supranee Buranapraditkul,\textsuperscript{1} Dongwan Yoo,\textsuperscript{3} Roongroje Thanawongnuwech,\textsuperscript{2} James A. Roth\textsuperscript{4} and Sanipa Suradhat\textsuperscript{2}

\textsuperscript{1}Interdisciplinary Program of Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok 10330, Thailand
\textsuperscript{2}Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
\textsuperscript{3}Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL 61802, USA
\textsuperscript{4}College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA

Correspondence
Sanipa Suradhat
Sanipa.s@chula.ac.th

Received 2 December 2011
Accepted 9 March 2012

Porcine reproductive and respiratory syndrome virus (PRRSV) infection induces interleukin (IL)-10 production and increased numbers of PRRSV-specific regulatory T-lymphocytes in infected pigs. In the present study, the roles of the nucleocapsid (N) protein in induction of IL-10 and CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} lymphocytes (T\textsubscript{reg}) were investigated. Transfection of porcine monocyte-derived dendritic cells (MoDCs) and pulmonary alveolar macrophages (PAMs) with a plasmid encoding N protein resulted in significant upregulation of IL-10 gene expression in the gene-transfected cells. Structural conformation, but not nuclear localization, of the expressed N protein was indicated to be essential for the ability to induce IL-10. Furthermore, the presence of recombinant N proteins in cultured PBMCs increased the number of IL-10-producing lymphocytes. Strong induction of IL-10-producing cells and T\textsubscript{reg} was observed when using N protein-pulsed MoDCs, suggesting an important role of MoDCs in induction of IL-10 and T\textsubscript{reg} by the N protein. Neutralization of IL-10 by addition of an anti-IL-10 antibody in the culture system resulted in marked reduction of PRRSV-induced T\textsubscript{reg} in the cultured PBMCs. Together, the data demonstrate the immunomodulatory properties of the PRRSV N protein and the linkage between IL-10 production and development of PRRSV-induced T\textsubscript{reg}. Our results reveal an immunomodulatory function of the PRRSV N protein that may contribute to the unique immunological outcome observed following PRRSV infection.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is currently one of the most important swine diseases, causing significant economic losses worldwide. The causative agent is porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped, positive-stranded RNA virus of the family Arteriviridae (Cavanagh, 1997). The major characteristics of PRRS include reproductive failure and respiratory disease that usually leads to compromised lung-defence mechanisms, followed by secondary complications (Thanawongnuwech \textit{et al.}, 2000; Thanawongnuwech \& Suradhat, 2010). PRRSV infection could delay and down-modulate adaptive immune functions, leading to reduced vaccine efficacy (De Bruin \textit{et al.}, 2000; Kitikoon \textit{et al.}, 2009; Suradhat \textit{et al.}, 2006; Thacker \textit{et al.}, 2000).

Several studies have indicated that PRRSV can negatively modulate the host immune system. PRRSV infection causes weak innate immune responses (van Reeth \& Nauwynck, 2000). Moreover, PRRSV infection results in the severe functional impairment of cells of the monocyte/macrophage lineage (reviewed by Thanawongnuwech \& Suradhat, 2010). Prolonged viraemia and persistent infection are common in PRRSV-infected pigs (Murtaugh \textit{et al.}, 2002). Interestingly, PRRSV infection enhances systemic interleukin (IL)-10 production in infected pigs, especially during acute infection (Chung \& Chae, 2003; Royaee \textit{et al.}, 2004; Suradhat \& Thanawongnuwech, 2003; Thanawongnuwech \& Thacker, 2003; Van Gucht \textit{et al.}, 2004). These findings lead to the notion that IL-10 production during PRRSV infection is a key event that leads to severe impairment of the immune system (Lopez \& Osorio, 2004; Meier \textit{et al.}, 2003; Meng, 2000). Antigenic stimulation of T-lymphocytes in the presence of IL-10 or IL-10-treated antigen-presenting cells (APCs) can induce regulatory T-lymphocytes (Sakaguchi, 2004). It has recently been shown that PRRSV can induce CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} regulatory T-lymphocytes (T\textsubscript{reg}) (Silva-Campa \textit{et al.}, 2009; Wongyanin
et al., 2010). However, it is not clear whether the induction of PRRSV-specific T<sub>reg</sub> is related to enhanced IL-10 production during the early phase of PRRSV infection.

Previously, a role for PRRSV structural protein(s) in enhanced IL-10 gene expression has been suggested (Suradhat et al., 2003). An association between PRRSV structural protein(s) and IL-10 upregulation has been demonstrated (Gómez-Laguna et al., 2010). Among the PRRSV structural proteins, the nucleocapsid (N) protein encoded by ORF7 is a highly conserved and the most abundantly expressed protein in PRRSV-infected cells (Snijder & Meulenberg, 1998). Interestingly, the induction of IL-10 occurs as early as 12 h following PRRSV infection, which is concurrent with N-protein expression in pulmonary alveolar macrophages (PAMs) isolated from infected pigs (Genini et al., 2008). It is possible that the N protein may be involved in induction of IL-10 in the infected host. In this report, we have examined the role of the PRRSV N protein in induction of IL-10 and T<sub>reg</sub>.

**RESULTS**

**Expression of PRRSV N genes results in IL-10 upregulation in PAMs**

PRRSV is grouped into two genotypes, US and EU. Amino acid sequence similarity between N proteins of the PRRSV US and EU genotypes is 57–64 % (Meulenberg, 2000). In the present study, PRRSV N proteins derived from US (pMASIA-ORF7) and EU (pXJ40-ORF7) genotypes were examined for their ability to induce IL-10 in N gene-transfected PAMs. Following transfection, expression of the PRRSV N protein in transfected PAMs was confirmed by immunofluorescence assay (IFA) (Fig. 1a). To examine whether the induction of IL-10 expression was specific for the PRRSV N protein, plasmids encoding PRRSV non-structural protein 1 (Nsp1) (pXJ41-Nsp1) or glutathione S-transferase (pXJ41-GST) were included in the study. The results indicated that transfection of pMASIA-ORF7 (US) and pXJ40-ORF7 (EU) increased the level of IL-10 gene expression in PAMs significantly (P<0.01). Induction of IL-10 expression was specific for the PRRSV N protein, as IL-10 induction was not observed in cells transfected with pXJ41-Nsp1 or pXJ41-GST. In addition, the level of IL-10 expression in pXJ40-ORF7 (EU)-transfected PAMs was not different from those obtained from pMASIA-ORF7 (US)-transfected cells, or cells cultured with lipopolysaccharide (LPS) (Fig. 1b).

**Effects of protein conformation and nuclear localization on the immunomodulatory properties of the PRRSV N protein**

Previous reports indicated that 11 aa at the C terminus of the N protein were crucial for maintaining its structural conformation. Deletion of these amino acids resulted in the loss of protein conformation (Doan & Dokland, 2003; Wootton et al., 1998). Furthermore, the N protein contains a nuclear-localization signal (NLS) that facilitates its transport to the nucleus. Mutations at aa 43 and 44 in the NLS of the N protein from 41PGKKNKKK to 41PGGNNKKK abrogate the nuclear-localization property of the protein (Lee et al., 2006). To investigate the role of protein conformation and nuclear localization on induction of IL-10, PAMs were transfected with plasmids encoding truncated N protein.

---

**Fig. 1.** Detection of PRRSV N protein expression in transfected PAMs and relative expression of IL-10 in PRRSV N protein gene-transfected PAMs. (a) PAMs (3D4/2) were transfected with the null plasmid pMASIA (left panel) and pMASIA-ORF7 (right panel) and subjected to IFA. (b) PAMs were transfected with the indicated plasmids. Levels of IL-10 gene expression were analysed by quantitative real-time RT-PCR and are expressed as means ± SEM (three replicates) of the relative expression subtracted from that of the cells transfected with the corresponding null plasmid. *Statistically significant difference of the mean percentage expression of the IL-10 gene between treatment group (ORF7-US and ORF7-EU) and control groups (GST and Nsp1) (repeated-measures ANOVA followed by Tukey multiple comparison test; P<0.01).
Transfection of PRRSV N genes results in IL-10 upregulation in porcine monocyte-derived dendritic cells (MoDCs)

To confirm the immunomodulatory role of the PRRSV N protein for leukocytes, porcine MoDCs were transfected with the plasmids encoding PRRSV full-length N protein (pMASIA-ORF7) or truncated N protein (pMASIA-ORF7t) and then analysed for N and IL-10 gene expression. The results demonstrated that both full-length and truncated PRRSV N genes were expressed successfully in the transfected MoDCs and could be detected 12 h post-transfection (Fig. 3a, b). In addition, transfection with the PRRSV N gene increased the level of IL-10 gene expression significantly in MoDCs from 24 h post-transfection ($P < 0.0001$). The peak of IL-10 gene expression was observed 24 h following transfection. Although there were no differences in the transfection efficiency, MoDCs transfected with pMASIA-ORF7t exhibited a significantly lower level of IL-10 gene expression than cells transfected with pMASIA-ORF7 (Fig. 3c). This finding was consistent with the results obtained from PAMs. In addition, production of IL-10 in the transfected MoDC population was confirmed by flow-cytometric analysis, starting from 24 h post-transfection. The level of IL-10-producing cells in the MoDC population transfected with pMASIA-ORF7, but not pMASIA-ORF7t, was significantly higher than the level of cells transfected with the null plasmid, pMASIA (Fig. 3d). Taken together, transfection of MoDCs with the PRRSV N gene resulted in increased IL-10 production.

Effects of *Escherichia coli*-expressed recombinant PRRSV N proteins on induction of IL-10-producing lymphocytes and $T_{reg}$ in cultured PBMCs

In the experiments described above, we demonstrated the immunomodulatory effect of the PRRSV N protein in the gene-transfection system. Thus, we further investigated whether *E. coli*-expressed recombinant PRRSV N protein would have same immunomodulatory properties as were observed in the transfection experiment. As PRRSV induced porcine $T_{reg}$ in cultured PBMCs, the effect of the N protein on $T_{reg}$ induction was also investigated in this set of experiments. Prior to the experiment, *in vitro* characterization of the expressed protein was performed. The altered protein conformation of the truncated N protein was confirmed in the Western blot analyses (Fig. 4a), as the protein was recognized by the swine anti-PRRSV polyclonal antibody, but not by the anti-N mAb SDOW17, which was reported to recognize a conformational epitope of the PRRSV N protein (Wootton et al., 1998). To determine the appropriate dose for *in vitro* culture, the levels of IL-10 gene expression in porcine PBMCs cultured with different amounts of the recombinant PRRSV N protein were determined. The dose–response was observed, and the optimal dose of 5 μg was chosen in the following experiment (Fig. 4b). Porcine PBMCs were then cultured in the presence of the recombinant N protein (ORF7) or truncated N protein (ORF7t) for 48 h prior to flow-cytometric analyses. The effects of the recombinant proteins on porcine PBMCs are demonstrated in Fig. 4(c, d). The incubation of PBMCs with full-length PRRSV N protein increased the numbers of IL-10-producing cells significantly ($P < 0.0001$), but did not increase $CD4^+CD25^+Foxp3^+$ lymphocytes ($T_{reg}$) in the cultured PBMC population. In contrast, the numbers of IL-10-producing cells and $T_{reg}$ in the PBMCs were not different from those of controls when cultured with the truncated PRRSV N protein, (Fig. 4c, d). These results demonstrated that the recombinant PRRSV N protein could induce IL-10, but not $T_{reg}$ in cultured porcine PBMCs.

Effects of *E. coli*-expressed recombinant N proteins on induction of IL-10$^+$ cells and $T_{reg}$ in the porcine MoDC culture system

Based on an earlier report (Wongyanin et al., 2010), PRRSV-infected MoDCs can efficiently induce IL-10-producing cells and $T_{reg}$. Thus, in the present study, the
Fig. 3. Mean percentage expression of the PRRSV N gene in MoDCs transfected with pMASIA-ORF7 or pMASIA-ORF7t, relative expression of the IL-10 gene in MoDCs and number of IL-10-producing cells in transfected MoDCs following transfection with the indicated plasmid. (a–c) Porcine MoDCs were transfected with pMASIA-ORF7 (▲) or pMASIA-ORF7t (▼). Negative-control groups included MoDCs alone (○) or transfected with the null plasmid pMASIA (■). The positive-control group was MoDCs cultured with 10 μg LPS ml⁻¹ (●). Results are expressed as means ± SEM of the relative expression values or percentage of IL-10-producing cells from three or four pigs per group. Superscript letters indicate statistically significant differences between the mean relative IL-10 gene expression of the indicated group, compared with the control group (pMASIA) (one-way ANOVA, followed by Dunnett’s multiple comparison test). (d) Dot-plot demonstrating the light-scattered profile of the gated MoDC populations (left panel), MoDCs labelled with the anti-MHC II and anti-swine IL-10 mAbs (middle panel) or the anti-MHC II mAb and the isotype control (right panel). Asterisks indicate statistically significant differences of the mean percentage positive cells between the indicated group and the control group (pMASIA) (repeated-measures ANOVA followed by Tukey multiple comparison test; *P<0.05; **P<0.01).
Fig. 4. Expression and characteristics of the recombinant N proteins, dose effects of the PRRSV N protein on the level of IL-10 gene expression in porcine PBMCs, and effects of the PRRSV N protein on induction of IL-10-producing cells and T<sub>reg</sub> in porcine PBMCs. (a) His×6-tagged full-length N protein (ORF7) and truncated N protein (ORF7t) were expressed in E. coli. The non-induced (−), unpurified (U) and 5 μg purified (P) protein products were subjected to SDS-PAGE (left panel). Western blot analysis was performed using polyclonal swine anti-PRRSV antibody (right upper panel). For verification of the protein conformation, a dot-blot analysis was performed using mAb SDOW17 (right lower panel). (b) Porcine PBMCs cultured with 1, 5 or 10 μg recombinant N protein. The negative-control group was PBMCs cultured with protein expressed from E. coli transformed with pQE31 (Neg). The positive control was PBMCs cultured with an m.o.i. of 0.1 of PRRSV. The levels of IL-10 gene expression were expressed as means ± SEM of the mean percentage expression from seven pigs. Asterisks indicate statistically significant differences of the mean percentage expression between groups (repeated-measures ANOVA followed by Tukey multiple comparison test; *P<0.05; **P<0.005; ***P<0.0001). (c, d) Porcine PBMCs were cultured with 5 μg of the indicated protein products or PRRSV. Data represent means ± SEM (n=6) of the percentage of positive cells from subtracting by the baseline obtained from cells cultured with protein elution buffer (NEB). ***Statistically significant differences between the indicated group and the negative-control groups (Neg) (repeated-measures ANOVA followed by Tukey multiple comparison test; P<0.0001).
immunomodulatory effect of the recombinant PRRSV N protein was evaluated in the MoDC culture system. Porcine MoDCs were cultured in the presence of recombinant PRRSV N protein prior to the addition of autologous peripheral blood lymphocytes (PBLs). The results indicated that the presence of either full-length or truncated PRRSV N protein increased the number of IL-10+ cells in the culture system significantly ($P<0.0001$). It should be noted that the numbers of IL-10-producing cells induced by the full-length PRRSV N protein were comparable to those obtained from PRRSV-cultured MoDCs. Interestingly, the truncated PRRSV N protein could also induce IL-10-producing cells to some extent in this cultured system, but the observed effect was not as strong as that induced by the full-length N protein (Fig. 5a). In addition, the full-length PRRSV N protein, but not the truncated protein, could also induce some Treg in the culture system. However, the number of Treg was significantly lower than the number induced by PRRSV (Fig. 5b). In summary, the recombinant N protein could induce both IL-10-producing cells and porcine Treg in the MoDC culture system. The results from the MoDC system confirmed the immunomodulatory role of the N protein in porcine lymphocytes, as was observed in the PBMC culture system. The strong induction of IL-10-producing cells in the MoDC system implies an important role for MoDCs in N protein-mediated IL-10 and Treg production.

Role of IL-10 on the induction of porcine Treg

In our experiments, PRRSV N protein was a weaker inducer than PRRSV for Treg. On the other hand, it is generally known that activation of T-lymphocytes in the presence of IL-10 can result in the induction of Treg. Thus, to investigate the effect of IL-10 on induction of Treg, anti-porcine IL-10 mAb was added to the PRRSV-infected MoDC culture to neutralize IL-10 bioactivity within the culture system (see Methods). To analyse the mechanism of Treg induction further, an anti-IL-10 mAb was added at the time of addition of PRRSV to MoDC culture (before), at the time of addition of PBL (after), or at both steps (both). The results demonstrate clearly that addition of the anti-IL-10 mAb significantly reduced the numbers of CD4+CD25+Foxp3+ Treg in the culture system ($P<0.001$).

![Fig. 5. Effects of the PRRSV N protein on induction of IL-10-producing cells (a) and Treg (b) in the MoDC culture system. Porcine MoDCs were cultured with 5 ng full-length (ORF7) or truncated (ORF7t) recombinant N protein expressed from E. coli transformed with the null plasmid, pQE31 (Neg) or PRRSV. Data represent means ± SEM ($n=6$) of percentage positive cells, as described in the legend to Fig. 4. Asterisks indicate statistically significant differences of the mean percentage positive cells between indicated group (repeated-measures ANOVA followed by Tukey multiple comparison test; **$P<0.001$; ***$P<0.0001$).](http://vir.sgmjournals.org)
responses (Dolganiuc et al., 2009). Interestingly, the HCV core protein (a homologue of the N protein) was observed for other viruses, including hepatitis C virus (HCV) (Aborsangaya et al., 2007; Barrett et al., 2008) and severe acute respiratory syndrome coronavirus (SARS-CoV) (Hao et al., 2005; Zhao et al., 2005; Zheng et al., 2009). Interestingly, the HCV core protein (a homologue of the PRRSV N protein) is linked to increased IL-10 production in human PBMCs. In addition, it has been reported that the induction of IL-10 by HCV-induced APCs can alter the development of protective T-cell responses (Dolganuic et al., 2003), which was evident by impaired gamma interferon production and HCV-specific T-cell proliferation in the early phase of infection (Lechner et al., 2000; Thimme et al., 2001). Interestingly, IL-10 genes were upregulated in splenocytes of mice immunized with either the recombinant SARS-CoV N protein or a plasmid encoding the SARS-CoV N gene (Zheng et al., 2009).

0.0001), whereas addition of the isotype-control antibody did not show any effect on the Treg numbers (Fig. 6). The results indicated that IL-10 played a significant role in the development of PRRSV-specific CD4+CD25+Foxp3+ Treg, regardless of the time of IL-10 neutralization.

**DISCUSSION**

In the present study, upregulation of IL-10 gene expression was observed only in MoDCs and PAMs transfected with the plasmid encoding the N protein. Moreover, the recombinant N protein produced in the E. coli expression system also induced IL-10 gene expression and increased the number of IL-10-producing cells in both the PBMC and MoDC culture systems. Together, these results provide convincing evidence that the PRRSV N protein plays an important role in the induction of IL-10. Production of the N protein during PRRSV infection may be the major factor contributing to IL-10 production.

Besides PRRSV, induction of IL-10 by N protein has been observed for other viruses, including hepatitis C virus (HCV) (Aborsangaya et al., 2007; Barrett et al., 2008) and severe acute respiratory syndrome coronavirus (SARS-CoV) (Hao et al., 2005; Zhao et al., 2005; Zheng et al., 2009). Interestingly, the HCV core protein (a homologue of the PRRSV N protein) is linked to increased IL-10 production in human PBMCs. In addition, it has been reported that the induction of IL-10 by HCV-induced APCs can alter the development of protective T-cell responses (Dolganuic et al., 2003), which was evident by impaired gamma interferon production and HCV-specific T-cell proliferation in the early phase of infection (Lechner et al., 2000; Thimme et al., 2001). Interestingly, IL-10 genes were upregulated in splenocytes of mice immunized with either the recombinant SARS-CoV N protein or a plasmid encoding the SARS-CoV N gene (Zheng et al., 2009).

Previously, it has been shown that mutations at aa 43 and 44 in the NLS abrogated nuclear localization of the N protein (Lee et al., 2006; Pei et al., 2008). The results of our transfection experiment indicate that IL-10 induction was still maintained with the NLS-mutant N protein. This finding implies that the molecular mechanism of IL-10 induction occurs in the cytoplasm. It is possible that the N protein may activate a transcription factor in the cytoplasm that may lead to induction of IL-10. An earlier study indicated that the deletion of 11 aa at the C terminus of the N protein caused a change in protein conformation (Wootton et al., 1998). In our study, the truncated N protein could still induce IL-10 in MoDCs and PAMs. However, the level of induction was significantly lower than that of the wild-type N protein. This finding implies that the protein conformation plays an important role in the induction of IL-10, possibly by facilitating the interaction between the N protein and presently unidentified molecules in the cell. The underlying mechanism of IL-10 induction by the N protein is currently under investigation in our laboratory.

Antigenic stimulation of T-lymphocytes by immature or IL-10-treated APCs can result in the development of antigen-specific adaptive Treg (Sakaguchi, 2004; Wang & Wang, 2007). The findings in this present study indicate that the N protein does not seem to have a capability to induce Treg directly, but may help to generate a micro-environment suitable for the induction of PRRSV-specific Treg. It is possible that interaction between APCs and the N protein, and/or viral induced IL-10, may lead to an alteration of the APC functions that results in the development of PRRSV-specific Treg. The finding that N protein-activated MoDCs induced a lower number of Treg than PRRSV implies that other viral protein(s) may also contribute to the induction of PRRSV-specific Treg. Alternatively, the N protein may not contain the immunodominant epitope recognized by PRRSV-specific Treg. Further studies investigating the Treg epitope(s) will be crucial for understanding the interactions between PRRSV and the host immune system.

PRRSV productively infects blood-derived macrophages, MoDCs and immature and mature DCS, and can negatively modulate the functions of these cells (Charerntantanakul et al., 2006; Park et al., 2008; Wang & Wang, 2007). Induction of IL-10 has been observed in PRRSV-infected DCS (Park et al., 2008; Peng et al., 2009). IL-10 is known to have potent immunosuppressive effects via inhibition of both APC maturation and T-cell activation (Moore et al., 2001). In our study, when IL-10 was neutralized during the time of PRRSV infection, the number of PRRSV-induced Treg decreased. This result implies that IL-10 from PRRSV-infected MoDCs plays an important role in the modulation of MoDC functions that result in the production of PRRSV-induced Treg. Interestingly, when IL-10 was neutralized during the MoDC–PBL interactions, the number of Treg also decreased. This finding suggests that IL-10 produced from PRRSV-infected MoDCs plays an

---

**DISCUSSION**

In the present study, upregulation of IL-10 gene expression was observed only in MoDCs and PAMs transfected with the plasmid encoding the N protein. Moreover, the recombinant N protein produced in the E. coli expression system also induced IL-10 gene expression and increased the number of IL-10-producing cells in both the PBMC and MoDC culture systems. Together, these results provide convincing evidence that the PRRSV N protein plays an important role in the induction of IL-10. Production of the N protein during PRRSV infection may be the major factor contributing to IL-10 production.

![Figure 6](https://example.com/figure6.png)

**Fig. 6.** Effects of anti-IL-10 mAb on PRRSV-induced Treg. Data represent means ± SEM (n=4) of percentage positive cells from cells cultured with PRRSV subtracted from the percentage positive cells from cells cultured with mock-infected cell lysate. *Statistically significant difference between the means of the treatment group and control group (ANOVA followed by Tukey multiple comparison test; P<0.0001).
important role during T_{reg} generation in the culture. These results also indicate that the induction of PRRSV-specific T_{reg} was dependent on the interaction between PRRSV-infected APCs and lymphocytes. Therefore, it is likely that increased IL-10 production can contribute to the development of viral-specific T_{reg} in vivo.

Although the induction of T_{reg} and IL-10 by PRRSV and N protein has been demonstrated in the present study, previous evidence suggests that not all PRRSV strains are able to induce IL-10 (Silva-Campa et al., 2009, 2010; Subramaniam et al., 2011). To verify whether induction of T_{reg} and IL-10 by PRRSV is important for pathogenesis of PRRSV, correlations of time to persistence, clinical severity and the ability of different PRRSV strains to induce T_{reg} and IL-10 should be investigated. A recent study indicates that a modified live vaccine strain can induce T_{reg} in vaccinated pigs equally as well as the virulent parent strain (LeRoith et al., 2011); thus, understanding the mechanism of T_{reg} induction in the PRRSV model will be crucial for the development of a better vaccine in the future.

Taken together, our data demonstrate the immunomodulatory properties of the PRRSV N protein and the linkage between IL-10 production and the development of PRRSV-induced T_{reg}. Our study reveals an immunomodulatory function of the PRRSV N protein, which may partly explain the immunological outcome following PRRSV infection.

**METHODS**

**Viruses and cells.** The US genotype Thai PRRSV strain 01NP1 (passage no. 15), isolated from PRRSV-infected pigs (Thanawongnuwech et al., 2004), was provided by the Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University. The virus was amplified and titrated in MARC-145 cells as described previously (Thanawongnuwech et al., 1998). The porcine PAM cell line 3D4/2 was purchased from ATCC (CRL 2845).

**Antibodies and secondary conjugates.** The directly conjugated mAb anti-swine CD25–PE (PGBL25A, IgG1) was provided by Dr J. A. Roth (Iowa State University, Ames, IA, USA). Anti-swine CD4–FITC (74-12-4, IgG2b) conjugate, anti-human CD80–PE (L307.4, IgG1), CD86–allophycocyanin (2331, IgG1), and anti-SLA-DR (1053H2-18, IgG2a) were purchased from BD Biosciences. Anti-human Foxp3–APC (236A/E7, IgG1) was purchased from eBioscience. Biotinylated anti-swine IL-10 mAb (148801, IgG2b) and the IgG2b isotype control (236A/E7, IgG1) was purchased from eBioscience. Biotinylated anti-human Foxp3–APC (236A/E7, IgG1) was purchased from BD Biosciences. Anti-human Foxp3–APC (236A/E7, IgG1) was purchased from eBioscience. Biotinylated anti-swine IL-10 mAb (148801, IgG2b) and the IgG2b isotype control (236A/E7, IgG1) was purchased from BD Biosciences.

**Plasmids for transfection experiments.** The pXJ40 vector containing ORF7-EU (Lelystad virus), the pXJ41 vector containing the PRRSV-NSP1 gene (Song et al., 2010) or GST gene, pCINEo vector and pCINEo-NLS (GG), containing substitution of glycine at the NLS (aa 43 and 44) were provided by Dr Dongwan Yoo (University of Illinois at Urbana-Champaign, Urbana, IL, USA).

For cloning of the plasmid encoding the PRRSV N protein from the Thai PRRSV 01NP1 strain, a specific primer set was designed for cloning genes encoding full-length PRRSV N protein; ORF7 (ORF7-F-TF, 5′-AAAAAAGATTCCTGACATCAAAACTACGCGCA-AG-3′; ORF7-R-TF, 5′-AAAAAAGATTCCTGACATCAAAACTACGCGCA-AG-3′). For cloning of the gene encoding truncated PRRSV N protein (ORF7t), a reverse primer containing a stop codon at aa 112 of the N protein product (ORF7t-R, 5′-TCAACAGATGATGCGATGGTT-3′) was used. The PCR products were cut by EcoRI restriction endonuclease (New England Biolabs) and subcloned into the pMASIA expression vector (a gift from Dr S. van Drunen Little van den Hurk, Virus and Infectious Diseases Organization, University of Saskatchewan, SK, Canada). The pMASIA vectors containing ORF7 or ORF7t genes were referred to as pMASIA-ORF7 or pMASIA-ORF7t, respectively.

**Cloning and recombinant protein production.** For production of the recombinant N protein, a specific primer set (ORF7-F, 5′-ATGCAATAAACAAGC-3′; ORF7-R, 5′-TGCTGAGGGTGATGCTGT-3′) was designed to clone the full-length PRRSV ORF7 gene from the US genotype PRRSV strain 01NP1 (Thanawongnuwech et al., 2004). For cloning of the truncated ORF7 gene (ORF7t), a reverse primer containing a stop codon at aa 112 of the N protein product (ORF7t-R, 5′-TCAACAGATGATGCGATGGTT-3′) was used. The ORF7 and ORF7t genes were PCR-amplified and cloned into the pGEM-T Easy vector (Promega), and referred to as pGEMT-ORF7 and pGEMT-ORF7t, respectively. Plasmids were amplified in E. coli strain JM109 (Promega). Subsequently, pGEMT-ORF7 and pGEMT-ORF7t were digested by SplI and Sall restriction endonucleases (New England Biolabs). The purified fragments were subcloned into the pQc31 vector (Qiagen) and referred to as pQc31-ORF7 and pQc31-ORF7t, respectively.

Recombinant protein expression was performed by transformation of the expression plasmids into E. coli strain M15 followed by induction with IPTG (Fermentas) at a final concentration of 1 mM for 4 h. Recombinant proteins were purified using Ni-NTA resin (Invitrogen), quantified using a Bradford assay (Bio-Rad) according to the manufacturer’s protocol, and stored at −20 °C until needed.

**Transfection experiments.** For transfection of MoDCs, cells were placed in a 96-well plate at 5 × 10^5 cells in 200 μl per well. Subsequently, cells were transfected with 0.5 μg pMASIA, pMASIA-ORF7 or pMASIA-ORF7t using Lipofectamine 2000 (Invitrogen) for 24 h at 37 °C in a 5% CO_2 incubator according to the manufacturer’s protocol. At the end of the incubation period, cells were harvested and washed once with Ca^{2+}-free, Mg^{2+}-free PBS (PBSA). The positive-control group was MoDCs cultured in the presence of 10 μg LPS ml⁻¹ (Sigma-Aldrich). The negative-control group was cultured cells alone.

For transfection of PAMs, a porcine PAM cell line (ATCC 3D4/2) was split into a 12-well plate at 3 × 10^5 cells in a volume of 1 ml per well and incubated overnight to allow complete attachment on the plate. Cells were transfected with 2 μg pMASIA, pMASIA-ORF7, pMASIA-ORF7t, pXJ40, pXJ40-ORF7 EU (LV), pXJ41, pXJ-GST, pXJ-Nsp1, pCINEo, or pCINEo-NLS (GG) using Lipofectamine 2000 (Invitrogen) and incubated for 24 h at 37 °C in a 5% CO_2 incubator according to the manufacturer’s protocol. At the end of the incubation period, cells were harvested and washed once with PBSA. The positive-control group was PAMs cultured with 10 μg LPS ml⁻¹. The negative-control group was cultured PAMs alone.

**RT-PCR and quantitative real-time PCR analysis.** Total RNA was extracted from cells (PBMCs or MoDCs) using a Nucleospin RNA II extraction kit (Macherey-Nagel). From PAMs, total RNA was extracted using TRIzol LS reagent (Invitrogen). Total RNA (250 ng) was used for cDNA synthesis using an Omniscript RT kit (Qiagen). Semi-quantitative PCR for analysis of IL-10 gene expression was performed using the previously published protocol (Suradhat et al., 2003). Quantitative PCR for detection of porcine Foxp3,
GAPDH and IL-10 gene expression was performed using the previously published primer sets (Käser et al., 2008; Suradhat et al., 2003) and protocol (Wongyanin et al., 2010). Results are expressed as the mean± SEM of the relative increments of mRNA between the treatments and the cell control (PBMCs or MoDCs cultured alone, at 0 h).

**IFA.** Transfected PAMs were washed three times with PBSA, fixed with 4 % formaldehyde in PBSA for 10 min, and permeabilized with 0.1 % NP-40 (Sigma-Aldrich) in PBSA for 15 min at room temperature. After blocking with 1 % BSA in PBSA, the cells were incubated with FITC-conjugated anti-N protein mAb (SDOW17–FITC) (Rural Technologies, Inc.) for 1 h. The presence of the N protein was determined with a fluorescent microscope at ×20 (Nikon Eclipse TS 100).

**Western blot analysis.** Proteins were separated by SDS-PAGE (12 % acrylamide) and transferred to PVDF membrane (PALL) at 100 mA for 90 min. After blocking with 5 % BSA (Merck) in PBSA, the membrane was incubated with porcine hyperimmune serum as the swine anti-PRRSV polyclonal antibody for 1 h at room temperature, washed three times with PBST [0.05 % Tween-20 (Sigma-Aldrich) in PBSA], followed by incubation with 1:500 goat anti-swine IgG (H + L)–HRP conjugate (KPL) in 1 % BSA in PBSA for 1 h. At the end of incubation period, 3,3′-diaminobenzidine (DAB) substrate (Sigma-Aldrich) was added for colour development.

For dot-blot analysis, 5 μg purified recombinant protein was spotted onto a nitrocellulose membrane (PALL). Protein analysis was performed using a 1:500 dilution in 1 % BSA in PBSA of the anti-PRRSV N protein mAb (SDOW17) for 1 h at room temperature. The membrane was washed three times with PBST followed by the incubation with 1:500 dilution in 1 % BSA in PBSA of the rabbit anti-mouse IgG–HRP conjugate (DakoCytomation). Colour was developed using DAB substrate (Sigma-Aldrich).

**Isolation and in vitro activation of PBMCs and PBLs, and generation of MoDCs.** The protocols used for isolation of porcine PBMCs and PBLs, and for generation of porcine MoDCs are described elsewhere (Wongyanin et al., 2010). For in vitro activation, 1 ml single-cell suspension of the appropriate cell population, i.e. PBMCs or PBLs (6 × 10^6 cells ml^−1), was cultured in a well of 24-well plate with an m.o.i. of 0.1 of PRRSV or 5 μg of the indicated recombinant protein at 37 °C in a 5 % CO₂ incubator for 48 h. For MoDC in vitro activation, the generated MoDCs were further cultured in the presence of an m.o.i. of 0.1 of PRRSV or 5 μg of the indicated recombinant antigens at 37 °C in a 5 % CO₂ incubator for 24 h. Mock-infected MARC-145 cells or elution buffer were used as controls.

**In vitro neutralization of IL-10.** MoDCs were cultured with PRRSV or mock-infected cell lysate for 24 h. Following incubation, autologous PBLs (5 × 10^6 cells in a volume of 1 ml per well) were added into the culture system and incubated further for 48 h prior to fluorescent labelling. For neutralization of porcine IL-10 bioactivity, the anti-porcine IL-10 mAb (clone #148801, R&D Systems) was added into the MoDC culture system at the time of infection with PRRSV (before), when autologous PBL was added (after), or at both steps (both) at 5 μg ml^−1. In the control system, anti-IL-10 mAb was replaced with the IgG2b isotype-control antibody (clone #20116, R&D Systems).

**Fluorescent labelling of porcine leukocytes.** To analyse intracellular molecules, the protein transport inhibitor monensin (GolgiStop; BD Biosciences) was added to the cell culture after 36 h incubation. Following 48 h in vitro stimulation, cells were harvested, washed once with PBSA and then resuspended in PBSA supplemented with 0.5 % BSA and 0.1 % sodium azide, referred to as FACS buffer. The PBMCs were distributed into a round-bottomed 96-well plate at approximately 2 × 10^6 cells per well, followed by centrifugation at 1000 g for 5 min and subjected to immunofluorescent staining. The surface molecules CD4 and CD25 were stained with 1:50 anti-CD4–FITC and 1:25 anti-CD25–PE antibodies diluted in FACS buffer and incubated at 4 °C in the dark for 40 min. In an MoDC experiment, porcine MHC class II molecules were labelled with 1:100 anti-SLAM mAb. The cells were then permeabilized and fixed with 100 μl per well of 50 % Reagent A (Leucoperm; Serotec) diluted in FACS buffer at room temperature in the dark for 15 min.

Intracellular staining of the Foxp3 and IL-10 molecules was performed by the incubation of cells with 1:5 anti-human Foxp3–allophycocyanin and 1:50 biotinylated anti-swine IL-10 antibodies diluted in Reagent B (Leucoperm; Serotec), and incubated at room temperature in the dark for 60 min. Subsequently, the cells were stained with 1:100 secondary conjugate streptavidin–PE–Cy5 diluted in FACS buffer and incubated at 4 °C in the dark for 40 min. All staining reactions were performed at a final volume of 50 μl per well. At the final stage, the pellet was resuspended in 200 μl FACS buffer in a 96-well plate, protected from the light and kept at 4 °C until analysed by flow cytometry.

**Statistical analyses.** Data were analysed using analysis of variance (ANOVA) followed by post-test, as indicated in the figure legends. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software Inc.).

**ACKNOWLEDGEMENTS**

This work was supported by the Thailand Research Fund (DB5280016), the 90th Anniversary of Chulalongkorn University, Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University, and by the National Research Initiatives of the US Department of Agriculture Cooperative State Research Education and Extension Service, grant no. 2008-35204-04634 awarded to D. Y. P. W. is supported by the Royal Golden Jubilee PhD Program (PhD 0178/2548) and Postdoctoral Fellowship, Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University.

**REFERENCES**


reproductive and respiratory syndrome virus infection on the development of the immune response against pseudorabies virus. Vet Immunol Immunopathol 76, 125–135.


