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Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus

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ABSTRACT

Delayed development of virus-specific immune response has been observed in pigs infected with the porcine reproductive and respiratory syndrome virus (PRRSV). Several studies support the hypothesis that the PRRSV is capable of modulating porcine immune system, but the mechanisms involved are yet to be defined. In this study, we evaluated the induction of T regulatory cells by PRRSV-infected dendritic cells (DCs). Our results showed that PRRSV-infected DCs significantly increased $Foxp3^{+}CD25^{+}$ T cells, an effect that was reversible by IFN-α treatment, and this outcome was reproducible using two distinct PRRSV strains. Analysis of the expressed cytokines suggested that the induction of $Foxp3^{+}CD25^{+}$ T cells is dependent on TGF-β but not IL-10. In addition, a significant up-regulation of Foxp3 mRNA, but not TBX21 or GATA3, was detected. Importantly, our results showed that the induced $Foxp3^{+}CD25^{+}$ T cells were able to suppress the proliferation of PHA-stimulated PBMCs. The T cells induced by the PRRSV-infected DCs fit the $Foxp3^{+}CD25^{+}$ T helper 3 (Th3) regulatory cell phenotype described in the literature. The induction of this cell phenotype depended, at least in part, on PRRSV viability because IFN-α treatment or virus inactivation reversed these effects. In conclusion, this data supports the hypothesis that the PRRSV succeeds to establish and replicate in porcine cells early post-infection, in part, by inducing Th3 regulatory cells as a mechanism of modulating the porcine immune system.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is currently considered to be the most significant and economically important infectious disease affecting swine industry worldwide with economic losses estimated at ~560 millions dollars per year (Neumann et al., 2005). The causative agent is PRRS virus (PRRSV) (Meulenberg et al., 1997), which belongs to the order Nidovirales, family *Arteriviridae* and genus *Arterivirus* (Cavanagh, 1997). Two PRRSV genotypes, the American and the European, have been described and they share approximately 55 to 65% nucleotide identities but high variability has also been described (Andreyev et al., 1997; Mateu et al., 2006).

The PRRSV can replicate and persist in pigs for long periods of time after the initial infection and that persistently infected animals can shed infectious virus, but eventually the virus is cleared by most of the infected animals by 150 days post-infection (dpi) or shortly thereafter (Allende et al., 2000). PRRSV-specific cellular immune response evaluated by the antigen-specific proliferation of PBMC is evident until 4 wk post-infection (PI) and is detectable 9–14 wk PI (Molitor, Bautista, and Choi, 1997). In addition, low-levels of IFN-γ producing cells are detected until 2 wk PI and increase significantly at 10 wk PI (Batista et al., 2004; Meier et al., 2003). The mechanisms involved in this unusual and delayed immune responses are still unknown, but available evidence suggests that the PRRSV is able to modulate the porcine immune system, at least during the first few weeks PI (Mateu and Díaz, 2008; Murtaugh et al., 2002).

It has been described that viruses are able to evade the immune response by utilizing a wide range of mechanisms including antigenic variation, infection of immune system cells, production of cytokine-like immunosuppressive proteins, modulation of DC function, and the induction of regulatory T cells (Tregs). Given the importance of DCs in initiating antiviral immune responses, PRRSV interference of DCs function represents an immune evasion mechanism that could confer advantages to this virus by inhibiting or reducing the DC-mediated immune response (Banchereau and Steinman, 1998; Chang et al., 2008; Charerntantanakul et al., 2006; Flores-Mendoza et al., 2008; Loving et al., 2007; Wang et al., 2007). This viral advantage can be reverted by IFN-α treatment, which is a potent antiviral molecule (Loving et al., 2007).

Induction of Tregs is a way that viruses such as HIV, HCV, EBV, CMV, HTLV, HBV, and FIV use to suppress or evade the immune

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response and in this way persist in the host (reviewed in Li et al., 2008). The conventional phenotype of Tregs is $CD4^+CD25^+Foxp3^+$, and it has been classified into natural and inducible Tregs, both in human and mice (Belkaid, 2007; Shevach, 2006). In addition, inducible Tregs can be classified according to the cytokines they produce and several subtypes have been described: 1) Treg 1 (TR1) cells which secrete IL-10; 2) T helper 3 (Th3) cells which secrete TGF-β; and 3) converted $Foxp3^+$ Tregs (Belkaid, 2007; Shevach, 2006). Inducible Tregs acquire their function following infection or exposure to other stimulus (Belkaid, 2007).

In pigs, a population of Tregs has recently been described in blood and tissues. This phenotype of porcine Tregs is heterogeneous, and the two main populations described are $CD4^+CD25^+Foxp3^+CD8\alpha^-$MHC-$II^-$ and $CD4^+CD25^+Foxp3^+CD8\alpha^+$MHC-$II^-$. The first population is described as resting Tregs and could represent natural Tregs. The second population represents activated or memory-like Tregs and could correspond to pathogen-induced Tregs (Kaser et al., 2008). However, no other reports have described natural or induced Tregs in pigs. Given that delayed development of virus-specific immune response has been observed in pigs infected with the PRRSV and that DCs plays a key role in priming immune responses, we evaluated the induction of T regulatory cells by PRRSV-infected DCs.

Results

Induction of $Foxp3^+CD25^+$ lymphocyte phenotype by PRRSV-infected DCs

In vitro induction of $Foxp3^+CD25^+$ T cells by PRRSV-infected DCs was evaluated by two color flow cytometry. Co-culture of lymphocytes with PRRSV-infected DCs for five days increased the percentage of $Foxp3^+CD25^+$ T cell subset and this effect was reversed significantly by IFN-α treatment (Fig. 1A, I–III). Compared to mock-treated DCs, a $Foxp3^+CD25^+$ T cell subset was significantly increased following co-culture of lymphocytes with CIAD008-infected DCs ($p<0.01$) or the NVSL-infected DCs ($p<0.01$) (Fig. 1B). Interestingly, addition of IFN-α significantly reduced the induction of the $Foxp3^+CD25^+$ T cell subset by both the CIAD008-infected DCs ($p<0.01$) and the NVSL-infected DCs ($p<0.05$) (Fig. 1B). In addition, heat inactivation (HI) of both PRRSV strains abrogated induction of the $Foxp3^+CD25^+$ T cell subset suggesting that virus viability is required (Fig. 1B). Previous reports have mentioned that IFN-α inhibits PRRSV replication but not infection of susceptible cells (Loving et al., 2007). To confirm that IFN-α blocks PRRSV replication in infected DCs, PRRSV viral load was quantified by real-time PCR and no changes were observed following

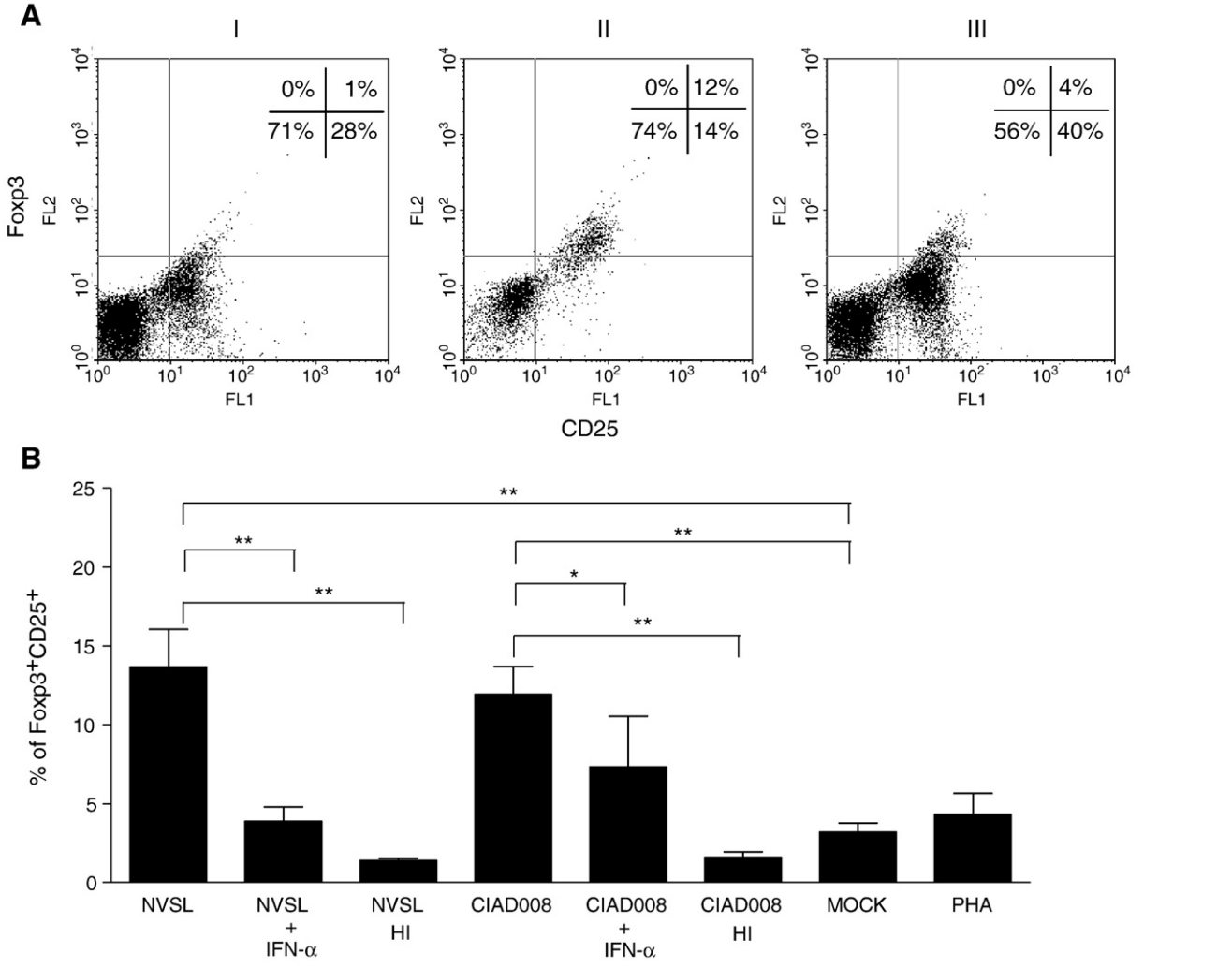

Fig. 1. Effect of two distinct PRRSV strains on induction of Foxp3+$CD25^+$ T cells. (A) Expression profile of $Foxp3^+$, $CD25^+$, and $Foxp3^+CD25^+$ was analyzed by flow cytometry in lymphocytes co-cultured for five days with PRRSV-infected (strain NVSL) DCs. I) mock-treated DCs; II) PRRSV-infected DCs; and III) PRRSV-infected DCs treated with IFN-α before and after infection. (B) The percentage of $Foxp3^+CD25^+$ T cells induced by NVSL-or CIAD008-infected DCs was quantified by flow cytometry using cells from five-day co-cultures. The data is from six independent experiments ($n=6$ pigs). Data analysis was done using a paired *t*-test and significant differences are shown (*$p<0.05$, and **$p<0.01$). HI, heat-inactivated virus.

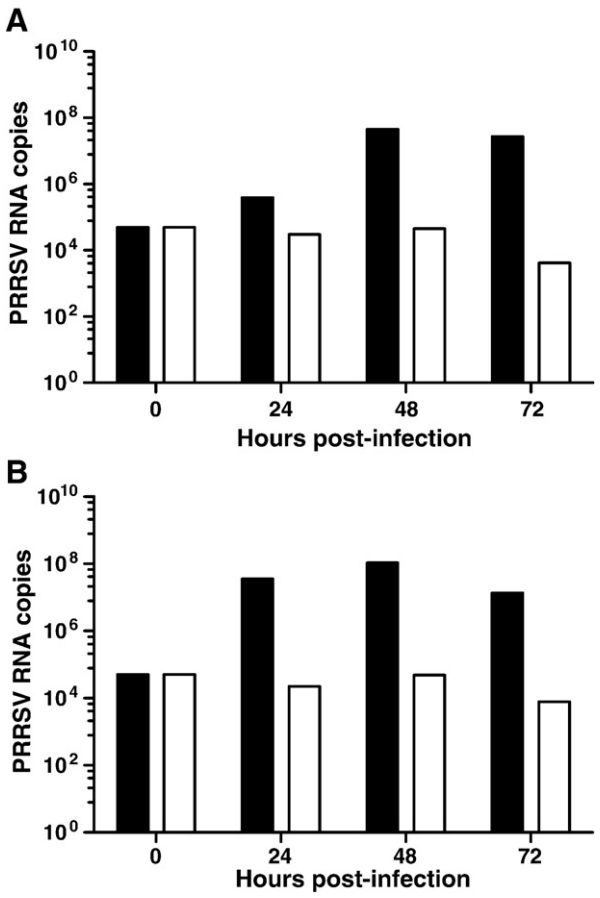

Fig. 2. Effect of IFN-α on PRRSV-infected DCs. PRRS viral RNA was quantified by real-time PCR at different time points after infection of DCs with PRRSV strain (A) NVSL; and (B) CIAD008 without IFN-α (black bars) or with IFN-α (white bars) treatment. PRRSV load is expressed as RNA copies-log/reaction.

treatment (Fig. 2). This outcome supports previous observations that PRRSV is capable of modulating porcine immune system, including reduced lymphocyte proliferation induced by PRRSV-infected DCs (Flores-Mendoza et al., 2008; Wang et al., 2007) and is consistent with the documented evidence that induction of T regulatory cells is a frequent event during viral infections, and can be induced both *in vivo* and *in vitro* (Balkow et al., 2007; Billerbeck et al., 2007; Estes et al., 2006; Granelli-Piperno et al., 2004; Krathwohl et al., 2006; Li et al., 2008).

Up-regulation of TGF-β expression by PRRSV

Up-regulation of IL-10 and TGF-β mRNA in lymphocytes co-cultured with PRRSV-infected DCs was evaluated by real-time PCR, whereas ELISA was used to quantify expressed proteins in the supernatants. Compared to mock treatment, TGF-β mRNA, but not IL-10 mRNA, was significantly ($p<0.05$) up-regulated in lymphocytes co-cultured with DCs infected with the CIAD008 or the NVSL PRRSV strains and both strains had similar effects on TGF-β mRNA up-regulation (Figs. 3A, B). Treatment with IFN-α showed a small but not significant reduction in the TGF-β mRNA expression (Fig. 3B). No IL-4 and IFN-γ mRNA changes were detected (Figs. 3C, D). Surprisingly, addition of IFN-α to lymphocytes co-cultured with the CIAD008-infected, but not the NVSL-infected DCs, significantly ($p<0.05$) up-regulated IFN-γ mRNA expression (Fig. 3D).

Compared to mock treatment, co-culture of lymphocytes with CIAD008- or NVSL-infected DCs, significantly ($p<0.05$) increased production of TGF-β, but not IL-10, protein and this outcome was reversed by addition of IFN-α (Fig. 4). This data suggests that PRRSV-infected DCs preferentially induce TGF-β-secreting immunosuppressive T lymphocytes which fit the previously described T helper (Th3) regulatory T lymphocyte phenotype (Belkaid, 2007; Shevach, 2006).

Up-regulation of Foxp3 mRNA

Real-time PCR was used to evaluate the mRNA profiles of the Foxp3, TBX21, and GATA-3 transcription factors in lymphocytes co-cultured with PRRSV-infected DCs. Compared to mock-treated DCs, NVSL-infected DCs significantly ($p<0.05$) up-regulated expression of Foxp3 mRNA (Fig. 5A). Although the CIAD008-infected DCs induced higher Foxp3 mRNA expression than NVSL-infected DCs, the difference was not significant ($p<0.08$) due to high variability (Fig. 5A) but the outcome was consistent with the finding that $Foxp3^+CD25^+$ T lymphocyte profile induced by the CIAD008-infected DCs was less affected by IFN-α treatment (Fig. 1B). This outcome suggests that Tregs induced by PRRSV are a consequence of Foxp3 up-regulation which is consistent with the previous demonstration that Foxp3 is required for the induction of Tregs (Billerbeck et al., 2007). The mRNA profile of the TBX21 and GATA-3 transcription factors which are responsible for the polarization of the immune response into Th1 or Th2, respectively, was not significantly changed following co-culture of lymphocytes with the PRRSV-infected DCs, and treatment with IFN-α did not have any effect (Figs. 5B, C). This outcome is consistent with previous results obtained in similar studies conducted in mice (Wei et al., 2007).

Suppressor activity of Tregs

An *in vitro* suppression assay was used to test whether Tregs induced by the PRRSV-infected DCs have suppressor activity on PHA-stimulated lymphocytes. Compared to mock treatment, lymphocytes previously co-cultured with the CIAD008- or the NVSL-infected DCs had a 58% suppressive effect on the proliferation of PHA-stimulated lymphocytes but IFN-α treatment did not reverse the suppressor activity (Fig. 6). This outcome is consistent with the previous demonstration that Tregs have suppressive activity on immunocompetent cells and function through several mechanisms (Bettelli et al., 2005; Miyara and Sakaguchi, 2007).

Discussion

In this study, a significant $Foxp3^+CD25^+$ T cell subset was induced in lymphocytes by the CIAD008- or the NVSL-infected DCs. The induced $Foxp3^+CD25^+$ T cell subset was associated with a significant up-regulation of TGF-β, but not IL-10, mRNA and protein expression. No significant IL-4 or IFN-γ mRNA changes were detected. The induced porcine $Foxp3^+CD25^+$ T cell phenotype closely resemble the TGF-β-secreting Th3 regulatory T cells described in humans and mice (Belkaid, 2007; Miyara and Sakaguchi, 2007; Shevach, 2006). This outcome supports the hypothesis that PRRSV induces regulatory T cells (Th3 Tregs cells) which, in part, could play a role in the documented delay in cellular immune responses in PPRSV-infected pigs early post-infection (Batista et al., 2004; Mateu and Díaz, 2008; Molitor et al., 1997; Murtaugh et al., 2002). This would be consistent with the previous demonstrations that viruses, such as HIV, HCV, EBV, CMV, HTLV, HBV, and FIV, modulate host immune responses by inducing Tregs and in this way persist in the host (Granelli-Piperno et al., 2004; Li et al., 2008). In pigs, natural $CD4^+CD25^{high}$ Tregs have been described, but to the best of our knowledge, this study describes *in vitro* pathogen-induced porcine Th3 Tregs for the first time (Kaser et al., 2008). Studies are underway to determine whether Th3 Tregs are induced *in vivo* upon PRRSV infection and if so, determine their profile during the ~30 days post-infection when most infected pigs shed infectious PRRSV. Understanding the role, if any, played by PRRSV-induced Tregs in delaying cellular immune responses early

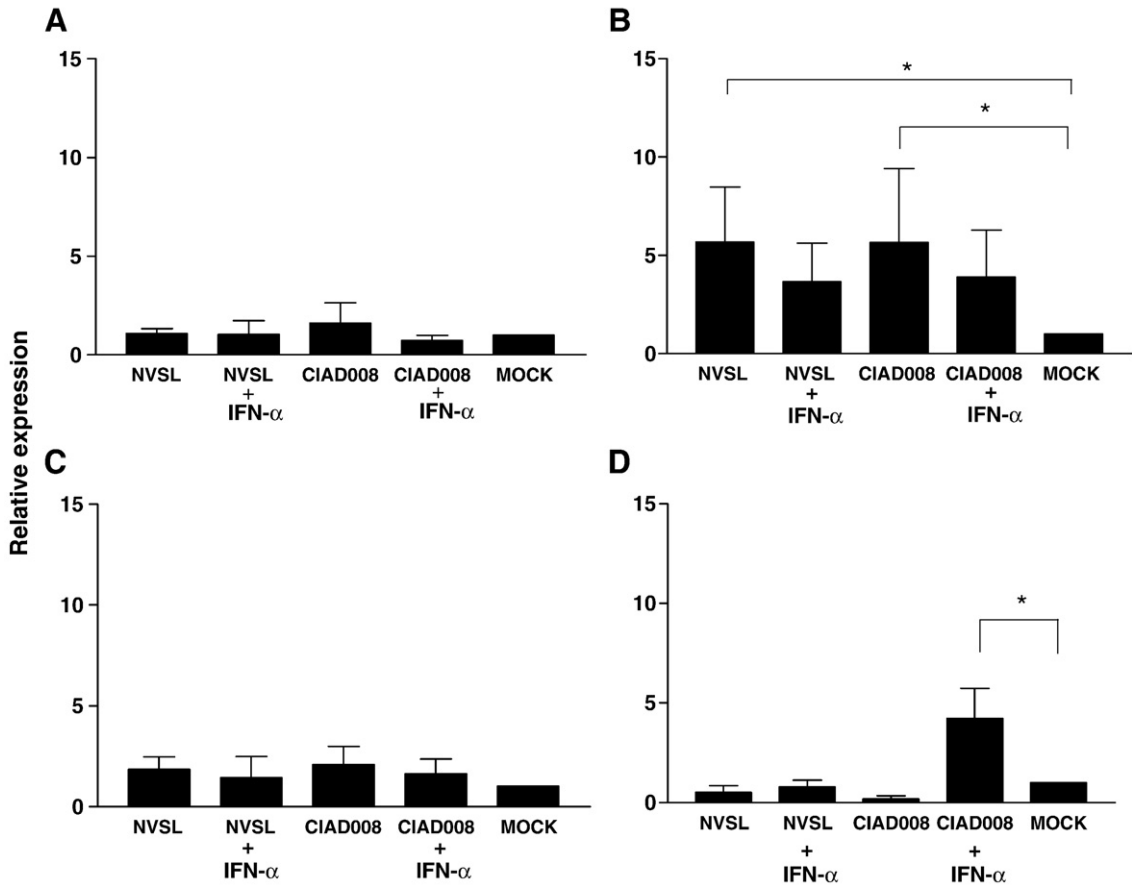

Fig. 3. Up-regulation of cytokine mRNA by PRRSV-infected DCs. Up-regulated of cytokine mRNA was evaluated by real-time PCR expression using cells from 24 h co-cultures. (A) IL-10; (B) TGF-β; (C) IL-4; and (D) IFN-γ. The data presented is the Mean ± SE of five independent experiments. Relative expression for each cytokine was calculated using the formula $2^{-\Delta\Delta Ct}$. Data analysis was done using a Kruskal–Wallis *Z* test and a $p<0.05$ was considered significant. *$p<0.05$.

post-infection is necessary for the development of contemporary vaccines and control strategies. In addition, determination of the immune mechanisms and factors involved in enabling infected pigs to naturally overcome the PRRSV-induced immuno-modulation and then clear the virus, will pave the way for the development of efficacious vaccines.

Induction of the Foxp3$^+$CD25$^+$ T cell subset by the CIAD008- or the NVSL-infected DCs was completely abolished by heat inactivation of the virus, suggesting that virus viability and hence the ability to infect host cells is required. This is consistent with previous findings that viability of pathogens is required for the induction of Tregs (Balkow et al., 2007). Interestingly, IFN-α treatment significantly reduced the induction of the Foxp3$^+$CD25$^+$ T cells by the NVSL- ($p<0.01$) or CIAD008-infected DCs ($p<0.05$) (Fig. 1B). Treatment of CIAD008-infected DCs with IFN-α inhibited virus replication (Fig. 3), but it had a minimal effect on Foxp3 expression and the induction of Foxp3$^+$CD25$^+$ T cells, suggesting that infection of DCs is sufficient to induce Tregs (Fig. 5A). The different susceptibility to IFN-α treatment has been reported to occur in field strains of PRRSV but the ability to withstand IFN-α-mediated clearance early post-infection does not seem to be necessary for the establishment of persistence (Díaz et al., 2006; Lee et al., 2004).

Naturally existing and pathogen-induced Tregs can inhibit priming of productive immune responses required for pathogen clearance, but Tregs can also play a role in the control of pathogen-induced inflammatory diseases (Belkaid, 2007; Sehrawat et al., 2008). The Tregs induced by the NVSL- or the CIAD008-infected DCs had a significant suppressive effect on the proliferation of PHA-stimulated PBMC. This outcome suggested that the induced Tregs function in a broad, non-pathogen-specific fashion and could function through the previously described mechanisms (Bettelli et al., 2005; Miyara and Sakaguchi, 2007). Consequently, the PRRSV-induced Tregs may hinder the ability of infected pigs to mount productive immune responses against PRRSV. Interestingly, the induction of the Foxp3$^+$CD25$^+$ by the PRRSV-infected DCs and the up-regulation of TGF-β expression could be reverted by IFN-α, but the suppressor activity of the induced Tregs was not affected, suggesting that the effector function(s) of the Tregs is not influenced by IFN-α.

It has been shown that PRRSV infection induces polyclonal B cell activation in piglets which results in lymphoid hyperplasia, hypergammaglobulinemia, and autoimmunity (Butler et al., 2007; Lemke et al., 2004). There is no evidence that PRRSV infects B cells and the mechanism(s) involved in the selection of the B cells that proliferate upon infection has not been studied (Therrien et al., 2000). Surprisingly, the PRRSV-responsive B cells constitute a minor population and thus it can be hypothesized that the virus directly or indirectly creates favorable conditions to facilitate proliferation of this B cell sub-population that is normally deselected during development (Butler et al., 2007). It is speculated that a PRRSV-derived super antigen-like product engages a minor subset of naive B cells expressing identical hydrophobic H chain third complementary region (HCDR3) and directs T-independent, non-antigen-specific, B cell proliferation without repertoire diversification (Butler et al., 2007; Butler et al., 2008). It is likely that PRRSV-induced Tregs are indirectly responsible for this polyclonal B cell activation given that PRRSV infection hinders induction of productive immune responses capable of mediating virus clearance early post-infection. We hypothesize that PRRSV-induced Tregs delays development of PRRSV-specific immune

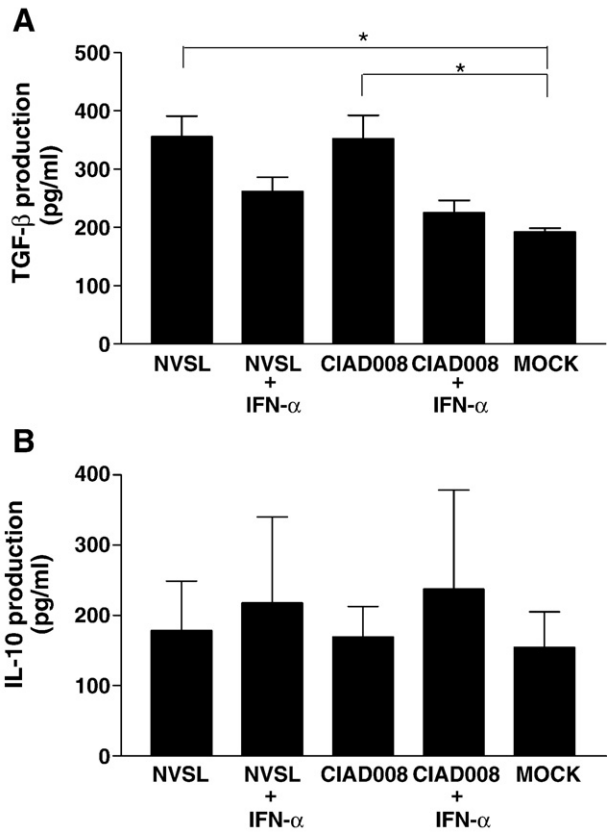

Fig. 4. Cytokine production. TGF-β and IL-10 in the supernatants of three-day old co-cultures of the lymphocytes and the PRRSV-infected DCs were quantified by ELISA. Differences among treatments ($n=6$ for TGF-β and $n=3$ for IL-10) were evaluated using one-way ANOVA. *Significant differences ($p<0.05$) were detected when compared to mock treatment.

effectors and this allows rapid virus expansion which in turn directs T-independent proliferation of the naïve HCDR3^{+} B cell population responsible for the PRRSV-induced pathology. In conclusion, the data from this study supports the hypothesis that the PRRSV succeeds to establish and replicate in porcine cells early post-infection, in part, by inducing Th3 regulatory cells as a mechanism of delaying PRRSV-specific porcine immune response.

Materials and methods

Viruses and cells

The PRRSV NVSL 97-7895 (GenBank accession no. AY545985) and CIAD008 (GenBank accession no. DQ250071) strains were used. The viruses were propagated in MARC-145 cells using Dulbecco's modified Eagle medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO), 100 IU penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (Sigma, St Louis, MO, USA) (complete DMEM). Once the cytopathic effect was apparent, cell cultures were freeze-thawed twice and the cell lysates were centrifuged at 650 ×*g* at 4 °C for 20 min. The supernatant containing the virus was collected, titrated, and stored at −70 °C.

Isolation of porcine PBMCs

Peripheral blood from PRRSV-free pigs was collected into heparin-coated collection tubes (Becton-Dickinson, BD), diluted 1:2 with DMEM (GIBCO), overlaid on Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden), and centrifuged at 500 ×*g* for 20 min. PBMCs were washed three times in DMEM, and resuspended in complete DMEM.

Generation of monocyte-derived DCs

Generation of DCs was conducted as previously reported (Flores-Mendoza et al., 2008). Briefly, freshly isolated PBMCs were placed into tissue culture flasks (Corning) and incubated overnight in complete DMEM at 37 °C in 5% CO_2 to allow monocytes to adhere. Non-adherent cells were removed by washing with DMEM and frozen for use in co-culture experiments. Adherent cells were cultured in complete DMEM containing 20 ng ml^{-1} of recombinant porcine GM-CSF (rpGM-CSF) and 20 ng ml^{-1} of recombinant porcine-interleukin 4 (rpIL-4) at 37 °C in 5% CO_2. Cells were incubated for 5 days with replacement of 50% of media on day 3. DCs were harvested on day 5 using cell dissociation

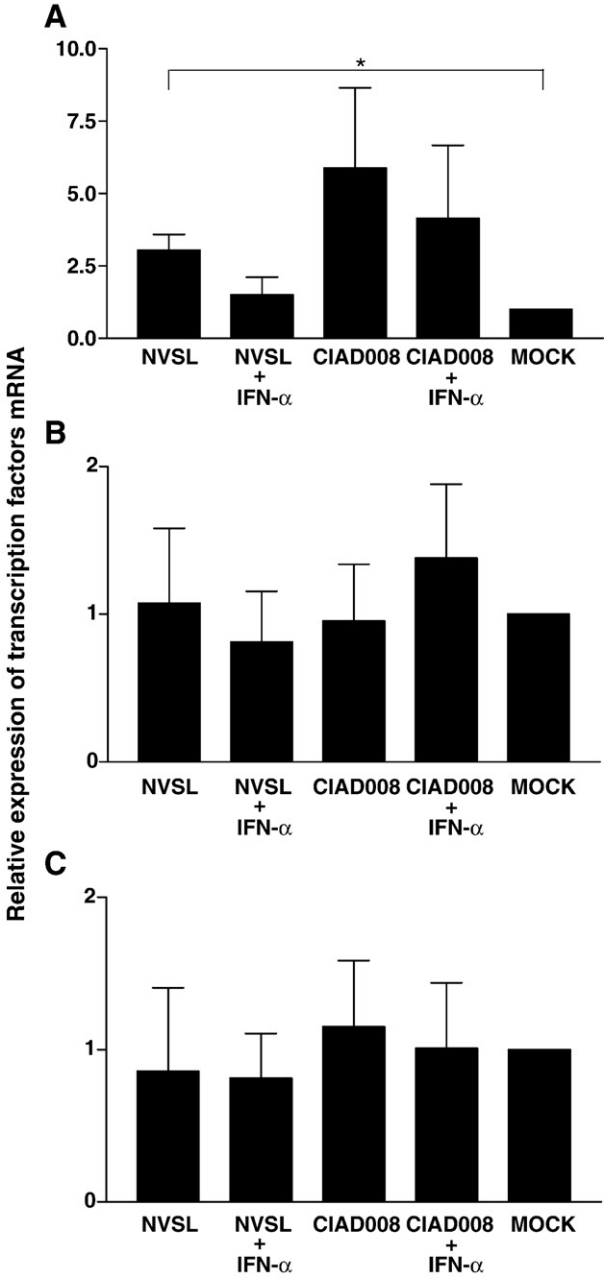

Fig. 5. Up-regulation of transcription factors. The relative expression of Foxp3, TBX21, and GATA-3 was evaluated in lymphocytes co-cultured for 24 h with PRRSV-infected DCs with or without IFN-α treatment. Relative expression for each cytokine was calculated using formula $2^{-\Delta\Delta Ct}$. The results presented are from five independent experiments and the data analysis was done using a Kruskal–Wallis *Z* test. A significant difference was detected when compared to mock treatment (*$p<0.05$).

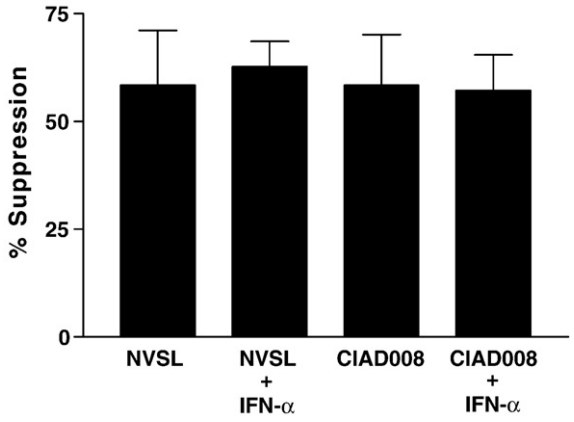

Fig. 6. Suppressor activity of Tregs induced by PRRSV-infected DCs. Suppressor activity of Tregs induced by PRRSV-infected DCs was evaluated by determining the reduction in proliferation of CFSE-labeled PHA-stimulated PBMC co-cultured with lymphocytes previously co-cultured for five days with PRRSV-infected DCs. Data presented is the Mean ± SE of six independent experiments. The percentage of suppression was calculated as follows: % suppression = 100 × [1 − (% proliferation w/PRRSV / % proliferation w/mock)].

enzyme-free Hanks'-based buffer (Gibco) and resuspended in complete DMEM with 10% of FBS.

Infection of DCs with PRRSV

Monocyte-derived DCs were infected with PRRSV at a multiplicity of infection (m.o.i.) of 0.1 for 1 h at 37 °C in complete DMEM containing 50 mM 2-mercaptoethanol (Sigma), washed three times at 200 g at 4 °C, and resuspended in fresh medium. Infected DCs (5×10^4) were seeded into 96-well tissue culture plates (Corning) and after 24 h, 5×10^5 lymphocytes (obtained by washing non-adherent cells after over night culture of PBMC) were added. In some experiment, DCs were pulsed with heat-inactivated (1 h at 56 °C) PRRSV, or treated with 200 U rpIFN-α (PBL Biomedical Laboratories) for 30 min prior to infection and during the co-culture with lymphocytes.

Flow cytometry

Co-expression of the CD25 and the Foxp3 markers by lymphocytes after 5 days of co-culture with PRRSV-infected DCs was evaluated by flow cytometry. Cells were harvested and stained with mouse anti-porcine CD25 (Serotec), followed by FITC-labeled goat anti-mouse IgG (Southern Biotech). For intracellular staining, the cells were fixed with 200 μl of 4% paraformaldehyde at 4 °C in the dark for 20 min followed by two washes with wash buffer (PBS containing 1% BSA and 0.02% Sodium azide), resuspended in 500 μl of wash buffer containing 0.1% saponin (permeabilization buffer) and incubated for 20 min at 4 °C in the dark. The cells were then washed once with permeabilization buffer and stained with mouse anti-human Foxp3 (Alexa Fluor®647-conjugate, clone 221D/D3, Serotec) for 30 min. Finally, the cells were washed twice and resuspended in wash buffer containing 1% paraformaldehyde. Flow cytometric analysis was conducted on the whole lymphocytes using FACSCalibur cytometer (BD) and analyzed using the WinMDI 2.9 software.

ELISA

Supernatants from a three-day co-culture of lymphocyte and PRRSV-infected DCs were collected, and the levels of secreted IL-10 and TGF-β were quantified using a commercial ELISA Kit according to the manufacturer's recommendations (Biosource, Camarillo, CA). Results are expressed as pg/ml.

Real-time PCR

IL-10, TGF-β, TBX21, GATA-3 and Foxp3 mRNA expression were quantified by real-time PCR. Viral load in PRRSV-infected DCs was also quantified by real-time PCR as previously described (Christopher-Hennings et al., 2006). Total RNA was extracted from co-cultures of lymphocytes and the PRRSV-infected DCs after 24 h using RNAeasy Protocol Mini Kit (Qiagen) according to the manufacturer's instructions. Real-time PCR was performed with one-step QRT-PCR Core Reagent Kits Brilliant® Master Mix (Stratagene, La Jolla, CA) and a SmartCycler system (Cepheid, Sunnyvale, CA). Amplification conditions were as follows: one cycle at 50 °C for 30 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Primers and probes are listed in Table 1. Ct values from different treatments were normalized against an endogenous control gene (peptidylprolyl isomerase A, PPIA) and differences in Ct values between the monocyte-depleted lymphocytes co-cultured with the PRRSV-treated DCs vs the lymphocytes co-cultured with mock-treated DCs were evaluated using the $2^{-\Delta\Delta CT}$ formula. Results are expressed as relative increments of mRNA between the treatment and control co-cultures.

Suppression activity assay

Suppressor activity of PRRSV-induced Tregs was tested by evaluating proliferation of PHA-treated (10 mg/ml) PBMCs (5×10^5) stained with CFSE and then co-cultured with five-day cultured lymphocytes (1×10^5) exposed to the PRRSV-infected DCs, with or without IFN-α treatment. Lymphocytes exposed to mock-treated DCs were used as negative control. Proliferation was assessed by flow cytometry using the fluorescent dye carboxifluorescein succinimidyl ester (CFSE; Molecular Probes). Briefly, PBMCs (10×10^6) were stained with 0.1 μM CFSE in RPMI-1640 medium for 10 min at 37 °C in the dark and, after addition of 10 ml RPMI-1640 containing 10% FBS (complete RPMI), the cells were centrifuged at 400 ×g for 10 min. 5×10^5 CFSE labeled-PBMC were co-cultured with 1×10^5 five-day cultured lymphocytes exposed to PRRSV-infected or rpIFN-α-treated DCs. Results are expressed as Mean ± SE percentage of suppression determined with the formula: % suppression = 100 × [1 − (% proliferation w/PRRSV / % proliferation w/mock)] (Brusko et al., 2007).

Table 1
Primer and probe sequences.

Gene	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Probe sequence (5′ to 3′)
IL-10[a]	TGAGAACAGCTGCATCCACTTC	TCTGGTCCTTCGTTTGAAAGAAA	TET-CAACCAGCCTGCCCCACATGC-BHQ1
TGF-β[a]	AGGGCTACCATGCCAATTTCT	CCGGGTTGTGCTGGTTGTACA	TET-CCTAGACACTCAGTACAGCAAGGTCCTGGC-BHQ1
TBX21[a]	TGGACCCAACTGTCAATTGCT	ACGGCTGGGAACGGGATA	TET-ACCACTACTCTCCTCTCCTCCCCAACCAGT-BHQ1
GATA-3[a]	TCTAGCAAATCCAAAAAGTGCAAA	GGGTTGAACGAGCTGCTCTT	TET-TCCTCCAGCGTGTCGTGCACCT-BHQ1
Foxp3[a]	CCCTGCCCTTCTCATCCA	GTGGCCCGGATGTGAAAA	TET-AGCCAGAGGACTTCCTCAAGCACTGCC-BHQ1
PPIA[b]	GCCATGGAGCGCTTTGG	TTATTAGATTTGTCCACAGTCAGCAAT	TET-TGATCTTCTTGCTGGTCTTGCCATTCCT-BHQ1

[a] Sequences from Porcine Immunology and Nutrition database (http://www.ars.usda.gov/Services/docs.htm?docid=6065).
[b] PPIA (peptidylprolyl isomerase A).

Statistical analysis

Data was analyzed using paired student *t*-test or one-way analysis of variance (ANOVA). Differences among treatments were determined by Tukey's test ($p<0.05$). Kruskal–Wallis test was used to assess effect of treatments in relative expression of cytokines. Data were analyzed using NCSS 2007 software.

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