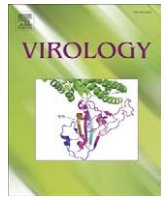




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## European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells

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### ABSTRACT

The aim of this study was to characterize the immune responses of DCs after infection with four different EU strains of PRRSV and whether they show any ability to immunomodulate T cells activation. Our results show that all EU strains can efficiently infect and replicate in DCs. Nevertheless, SLA-II levels remained unaltered in DC infected by all EU PRRSV strains, whereas SLA-I expression was only reduced when strain 2992 was used. IL-10 production was induced by three EU PRRSV strains, being strain 2992 the highest inducer. However, no induction of Treg cells, measured by CD25 and Foxp3 expression on lymphocytes co-cultured with infected DCs, was found. TGF- $\beta$  induction was not detected in DC infected with any EU strain tested. In conclusion, DCs infected with EU PRRSV strains exhibited an unbalanced ability to stimulate T cell response and was strain dependent. However, Treg cells were not induced, at least *in vitro*.

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### Introduction

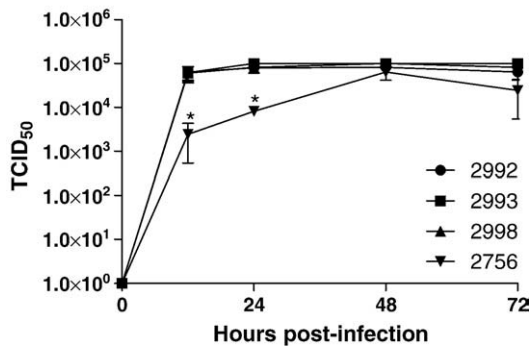
Porcine reproductive and respiratory syndrome (PRRS) is the most significant and economically important infectious disease affecting swine worldwide. The economic losses caused by the virus ascend to 560 million dollars per year (Neumann et al., 2005). The causative agent is a virus, named PRRSV (Meulenber et al., 1997), which belongs to the order Nidovirales, family Arteriviridae and genus Arterivirus (Cavanagh, 1997). Two genotypes of PRRSV have been described: the American and European (EU). Both genotypes share approximately 55–70% nucleotide identities (Andreyev et al., 1997; Mateu et al., 2006; Meng et al., 1994; Nelsen et al., 1999). In addition, pathogenic variations between American and EU PRRSV strains have been described. Some strains of American PRRSV are capable of replicating in pulmonary alveolar macrophages (PAM) and cell lines (CL2621, MARC-145 and CRL11171), whereas EU strains are preferentially propagated in PAM. Therefore, differences of cell susceptibility between EU and American PRRSV may indicate variants of pathogenesis between genotypes (Meng, 2000). In addition, several reports indicate that the EU genotype probably induces a lower viremia than the American strains in experimentally infected pigs (Batista et al., 2004; Diaz et al., 2005, 2006).

The development of adaptative immune responses for both genotypes is delayed, but the kinetics are different. Cell mediated immune (CMI) response of pigs infected with American PRRSV strains appears transiently at 2–8 weeks after infection and becomes more pronounced months later (Murtaugh et al., 2002; Xiao et al., 2004). Low levels of IFN- $\gamma$ -secreting cells are detectable at 2–4 weeks post-infection (PI) and a gradual increase in the intensity of the IFN- $\gamma$  response ensues at 5 months (Batista et al., 2004; Meier et al., 2003, 2004). In contrast, IFN- $\gamma$ -secreting cells in EU PRRSV-infected pigs are detectable even 7 days post-infection (PI) (Diaz et al., 2005, 2006), suggesting that the development of the immune response during American or EU PRRSV infection might be different, probably reflecting the interaction of PRRSV with the immune system.

The mechanisms involved in this unusual delayed immune response are still unknown, but apparently PRRSV is able to modulate immune responses at least during the initial PI weeks (Flores-Mendoza et al., 2008; Mateu and Diaz, 2008; Murtaugh et al., 2002). One possible mechanism proposed to explain the delayed immune response is the infection and immunomodulation of dendritic cell (DCs) function by PRRSV. It is widely accepted that American PRRSV strains infect monocyte-derived DCs and decrease the expression of molecules such as CD80/86, CD11d/c, CD14, and class I and II MHC (Flores-Mendoza et al., 2008; Loving et al., 2007; Wang et al., 2007). However, the ability of PRRSV to increase IL-10 production in antigen presenting cells (APC) is still controversial. Wang et al. (2007) reported no increase in IL-10 production in PRRSV-infected DCs. Con-

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**Fig. 1.** EU PRRSV infects and replicates in monocyte-derived DCs. Porcine DCs were generated from monocytes and infected with four different strains of EU PRRSV (2992, 2993, 2998 and 2756) at m.o.i. of 0.1. Supernatants from cultures were taken at 14, 24, 48 and 72 h PI and PRRSV was quantified in alveolar macrophages. The results are expressed as TCID<sub>50</sub>. Data represent the mean  $\pm$  SEM of  $n = 5$ . \* represents significant differences between virus.

versely, Charerntantanakul et al. (2006) observed that IL-10 expression was increased in monocytes and Flores-Mendoza et al. (2008) reported that mature PRRSV-infected DCs show an increment of IL-10. As a consequence, DCs infected with the American strain of PRRSV remain in immature state and produce cytokines to promote the induction of regulatory T cells (Silva-Campa et al., 2009). Therefore, PRRSV infection provokes impaired antigen presentation and induces the production of anti-inflammatory cytokines like IL-10 and TGF- $\beta$ .

Regulatory T cells (Tregs) are responsible for controlling the immune response and maintaining homeostasis, suppressing or controlling the function of effectors and immunocompetent cells. Tregs are classified into natural and induced, which control the immune response against themselves or exogenous antigen, respectively (Belkaid, 2007; Miyara and Sakaguchi, 2007). Recently, Tregs have been related with chronic or persistent infection establishment caused by viruses such as human immunodeficiency virus, hepatitis virus C and B, cytomegalovirus, and Epstein-Barr virus (Kinter et al., 2004; Peng et al., 2008; Rieger et al., 2006; Smyk-Pearson et al., 2008;

Vahlenkamp et al., 2005; Voo et al., 2005). As a whole, Tregs are one of the mechanisms used by pathogens to escape from the immune response.

DC infection by American PRRSV strains has been characterized in several ways; however, no data is available for EU PRRSV strains. Taking into account their different ability to induce or not IL-10-secreting cells from PBMCs of infected pigs (Diaz et al., 2005; Mateu and Diaz, 2008), the aim of the present study was to characterize the immune responses of DCs after infection with EU strains of PRRSV and their capacity to induce lymphocyte activation and Tregs cells *in vitro*.

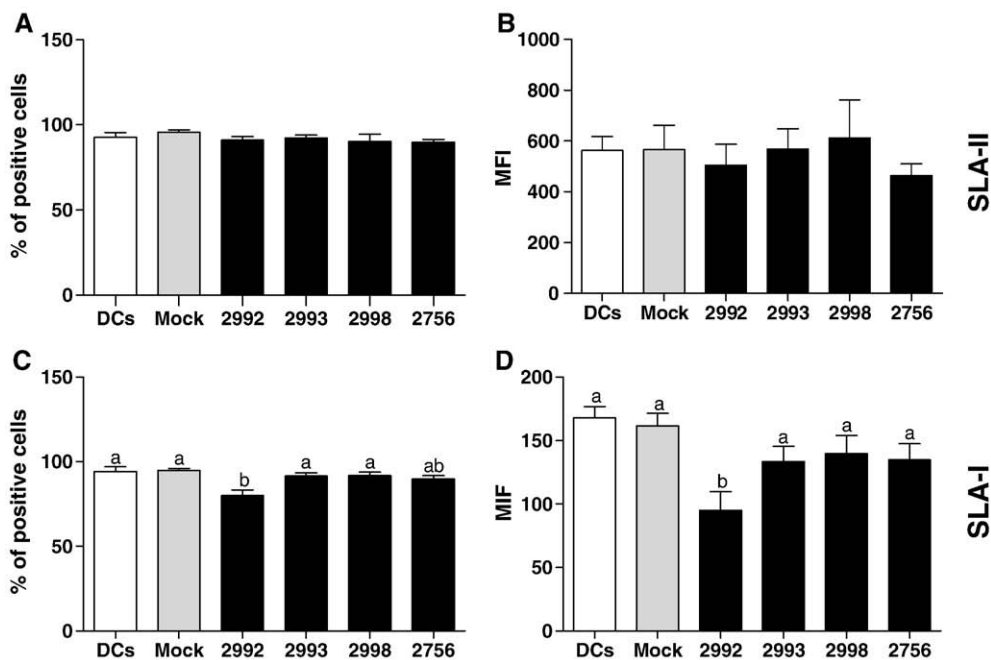
## Results

### EU PRRSV can infect and replicate in monocyte-derived DCs

Firstly, we aimed at determining if the EU genotype of PRRSV was able to productively infect DCs. Four different strains of PRRSV (2992, 2993, 2998 and 2756) were used to evaluate replication at different times PI. Fig. 1 showed that all strains of EU genotype of PRRSV were able to replicate in DCs, most of them showed a  $10^5$  TCID<sub>50</sub> titer at 14-h PI and this level remained until 72-h PI. However, strain 2756 differed from the other three since it replicated at a lower level as compared with the rest of the strains during the first 24-h PI ( $p < 0.05$ ). After 48-h PI, no significant differences were found among strains in their replication ability in DCs (Fig. 1). The variation in the replication kinetics might be related to different immunopathological effects of PRRSV UE strains.

### Expression of SLA-I and SLA-II on PRRSV-infected DCs

The effects of infection and replication of EU PRRSV strains on DCs were subsequently evaluated through expression of surface markers such as SLA-II and SLA-I after 24-h PI. The percentage of DCs expressing SLA-II compared with mock-treated DCs ( $95.7 \pm 2.5\%$ ) was not affected after PRRSV infection regardless of the strain used: 2992 ( $91 \pm 4.2\%$ ), 2993 ( $92.2 \pm 3.5\%$ ), 2998 ( $90.2 \pm 4.27\%$ ), and 2756 ( $90 \pm 3.3\%$ ) ( $p > 0.05$ ) (Fig. 2A). In addition, no changes were ob-

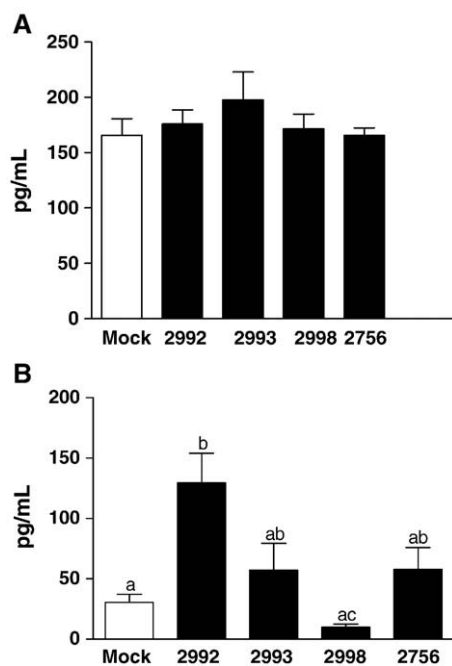


**Fig. 2.** Expression of SLA-I and SLA-II in PRRSV-infected DCs. The surface markers SLA-II and SLA-I were evaluated in DCs infected at m.o.i. of 0.1 at 24-h PI. On the left side, the percentage of positive cells for each marker is shown: (A) SLA-II and (C) SLA-I. The values on the right side represent the median fluorescent intensity (MFI) for (B) SLA-II and (D) SLA-I. The figure shows mean  $\pm$  SEM of  $n = 4$ . Statistical analysis was done using one-way ANOVA and mean differences among strains were determined by Tukey's test. Bars showing different letters represent values significantly different from each other ( $p < 0.05$ ).

served on SLA-II mean fluorescence intensity (MFI) after infection (Fig. 2B). In contrast, the percentage of cells expressing SLA-I decreased in DCs infected with strain 2992 ( $80 \pm 6.3\%$ ) as compared to mock-treated DCs ( $94.75 \pm 2.5$ ) ( $p < 0.05$ ) (Fig. 2C). Also, the MFI value of SLA-I molecules decreased in infected cells as compared to mock-treated cells ( $95 \pm 29$  and  $162.8 \pm 19$ , respectively) ( $p < 0.05$ ) (Fig. 2D). No significant changes were observed neither in the percentage nor in MFI values of SLA-I molecules in DCs infected with the other three strains analyzed: 2993 ( $91.5 \pm 3.9\%$ ), 2998 ( $91.75 \pm 4\%$ ) and 2756 ( $89.75 \pm 3.8\%$ ) (Figs. 2C and D). The fact that SLA-I and SLA-II expression remained unaltered after infection with three out of four EU PRRSV strains may indicate that infected DCs may remain in an “immature state,” resembling mock-treated DC.

#### Production of suppressor cytokines by infected DCs

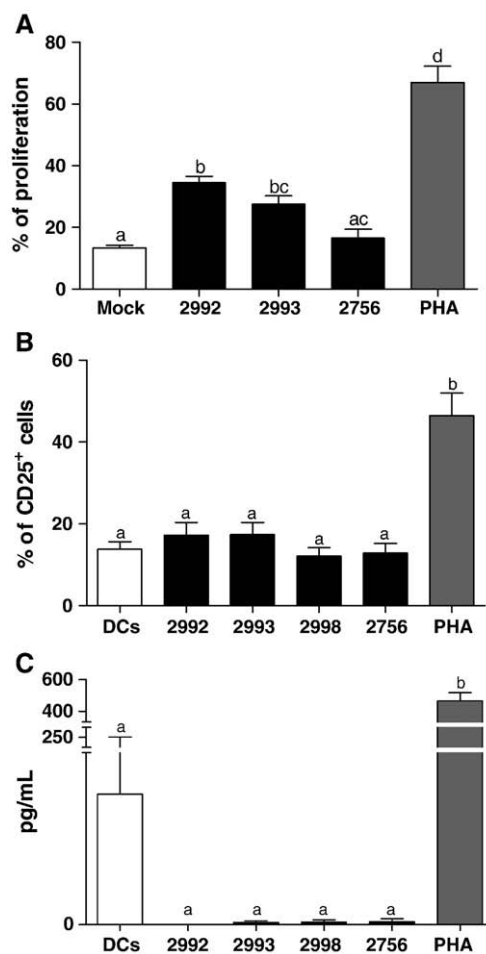
Dendritic cells are able to orchestrate immune responses not only through the expression of surface molecules, but also by secreting cytokines. Therefore, we investigated whether EU strains of PRRSV were able to modulate cytokine production in infected DCs through secretion of regulatory cytokines such as IL-10 and TGF- $\beta$ . The results showed that TGF- $\beta$  production was not induced by EU strains of PRRSV-infected DCs 2992 ( $176 \pm 28.6$  pg/dL), 2993 ( $197.5 \pm 6.8$  pg/dL), 2998 ( $171.5 \pm 32.7$  pg/dL), or 2756 ( $165.5 \pm 17.1$  pg/dL), as compared with mock-treated DCs ( $165.7 \pm 36.9$  pg/dL) ( $p > 0.05$ ) (Fig. 3A). Conversely, IL-10 induction was related to each EU PRRSV strain used. Dendritic cells infected with strain 2992 exhibited the highest IL-10 level ( $129.7 \pm 54$  pg/dL) ( $p < 0.05$ ), followed by DC infected with 2993 and 2756 strains ( $57.2 \pm 9.9$  pg/dL and  $57.7 \pm 18.2$  pg/dL, respectively), as compared with mock-treated cells ( $30.5 \pm 15.3$  pg/dL). However, IL-10 induction observed in DCs infected with 2993 or 2756 was not statistically significant ( $p > 0.05$ ) (Fig. 3B). IL-10 induction of DC infected with strain 2998 ( $9.85 \pm 6.3$  pg/dL) was below mock-infected DC level.



**Fig. 3.** Production of regulatory cytokines by infected DCs. TGF- $\beta$  (A) and IL-10 (B) production was quantified on DC supernatant infected with PRRSV strains at 24-h PI by ELISA. Differences among treatments ( $n = 5$ ) were evaluated using one-way ANOVA. Significant differences were detected by Tukey's test. Bars showing different letters represent values significantly different from each other ( $p < 0.05$ ).

#### Effect on lymphocyte proliferation induction by infected-dendritic cells

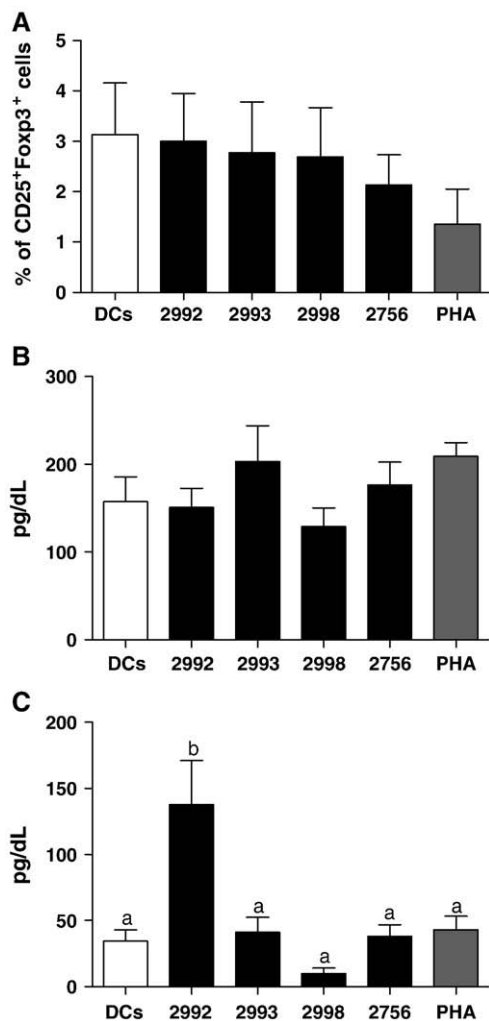
Secretion of IL-10 by PRRSV-infected DCs would bias immune responses in general, and particularly it would affect lymphocyte function. Thus, proliferation and IFN- $\gamma$  production by lymphocytes stimulated with infected DCs was determined. Proliferation of lymphocytes was analyzed using CFSE staining followed by flow cytometry at 5 days of co-culture. The results in Fig. 4 showed that PRRSV-infected DCs with strains 2992 and 2993 induced a significant increase of lymphocyte proliferation ( $34.4 \pm 5\%$  and  $27.5 \pm 6.6\%$ , respectively), as compared with lymphocytes stimulated with uninfected control DCs ( $13.4 \pm 1.9\%$ ) ( $p < 0.05$ ). The rest of PRRSV strains analyzed did not induce cell proliferation ( $p > 0.05$ ) (Fig. 4A). Although the proliferative response induced by DCs infected with strains 2992 and 2993 could indicate an apparent activation of lymphocytes, no changes in the percentage of lymphocyte CD25 expression were observed as compared with levels observed in lymphocytes stimulated with control DCs: 2992 ( $17.8 \pm 10.4\%$ ), 2993 ( $17.4 \pm 9.7$ ), 2998 ( $12.1 \pm 5.9$ ), 2756 ( $12.9 \pm 7.3\%$ ) and control ( $13.8 \pm 6.2\%$ ) (Fig. 4B). Moreover, infected DCs with any EU PRRSV strain were unable to induce IFN- $\gamma$  production in T cells (Fig. 4C).



**Fig. 4.** Effect on lymphocyte activation by infected DCs. Infected DCs (24-h PI) were co-cultured with syngeneic lymphocytes (ratio 1:10, respectively). (A) Five days after, co-culture proliferation was evaluated using CFSE. Lymphocytes co-cultured with mock-treated DCs were used as negative control, whereas PHA stimulated ( $10 \mu\text{g/mL}$ ) lymphocytes served as positive control. Data represent the mean  $\pm$  SEM ( $n = 6$ ). Differences among treatments were determined using a t-test ( $p < 0.05$ , compared with negative control). (B) IFN- $\gamma$  production was quantified by ELISA from 3-day co-cultures. (C) CD25<sup>+</sup> cells were evaluated by flow cytometry after 5 days of co-culture. Graphs represent the mean  $\pm$  SEM of  $n = 5$ ; data were analyzed by one-way ANOVA and Tukey's test for mean differences. Bars showing different letters represent values significantly different from each other ( $p < 0.05$ ).

### Induction of regulatory T cells (CD25<sup>+</sup>Foxp3<sup>+</sup>)

The immature state observed in DCs after PRRSV infection, the induction of IL-10 by some PRRSV strains and the absence of T cell activation suggested a possible induction of regulatory T cells by EU PRRSV-infected DCs. Thus, expression of CD25 and Foxp3 in autologous lymphocytes after co-culture with PRRSV-infected DCs was determined. No evidence of Tregs induction was found by means of any increment in CD25<sup>+</sup>Foxp3<sup>+</sup> T cell percentage (Fig. 5A), even when using DCs infected with strain 2992 which showed increased IL-10 production (Fig. 3B). In addition, production of regulatory cytokines, such as TGF- $\beta$  and IL-10, was determined in co-culture supernatants. No changes were found on TGF- $\beta$  production by lymphocytes stimulated with infected DCs with strain 2992 (150.5  $\pm$  65.2 pg/dL), 2993 (203  $\pm$  121.9 pg/dL), 2998 (129  $\pm$  51.6 pg/dL), or 2756 (176.3  $\pm$  79 pg/dL) in comparison to control uninfected DCs (157.4  $\pm$  84.4 pg/dL) (Fig. 5B). Again, only co-cultures with DCs infected with strain 2992 were capable of inducing IL-10 production, showing a 4-fold increment as compared with control DCs (137.8  $\pm$  124.6 vs. 34.5  $\pm$  31.7 pg/dL, respectively) ( $p < 0.05$ ). No significant increase was



**Fig. 5.** Induction of regulatory T cells (CD25<sup>+</sup>Foxp3<sup>+</sup>). (A) Expression profile of Foxp3<sup>+</sup>, CD25<sup>+</sup>, and Foxp3<sup>+</sup>CD25<sup>+</sup> was analyzed by flow cytometry in lymphocytes co-cultured for 5 days with PRRSV-infected DCs. In addition, regulatory cytokine profile was evaluated by quantification of (B) TGF- $\beta$  and (C) IL-10 was quantified by ELISA in the supernatants from 3-day co-cultures of lymphocytes and PRRSV-infected DCs. Data represent the mean  $\pm$  SEM of  $n = 11$ ; differences were determined using one-way ANOVA and Tukey's test. Bars showing different letters represent values significantly different from each other ( $p < 0.05$ ).

observed in co-cultures stimulated with DCs infected with any other strain tested: 2993 (41.7  $\pm$  42.5 pg/dL), 2998 (9.9  $\pm$  14.5), and 2756 (38.1  $\pm$  32.2) (Fig. 5C).

### Regulation of mRNA expression of regulatory cytokines in lymphocytes

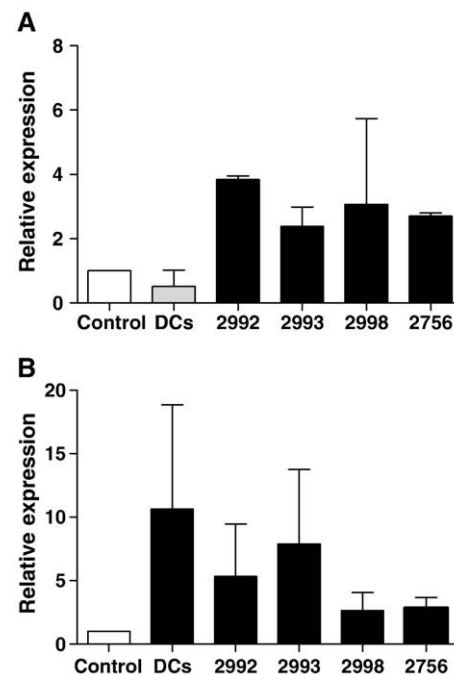
To evaluate the effects on cytokine regulation at the transcriptional level in lymphocytes stimulated with PRRSV-infected DCs, mRNA expression of regulatory cytokines was quantified by real-time PCR from co-cultures. Fig. 6A showed that mRNA expression of IL-10 was higher using strain 2992 (3.8-fold increment) and this is in agreement with the results obtained in Fig. 3B. IL-10 mRNA increment expression from samples infected with the other strains ranged between 3- and 2.3-fold increments. Fig. 6B showed that TGF- $\beta$  mRNA levels remained unaltered after infection with any of the strains used.

### Expression of regulatory Foxp3 transcription factor in lymphocytes

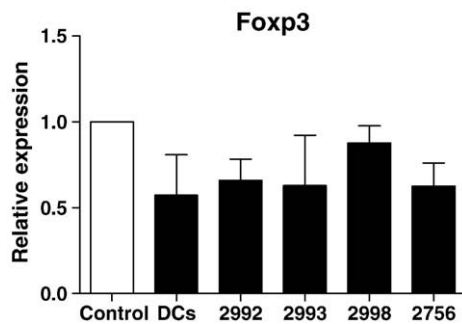
To further characterize the type of response induced by PRRSV-infected DCs, expression of Foxp3 transcription factor was analyzed in T cells from co-culture experiments by quantitative real-time PCR. Fig. 7 showed that Foxp3 mRNA levels did not vary in DC infected with any of the EU PRRSV strains. This result was in agreement with the data previously obtained using flow cytometry (Fig. 5).

### Discussion

In this study, we evaluated the effects of four EU PRRSV strains on DCs function. Our results indicated that EU PRRSV strains were capable of efficiently replicating in monocyte-derived DCs and only one EU PRRSV strain down-regulated SLA-I expression in infected DCs, although there was a tendency to decrease the expression of this



**Fig. 6.** Regulation of mRNA expression of regulatory cytokines in lymphocytes. Regulatory cytokine mRNA relative expression was evaluated by real-time PCR expression using cells from 24-h co-cultures. Relative expression of (A) IL-10 and (B) TGF- $\beta$  was calculated using the  $2^{-\Delta\Delta Ct}$  method. Non-stimulated lymphocytes were used as control. Data presented correspond to the mean  $\pm$  SEM of five independent experiments. Data analysis was done using a Kruskal-Wallis Z test and a  $p < 0.05$  was considered significant.



**Fig. 7.** Expression of regulatory Foxp3 transcription factor on lymphocytes. Relative expression of Foxp3 was evaluated in lymphocytes co-cultured for 24-h with PRRSV-infected DCs. Relative expression was calculated as previously described using formula  $2^{-\Delta\Delta C_t}$ . Results presented are from three independent experiments and data analysis was done using a Kruskal-Wallis Z test and a  $p < 0.05$  was considered significant.

molecule in DCs infected with the other three strains. No alteration for SLA-II expression was detected in any of the EU PRRSV strains tested, at least at 24-h PI. These results may indicate that regardless of the strain used, DCs remain in an immature state of activation after EU PRRSV infection. Equivalent results have been previously reported in DCs infected with American PRRSV strains (Flores-Mendoza et al., 2008; Wang et al., 2007). One important issue in this study was to compare the immunomodulatory properties of several circulating EU PRRSV field strains by means of cytokine induction pattern in infected DCs. One previous report suggested differences in cytokine production by DCs infected with different EU PRRSV strains (Mateu and Diaz, 2008). Following the same line, the results in this study clearly showed that different strains of EU PRRSV were able to differentially induce an immunoregulatory cytokine, IL-10, whereas TGF- $\beta$  remained unaltered (Fig. 3).

PRRSV-infected dendritic cell effects on lymphocyte proliferation were also strain dependent. Similar studies using DCs infected with American strains of PRRSV demonstrated that infected DCs were unable to stimulate lymphocyte proliferation above mock-infected levels (Flores-Mendoza et al., 2008; Loving et al., 2007; Wang et al., 2007). However, our results showed that EU PRRSV strains behave differently as proliferation of autologous lymphocytes increased in two of the four EU PRRSV strains tested. Differences among American and EU strains may be due to multiple factors, including unaltered SLA-II expression in DCs infected with EU strains, at least at 24-h PI. In fact, as SLA-II expression remains invariable, DCs infected with EU strains apparently have the capacity to stimulate CD4 T cell response. Indeed, two (strains 2992 and 2993) out of four EU PRRSV strains were able to induce a significant increase in lymphocyte proliferation as compared to mock-treated cells. However, no significant differences were observed in CD25 (IL-2 $\alpha$  chain receptor) expression in lymphocytes although there was a slight increase in CD25 when strains 2992 and 2993 were tested. Moreover, IFN- $\gamma$  production was not induced in response to any EU PRRSV strains (Fig. 4). These results suggested that some EU strains of PRRSV were able to modulate DCs function to a certain extent inducing lymphocyte proliferation, slightly dependent on CD25, but independent from IFN- $\gamma$  production, at least below our detection level.

Impaired lymphocyte activation could be the result of absent co-stimulation. Several reports indicate that CD80/86 co-stimulatory molecule expression is diminished in DCs infected with American PRRSV strains. Co-stimulation is very important for Th1 polarization (Wang et al., 2007). Unfortunately, we were not able to evaluate the expression of this molecule in our study. However, not only surface-expressed co-stimulatory molecules are responsible for lymphocyte activation but also the cytokines secreted by infected cells have an important role on the development of T helper response (Banchemau and Steinman, 1998). To this regard, IL-10 and TGF- $\beta$  production was

determined on infected DCs. TGF- $\beta$  was not increased in infected DCs with any EU PRRSV strain. This result differs from the study using DCs infected with American strains, where the relative expression of TGF- $\beta$  mRNA increased with respect to the mock control (unpublished observations from our research group). In contrast, IL-10 was induced by some PRRSV strains. These results agree with Diaz et al. (2006) who observed a strong IL-10 production by some EU strains of PRRSV that correlated with strain virulence.

Taken together, our results on IL-10 production, immature state of DCs infected with EU PRRSV and impaired T cell response could indicate T regulatory cell induction by EU PRRSV. The existence of porcine Treg cells with phenotype CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> was recently reported (Kaser et al., 2008a). Similar to human and mouse, two subtypes are present in swine: natural (none or low IL-10 producers) and inducible Tregs (TGF- $\beta$  producer) (Kaser et al., 2008a, 2008b; Silva-Campa et al., 2009). Treg cells in human and mouse are induced by DCs directly through IL-10 and TGF- $\beta$  production in response to pathogens, more commonly associated to viral or parasitic infections (Amarnath et al., 2007; Belkaid, 2007; Li et al., 2008; Voo et al., 2005). Our results support the idea that CD25<sup>+</sup>Foxp3<sup>+</sup> T cell population is unaffected when lymphocytes are stimulated by DCs infected with any EU PRRSV strain. These results differ from our previous observations where Tregs were induced by DCs infected with American PRRSV strains (Silva-Campa et al., 2009). In addition, IL-10 induction was only detectable on supernatants of lymphocytes treated with DCs infected with the IL-10-inducing strain (2992). It is well known that IL-10 production is a common mechanism used by viruses to suppress or delay the host immune response in pigs (Redpath et al., 2001). Conversely, no changes in IL-10 were observed in supernatant from lymphocytes co-cultured with American PRRSV strain-infected DCs on similar experiments (Silva-Campa et al., 2009).

IL-10 has been shown to be a critical cytokine with pleiotropic and somehow contradictory properties, particularly in viral infections (Couper et al., 2008; Vicari and Trinchieri, 2004). The picture that is emerging is that in viral infections of low to moderate virulence, as occurs with EU PRRSV strains, IL-10 from DCs or macrophages drives the production of IL-10 by natural Tregs, preventing pathology but allowing long-term escape of virus from immune control. Alternatively, in high virulent infections giving rise to strong proinflammatory responses, as occurs with American PRRSV strains, IL-10 production from induced Tregs seems to be required. However, if the source and thus the timing of IL-10 secretion are inappropriate, overwhelming infection or severe tissue damage, respectively, will result. Given the available data on American and EU PRRSV strains, it is plausible to think that differences in IL-10 production in PRRSV-infected pigs could be due to the differential and close interaction of PRRSV strains with DCs.

Moreover, no induction of TGF- $\beta$  by EU PRRSV strains by either DCs or DCs-stimulated lymphocytes was found. This is an important finding if considering that one of the most remarkable differences between American and EU strains was the ability of TGF- $\beta$  induction. Royae et al. (2004) reported that IL-10 and TGF- $\beta$  gene expressions were increased at 2 weeks post-vaccination with the American PRRSV strain. In contrast, Diaz et al. (2005) reported no significant differences in TGF- $\beta$  production from pigs infected with EU PRRSV, whereas IL-10 levels increased in culture supernatants of PRRSV-stimulated PBMC obtained from infected pigs. Lack of Treg cell induction by EU PRRSV strain could be explained by the absence of TGF- $\beta$ , since this cytokine is essential for the de novo induction of Foxp3, which is directly involved in regulating the differentiation and function of Tregs (Belkaid, 2007; Coombes et al., 2007; Shevach, 2006; Walker et al., 2005).

In conclusion, our results show that DCs infected with EU PRRSV strains exhibited an unbalanced ability to stimulate T cell immune responses. Additionally, immunomodulation of T cell responses was strain dependent. However, Treg cells were not induced, at least in *in*

*vitro* conditions. All in all, these data suggested that induction of regulatory T cells in swine can be determined by TGF- $\beta$  presence but not IL-10, just as in humans and mice.

## Materials and methods

### Viruses and cells

Four EU PRRSV strains (2992, 2993, 2998 and 2756), isolated from Spanish farms between 2003 and 2006, were used in this study. Isolation was done in porcine alveolar macrophages (PAM). Macrophages were obtained from healthy pigs free from all major diseases including PRRSV, pseudorabies virus and classical swine fever virus and they were used at passage no. 3. Viruses were propagated in PAMs obtained as previously described (Mengeling et al., 1996) using Dulbecco's modified Eagle medium (DMEM; GIBCO) containing 10% heat-inactivated fetal calf serum (FCS), 50  $\mu$ M 2-mercaptoethanol, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Sigma) (complete DMEM). Once the cytopathic effect was apparent usually after 3 days in culture, cell cultures were freeze-thawed twice and cell lysates were centrifuged at 650 g at 4 °C for 20 min. Supernatant containing the virus was collected, titrated, and stored at -70 °C. Cells with the same treatment but without infection were used as control (mock).

### Isolation of porcine PBMCs

Porcine peripheral blood mononuclear cells (PBMC) were from blood of conventional PRRSV-free pigs and were separated by a gradient density centrifugation using Histopaque 1.077 (Sigma, Madrid, Spain). PBMCs were washed three times in DMEM, and resuspended in complete DMEM. Monocytes were depleted by adherence and lymphocytes were collected and frozen in complete medium containing 10% of dimethyl sulfoxide (Sigma).

### Generation of monocyte-derived DCs

DCs were generated as previously reported (Carrasco et al., 2001). Briefly, freshly isolated PBMCs were placed in tissue culture flasks and incubated overnight at 37 °C in 5% CO<sub>2</sub> to allow monocytes to adhere. Non-adherent cells (lymphocytes) 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 of recombinant porcine GM-CSF (rpGM-CSF) and 20 ng/mL of recombinant porcine-interleukin (rpIL-4) (R&D Systems) at 37 °C in 5% CO<sub>2</sub>. Cells were incubated for 5 days with cytokine-containing medium replacement on day 3. DCs were harvested on day 5 using cell dissociation enzyme-free Hank's-based buffer (Gibco) and resuspended in complete DMEM.

### PRRSV infection of DCs

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. In order to eliminate the non-absorbed virus, cells were washed three times at 200 g at 4 °C, and resuspended in fresh medium. Infected DCs were seeded onto 96-well ( $5 \times 10^4$ ) or 48-well ( $5 \times 10^5$ ) tissue culture plates. After 24 h, DCs

were collected for staining or added to each well with  $5 \times 10^5$  autologous lymphocytes for co-culture. To evaluate virus replication within the DCs, the supernatants of infected cells were collected at 14, 24, 48 and 72 h post-infection (PI) and titrated in alveolar macrophages (TCID<sub>50</sub>).

### Cell proliferation assay

Cell proliferation was evaluated by flow cytometry using the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes). Briefly, lymphocytes ( $10 \times 10^6$ ) were stained with 0.1  $\mu$ M CFSE in RPMI-1640 medium for 10 min at 37 °C in the dark. After addition of 10 mL RPMI-1640 containing 10% FBS (complete RPMI), the cells were centrifuged at 400g for 10 min, and the cell concentration was then adjusted to  $5 \times 10^6$  cells in complete RPMI. CFSE labeled-lymphocytes ( $5 \times 10^5$ ) were co-cultured with PRRSV-infected DCs at a ratio 1:10 (DCs:lymphocytes). Co-cultures were incubated at 37 °C in 5% CO<sub>2</sub> for 5 days.

### Flow cytometry analysis

The expression of swine leukocyte antigen (SLA) class I and II was evaluated with anti-SLA-I (clone 2Gi/VAN) and anti-SLA-II (clone DR 1F12). All antibodies were kindly donated by Dr. J. Dominguez (INIA, Madrid, Spain). To evaluate the induction of Tregs by PRRSV-infected DCs, expression of CD25 and Foxp3 was evaluated after 5 co-culture days. Cells were harvested and stained with mAb anti-CD25 (Serotec), followed by a FITC conjugated goat anti-mouse IgG (Jackson ImmunoResearch Europe Ltd.) and goat anti-mouse IgG-(ab) FITC conjugated (R&D Systems). Intracellular staining was performed as previously described (Silva-Campa et al., 2009), and the cells were fixed with 200  $\mu$ L of 4% paraformaldehyde at 4 °C in the dark, during 20 min, followed by two washes with PBS/1% BSA/0.02% sodium azide (wash buffer), resuspended in 300  $\mu$ L 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. The cells were acquired on Coulter® EPICS XL-MCL cytometer and analyzed using WinMDI 2.9 software.

### ELISA

Supernatants from a 3-day co-culture were collected and levels of secreted IFN- $\gamma$ , IL-10 (R&D systems), and TGF- $\beta$  (Biosource) were quantified using commercial ELISA kits, according to the manufacturer's recommendations.

### Real-time RT-PCR

IL-10, TGF- $\beta$ , and Foxp3 mRNA expression were quantified by real-time RT-PCR. Total RNA was extracted from 24-h co-cultures of lymphocytes and PRRSV-infected DCs using RNeasy Protocol Mini Kit (Qiagen) according to the manufacturer's instructions.

**Table 1**  
Primer and probe sequences.

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Probe sequence (5' to 3')
TGF- $\beta$ <sup>a</sup>	AGGGCTACCATGCCAATTCT	CCGGTGTGCTGGTGTGACA	TET-CAACCAGCCTGCCACATGC-BHQ1
IL-10 <sup>a</sup>	TGAGAACAGCTGCATCCACTTC	TCTGGTCTTCGTTTGAAGAAA	TET-CAACCAGCCTGCCACATGC-BHQ1
Foxp3 <sup>a</sup>	CCCTGCCCTTCTCATCCA	GTGGCCCGATGTGAAAA	TET-AGCCAGAGGACTTCCTCAAGCACTGCC-BHQ1
RPL32	TGGAAGAGACGCTGTGAGCAA	CGGAAGTTCTGTTACACAATGTAA	TET-ATTGTTCACATTAGCAGACATCAAGCTC-BHQ1

RPL32, ribosomal protein L32.

<sup>a</sup> Sequences from Porcine Immunology and Nutrition database (<http://www.ars.usda.gov/Services/docs.htm?docid=6065>).

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Real-time RT-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.  $C_t$  values from different treatments were normalized against the endogenous control gene RPL32 (ribosomal protein L32) (Dawson et al., 2004) and differences in  $C_t$  values of the lymphocytes co-cultured with the PRRSV-treated DCs vs. the lymphocytes co-cultured with mock-treated DCs were evaluated using the  $2^{-\Delta\Delta C_t}$  formula. Results are expressed as relative expression of mRNA between the treatment and control.

#### Statistical analysis

Data were analyzed using paired Student's t-test or one-way analysis of variance (ANOVA). Differences among treatments were determined by Tukey's test ( $p < 0.05$ ). Kruskal-Wallis test and Dunns for medians comparison were done for relative expression analysis. Analyses were performed with PRISM 5.02 software (GraphPad, San Diego, CA).

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