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Cytokine Expression by Macrophages in the Lung of Pigs Infected with the Porcine Reproductive and Respiratory Syndrome Virus

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Summary
Porcine reproductive and respiratory syndrome (PRRS) is caused by a virus that predominantly replicates in alveolar macrophages. The aim of the present study was to characterize the production of cytokines by subpopulations of pulmonary macrophages in pigs infected by the PRRS virus (PRRSV). Expression of interleukin (IL) 1α, IL-6 and tumour necrosis factor (TNF)-α correlated with the severity of pulmonary pathology and the numbers of pulmonary macrophages. Significant correlations were observed between PRRSV infection and the expression of IL-10, between the expression of IL-12p40 and interferon (IFN)-γ, and between the expression of TNF-α and IFN-γ. These findings suggest that PRRSV modulates the immune response by the upregulation of IL-10, which may in turn reduce expression of cytokines involved in viral clearance (e.g. IFN-α, IFN-γ, IL-12p40 and TNF-α). The results also suggest that expression of IFN-γ is stimulated by IL-12p40 and TNF-α, but not by IFN-α. All of these cytokines were expressed mainly by septal macrophages with weaker expression by alveolar macrophages, lymphocytes and neutrophils. There appears to be differential activation of septal and alveolar macrophages in PRRSV infection, with septal macrophages being the major source of cytokines.

Introduction
Macrophages play a significant role in the defence against pathogens by phagocytosis following recognition by surface pattern recognition receptors (PRRs), by antigen presentation involving class II molecules of the major histocompatibility complex (MHC II) and by production of cytokines (Mitchell and Kumar, 2004). Cytokines may also be synthesized by several other immune or non-immune cells including lymphocytes, neutrophils and fibroblasts. The expression of cytokines following engagement of PRRs by pathogen-associated molecular patterns (PAMPs) constitutes the main pathway involved in the activation of macrophages (Zhang and Mosser, 2008). Some cytokines may also act as inhibitors of macrophage activation. For example, interleukin (IL)-12, tumour necrosis factor (TNF)-α, interferon (IFN)-α and IFN-γ act as potent activators of macrophages, whereas IL-10 inhibits activation of these cells (Mitchell and Kumar, 2004).

IFN-γ and IL-12 are classically involved in the subtype of immune response mediated by Th1 lymphocytes, with both cytokines working in parallel (Biron and Sen, 2001). The proinflammatory cytokines, including IL-1α, TNF-α and IL-6, are of greatest importance during the innate immune response (Biron and Sen, 2001). IFN-α also participates in the innate response through antiviral activity, by inducing the differentiation of naive T cells into IFN-γ secreting effector cells and by down-regulation of IL-12 expression (Biron and Sen, 2001; Tizard,
In contrast, IL-10 is considered to be an immunosuppressive cytokine as it down-regulates the expression of several other cytokines including IL-1, TNF-α, IL-6, IL-10 itself, IL-12 and IFN-γ (Biron and Sen, 2001; Moore et al., 2001).

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically significant diseases of the swine industry (Neumann et al., 2005). The syndrome is characterized by interstitial pneumonia in growing pigs and reproductive failure in gilts (Rossow, 1998). PRRS is caused by a positive-strand enveloped RNA virus, known as PRRS virus (PRRSV), which belongs to the family Arteriviridae in the order Nidovirales (Fauquet et al., 2005). PRRSV replicates mainly in porcine alveolar macrophages and, to a lesser extent, in monocytes and dendritic cells (Molitor et al., 1997; Bautista and Molitor, 1999).

Several studies have examined the role of cytokines in the pathogenesis of PRRS (Van Reeth and Naughton, 2000); however, it is not clear how cytokines participate in macrophage activation during PRRSV infection or how they regulate development of the immune response to the virus. Thanawongnuwech et al. (2003) suggested that expression of IFN-γ by macrophages and lymphocytes may have an inhibitory effect on the replication of PRRSV. Another study of a PRRSV modified-live vaccine has shown that up-regulation of IL-10 expression is associated with a lower number of IFN-γ secreting cells amongst peripheral blood mononuclear cells (PBMCs) (Díaz et al., 2006).

The role of cytokines in the interstitial pneumonia described in PRRS has not yet been determined. Accordingly, the aim of the present study was to characterize the production of cytokines by subpopulations of pulmonary macrophages in pigs infected by PRRSV.

Materials and Methods

Virus, Animals and Experimental Design

Thirty-two specific pathogen free, 5-week-old pigs from a PRRSV seronegative farm were used in this study. Twenty eight animals were randomly assigned to groups of four and inoculated by the intramuscular route with 1 ml of the third passage of PRRSV field isolate 2982 (kindly provided by Dr. E. Mateu) at 10^4.0 TCID50. The virus was initially isolated in porcine alveolar macrophages from the serum of naturally infected piglets during a respiratory outbreak of PRRS affecting a Spanish farm. This field isolate has an open reading frame-5 sequence similarity of 93% with Lelystad virus (GenBank accession number EF429108). The inoculated animals were killed at 3, 7, 10, 14, 17, 21 and 24 days post-inoculation (dpi). Another group of four pigs were sham-inoculated controls. These animals were injected intramuscularly with 1 ml of sterile RPMI 1640 medium and killed at the end of the study (24 dpi). All animals were sedated with tiletamine-zolazepam (Zoletil®; Virbac, Barcelona, Spain) followed by a lethal dose of 5% sodium thiopental (Thiovet®; Vet Limited, Leyland, Lancashire). The experiment was carried out according to the guidelines of the European Union (Directive 86/609/EEC) and was approved by the local ethical committee of Centro de Investigación en Sanidad Animal (CISA-INIA; Valdeolmos, Madrid, Spain).

Clinical Signs, Gross Pathology and Pulmonary Histopathology

The pigs were monitored daily for clinical signs, including rectal temperature and a clinical respiratory score, as previously described (Halbur et al., 1995). During post-mortem examination, gross lung lesions were evaluated by visual inspection and each lung lobe was scored to reflect the approximate volume or percentage of the lung tissue affected (Halbur et al., 1995). Samples from the medial lobe of the right lung were fixed in 10% neutral buffered formalin and in Bouin’s solution, processed routinely and embedded in paraffin-wax. Sections (4 μm) of formalin-fixed tissue were stained with haematoxylin and eosin (HE) for microscopical examination.

Immunohistochemistry (IHC)

Since PRRSV is most frequently detected in the apical and medial lung lobes (Halbur et al., 1996), the medial lobe was selected for immunohistochemical examination. The avidin–biotin–peroxidase complex technique (ABC) was used for the detection of PRRSV, macrophages and cytokine proteins as described previously (Hsu et al., 1981). Formalin-fixed tissue was used for detection of macrophages and tissue fixed in Bouin’s solution for all other immunohistochemical reactions. Briefly, the sections were dewaxed and dehydrated through graded ethanol and the endogenous peroxidase activity was quenched in H2O2 3% in methanol for 30 min. The sections were washed with phosphate buffered saline (PBS; pH 7.4, 0.01 M) and incubated for 30 min at room temperature with 100 μl per slide of blocking solution in a humid chamber. Table 1 describes the primary antibodies and antigen retrieval methods applied. Primary antibodies were incubated overnight at 4 °C in a humid chamber. In each case the corresponding biotinylated secondary antibody was incubated for 30 min at room temperature. An
avidin–peroxidase complex (Vector Laboratories, Burlingame, California) was applied for 1 h at room temperature. Labelling was ‘visualized’ by application of the NovaRED/C212 substrate kit (Vector Laboratories). Sections were counterstained with Mayer’s haematoxylin, dehydrated and mounted. For negative controls, the primary antibody was replaced by blocking solution, normal serum and isotype-matched reagents of irrelevant specificity.

**Cell Counting**

The number of labelled cells was determined as described previously (Salguero et al., 2005). Briefly, the labelled cells were counted in 50 non-overlapping and consecutively selected high magnification fields of 0.20 mm². Results are expressed as the number of cells per mm². Immunolabelled cells were identified and counted morphologically as macrophages, lymphocytes or neutrophils. Pulmonary intravascular macrophages and interstitial macrophages were grouped together and described as ‘septal macrophages’.

**Statistical Analysis**

The numbers of macrophages, PRRSV-infected and cytokine-expressing cells were expressed as a mean ± SD. These values were evaluated for approximate normality of distribution by the Kolmogorov–Smirnov test. Differences between the means of control and inoculated animals were assessed by the Kruskal–Wallis test followed by the Mann–Whitney-U non-parametric test (GraphPad Instat 3.05). Correlation between the presence of lung lesions and the expression of virus, macrophages and cytokines was assessed by the Spearman test (GraphPad Instat 3.05). \( P < 0.05 \) was considered to represent a statistically significant difference.

**Results**

**Clinical Signs, Gross Pathology and Pulmonary Histopathology**

Control animals did not display clinical signs or significant gross or microscopical lung lesions. Although inoculated animals displayed no significant respiratory distress, they did develop dullness, weight loss and mild hyperthermia from 3 dpi. From 7 dpi until the end of the study, almost 50% of the pulmonary parenchyma of the inoculated animals was affected by interstitial pneumonia, and this was confirmed by microscopical examination of the tissue samples (Figs. 1A, 2A).

**Labeling of Macrophages and Expression of PRRSV Antigen**

MAC387 antibody defined macrophages in sections of lung tissue. The total number of macrophages increased in the lung of inoculated animals from 7 dpi onwards (Fig. 1B). This related primarily to an increase in the number of septal macrophages (Figs. 1B and 2B). The number of alveolar macrophages decreased to 7 dpi and recovered thereafter (Fig. 1B). The number of macrophages, as determined by expression of MAC387, was significantly correlated with the microscopical score of lung lesions \( (r = 0.85; \ P < 0.05) \) (Table 2).
There was no expression of PRRSV antigen in the lung of control animals. PRRSV antigen was detected in the lung of infected pigs from 3 dpi until the end of the study, peaking at 7 dpi (Fig. 1C). This antigen expression was detected mainly in the cytoplasm of macrophages, and was significantly higher in alveolar macrophages than in septal macrophages ($P < 0.05$) (Figs. 1C, 2C and 4A). Immunolabelled cells were observed not only in areas of interstitial pneumonia, but also in lung parenchyma without lesions.

**Tissue Expression of Proinflammatory Cytokines**

IL-1$\alpha$ was observed in the cytoplasm of alveolar and septal macrophages and neutrophils, the latter appearing to be a significant source of this cytokine (Fig. 1D). Expression of IL-1$\alpha$ was always higher in inoculated animals than in controls, and had a bi-modal peak at 7 and 14 dpi ($P < 0.05$) (Fig. 1D). The increase in IL-1$\alpha$ at 14 dpi was attributed primarily to neutrophils ($P < 0.05$) (Figs. 1D and 2D).
Fig. 2. (A) Photomicrograph of the medial lobe of the right lung from a pig inoculated with PRRSV field isolate 2982 and killed at 7 dpi. Interstitial pneumonia is characterized by severe septal infiltration with mononuclear cells and type 2 pneumocyte hypertrophy and hyperplasia. HE. Bar, 100 μm. (B) Marked infiltration of macrophages into the alveolar septa in the lung of a pig killed at 7 dpi. IHC. Bar, 100 μm. (C) Alveolar and septal macrophages expressing PRRSV antigen in a pig killed at 7 dpi. IHC. Bar, 25 μm. (D) Numerous septal macrophages and neutrophils expressing IL-1β in the pulmonary parenchyma of a pig killed at 7 dpi. There is marked thickening of the alveolar septa. IHC. Bar, 100 μm. (E) Alveolar and septal macrophages expressing IL-6 in the lung of a pig killed at 7 dpi. IHC. Bar, 25 μm. (F) Pulmonary parenchyma of a pig killed at 7 dpi, with a mild interstitial pneumonia, showing septal macrophages expressing TNF-α. IHC. Bar, 30 μm.
Expression of IL-6 and TNF-α also had a bimodal peak at 7 and 14 dpi ($P < 0.05$) (Fig. 1E and F). IL-6 expression remained elevated until the end of the study (Fig. 1E), but the expression of TNF-α was no different to that of control animals from 21 dpi (Fig. 1F). Septal macrophages were the main cell population involved in the expression of both IL-6 and TNF-α ($P < 0.05$) (Figs. 1E, F, 2E and F). Alveolar macrophages and lymphocytes also expressed these cytokines, but to a lesser extent (Fig. 1E and F).

The labelling of proinflammatory cytokines was observed mainly in areas of interstitial pneumonia with moderate to severe thickening of the alveolar septa. Few immunolabelled cells were observed in areas of the lung without lesions (Fig. 2D–F). The correlation between the lung lesion score, macrophage count and expression of proinflammatory cytokines is shown in Table 2. Table 3 shows the correlation between the expression of TNF-α and IFN-γ.

Expression of IFN-α and TNF-α also had a bimodal peak at 7 and 14 dpi ($P < 0.05$) (Fig. 1E and F). IFN-α expression was always higher in inoculated animals than in controls (Fig. 3A). The expression of IFN-α was significantly correlated with virus expression ($r = 0.86; P < 0.05$) (Table 3).

The kinetics of labelling for IFN-γ and IL-12p40 were similar throughout the study ($r = 0.95; P < 0.05$) (Table 3), with both cytokines peaking at 7 dpi and decreasing thereafter (Fig. 3B and C). These cytokines were expressed not only mainly by septal macrophages, but also by alveolar macrophages and lymphocytes (Fig. 4C, E and G). Inoculated animals always had more IFN-γ-expressing cells than controls.

The expression of IL-10 peaked at 7 dpi and decreased thereafter (Fig. 3D). IL-10 was expressed mainly in the cytoplasm of septal macrophages (Fig. 4B and D). The kinetics of expression of IL-10 were significantly correlated with that of the virus ($r = 0.77; P < 0.05$) (Table 3).

Consecutive sections immunolabelled for PRRSV antigen, IFN-γ and IL-10 showed co-localization of IFN-γ and PRRSV antigen, whereas the expression of IL-10 occurred in areas without expression of IFN-γ (Fig. 4A–C).

The number of septal macrophages expressing these cytokines was always greater than the number of labelled alveolar macrophages (Fig. 3). Immunolabelling for IFN-α, IFN-γ, IL-12p40 and IL-10 was associated with areas of mild to moderate interstitial pneumonia and was much less in areas of pulmonary parenchyma without lesions (Fig. 4). The correlations between the expression of PRRSV, IFN-α, IFN-γ, IL-10, IL-12p40 and TNF-α in the lung of PRRSV-infected pigs are shown in Table 3.

### Discussion

Several reports have described changes in cytokine expression during PRRSV infection, but these have not addressed local cytokine production within pulmonary lesions. The present study has characterized expression of cytokines by pulmonary macrophages...
in order to determine the role of these molecules in the pathogenesis of the respiratory form of PRRS.

The experimental infection did not lead to the animals developing respiratory symptoms, but dullness, weight loss, mild hyperthermia and lesions of the pulmonary parenchyma were observed. PRRSV replication peaked at 7 dpi and was mainly localized to alveolar macrophages, which are considered as the target cell for viral replication (Molitor et al., 1997; Batista and Molitor, 1999). No correlation was observed between the presence of viral antigen and the severity of the microscopical lung lesions. However, the microscopical lung lesions were significantly correlated with marked inflammatory infiltration of the septa and the number of infiltrating macrophages. Moreover, the lung lesions showed significant correlation with the expression of both IL-1α and IL-6, but not of TNF-α, and macrophage counts were correlated with the expression of IL-1α and TNF-α, but not of IL-6. These observations suggest that IL-1α may play a significant role in the development of interstitial pneumonia during PRRS. Nonetheless, when all the three proinflammatory cytokines were considered, a highly significant correlation was observed between both microscopical pulmonary lesions and macrophage counts.

Although PRRSV replicated mainly in alveolar macrophages, proinflammatory cytokines were expressed mainly by septal macrophages, especially IL-6 and TNF-α, from 14 dpi onwards. This fact indicates activation of septal macrophages, which may be induced by the synthesis of cytokines (Zhang and Mosser, 2008). Similar findings have been reported for other porcine viral diseases, including African swine fever, which triggers activation of interstitial macrophages expressing IL-1α and TNF-α after viral replication (Carrasco et al., 2002).

In the present study there was marked intra-alveolar infiltration of neutrophils expressing IL-1α at 14 dpi. The earlier increase of both IL-1α and TNF-α may have been responsible for the induction of this infiltration and the subsequent activation of these cells, since these cytokines are considered as neutrophil-chemoattractant and stimulant agents (Van Reeth and Nauwynck, 2000). Furthermore, IL-1α and TNF-α may induce the synthesis of IL-6 (Van Reeth and Nauwynck, 2000; Mitchell and Kumar, 2004); however, in our study no correlation was observed between the expressions of these cytokines, although the maximum expression of IL-6 temporally coincided with higher expression of IL-1α and/or TNF-α.
The interferons are known to play a significant role in the host immune response against viruses (Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). IFN-α participates in the innate immune response and is able to induce synthesis of IFN-γ (Biron and Sen, 2001; Tizard, 2008). In the present study, a significant correlation was observed between PRRSV replication and IFN-α expression, suggesting that PRRSV
directly induces expression of IFN-α by macrophages. However, PRRSV induces lower levels of IFN-α when compared with other porcine respiratory viral diseases, such as those caused by swine influenza virus or porcine respiratory coronavirus (Van Reeth and Nauwynck, 2000), which indicates that IFN-α expression may be insufficient to induce clearance of PRRSV.

The expression of IFN-γ by macrophages and lymphocytes has been previously reported in the lung of PRRSV-infected pigs (Thanawongnuwech et al., 2003). In that study, an increase in expression of IFN-γ was observed at 10 dpi for infection with highly virulent strains, whereas strains of low virulence induced a higher expression at the end of the study (28 dpi). In the present study, the expression of IFN-γ was undulating, showing a peak at 7 dpi, just when PRRSV replication was maximal. IFN-γ is known to protect macrophages in vitro against PRRSV replication (Bautista and Molitor, 1999); however, that viral replication occurred throughout the period of the present study may suggest that in this experimental infection the IFN-γ response was not strong enough to eliminate PRRSV infection.

The production of IFN-γ by pulmonary macrophages is induced by the expression of other cytokines including IL-12, TNF-α and IFN-α (Nguyen and Benveniste, 2002; Mitchell and Kumar, 2004; Tizard, 2008). In the present study there was good correlation between the expression of IL-12p40, TNF-α and IFN-γ, but poor correlation between expression of IFN-α and IFN-γ. Therefore, IL-12p40 and TNF-α might be the most significant cytokines involved in the induction of synthesis of IFN-γ in this experimental infection. Royaes et al. (2004) reported correlation between virus-specific IFN-α secreting cells and virus-specific IFN-γ secreting cells in pigs vaccinated with an attenuated, modified-live PRRSV vaccine. High antigenic and pathogenic differences have been related to European and North American PRRSV genotypes, and suggested to occur within a given genotype (Halbur et al., 1995; Mateu et al., 2003; Stadejek et al., 2006), which may be the cause of the discrepancies between the present study and that of Royaes et al. (2004).

Despite the expression of IFN-α, IFN-γ, IL-12p40 and TNF-α, PRRSV was still replicating in the lung of infected pigs at the end of the study. IL-10 is an immunomodulatory cytokine that is able to inhibit the synthesis and release of other cytokines (Biron and Sen, 2001; Moore et al., 2001). Therefore, the expression of IL-10 observed in the present study might be responsible for reduced expression of cytokines such as IFN-α, IFN-γ, IL-12p40 and TNF-α, that in turn may impair prolonged viral replication in the lung of infected animals. Interestingly, the expression of IL-10 was observed in areas of lung that showed no expression of IFN-γ. Moreover, the expression of IL-10 was significantly correlated with PRRSV replication. These results suggest that PRRSV may induce the expression of IL-10 and, therefore, the expression of IL-10 might inhibit the expression of other cytokines, allowing a prolonged viral replication in the lung. This idea is supported by the observed immunolabeling for IL-10 and IFN-γ in consecutive sections of the lung and by the correlation observed between the expression of IL-10 and IFN-α.

In conclusion, the results of the present study indicate that activation of septal and alveolar macrophages differs throughout PRRSV infection, and that the septal cells are the main source of cytokines. Proinflammatory cytokines are actively expressed at the onset of the interstitial pneumonia and there is direct correlation between this expression and the infiltration of the pulmonary interstitium by macrophages. Additionally, PRRSV appears able to modulate the local immune response by inducing the expression of IL-10 by macrophages, which may in turn reduce the levels of cytokines involved in viral clearance such as IFN-α, IFN-γ, IL-12p40 and TNF-α.

Conflict of Interest
The authors declare no competing financial interests.

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Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcpa.2009.07.004.

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