Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus

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Recent studies suggest that porcine reproductive and respiratory syndrome virus (PRRSV) may have immunomodulatory effects on the host immune system by upregulating interleukin (IL)-10 gene expression. To determine the effect of PRRSV on porcine cytokine gene expression in vivo, we infected pigs with either the European or North American strain of PRRSV and monitored cytokine gene expression in peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BALC) using a multiplex PCR assay. Our results showed that both European and North American strains of PRRSV significantly upregulated IL-10 gene expression in PBMC of infected pigs from 5 days post-infection (p.i.). In addition, upregulation of IL-10 and interferon (IFN)-γ gene expression was observed in BALC starting from 9 days p.i. The upregulation of cytokine gene expression in BALC was observed concurrent with an increased percentage of lymphocytes in the BALC population, suggesting a role for peripheral leukocytes in cytokine production in lungs. Our results showed that PRRSV infection resulted in an upregulation of IL-10 gene expression in vivo and that both European and North American strains induced comparable levels of IL-10 gene expression in the infected pigs, despite differences in the clinical signs. Our data support the notion that induction of IL-10 production may be one of the strategies used by PRRSV to modulate the host’s immune responses, and this may contribute to the unique clinical picture observed following PRRSV infection.

INTRODUCTION

Since its emergence in the late 1980s, porcine reproductive and respiratory syndrome virus (PRRSV) has been recognized worldwide as one of the most economically important pathogens of swine (Meng, 2000). PRRSV, an enveloped positive-stranded RNA virus, is a member of the family Arteriviridae in the order Nidovirales (Cavanagh, 1997). The major characteristics of porcine reproductive and respiratory syndrome (PRRS) include reproductive failure of sows and respiratory disease in pigs of all ages. Furthermore, infection with PRRSV results in the deterioration of local lung defences, leading to secondary bacterial infections, which are known as the porcine respiratory disease complex (Halbur, 1998). Two major and distinct genotypes of PRRSV have been reported, namely the European and North American types (Meng et al., 1995). The two genotypes are believed to have evolved on the two separate continents from a common ancestor. Despite the fact that the European and North American strains appear to share similarity in disease phenotype, they differ in virulence and are heterologous both antigenically and genotypically (Meng, 2000).

Several studies suggest that PRRSV may negatively modulate the host immune system (reviewed in Molitor et al., 1996; Lager & Mengeling, 2000). Following infection, PRRSV persists in infected pigs for up to 12 weeks and infectious virus can be shed during this stage (Will et al., 1997). This suggests that the immune response is not able completely to eliminate the virus from the infected host. Although PRRSV is highly contagious, virus replication appears to be limited mainly to phagocytic cell populations, including macrophages and activated monocytes (Molitor et al., 1996). Interestingly, there appears to be a weak innate immune response at the time of PRRSV infection by several pro-inflammatory cytokines, which are mostly undetectable or minimally increased following exposure to PRRSV (Van Reeth & Nauwynck, 2000; Murtaugh et al., 2002). This is supported by the mild clinical signs and pathological changes seen in the lungs following PRRSV infection compared with other respiratory virus infections (Van Reeth et al., 1999). The poor innate immune response is consistent with the induction of a delayed and ineffective adaptive immunity against the virus (Yoon et al., 1995; Bautista & Molitor, 1997; Lopez Fuertes et al., 1999; Murtaugh et al., 2002).

Cytokines play a crucial role in the induction and
modulation of immunological processes. Interleukin (IL)-10 is known to inhibit production of several pro-inflammatory cytokines. The inhibitory effects of IL-10 on the production of IL-1 and tumour necrosis factor (TNF) are crucial for its anti-inflammatory activities (Moore et al., 2001). We recently reported that PRRSV significantly induces IL-10 gene upregulation in vitro in porcine peripheral blood mononuclear cells (PBMC), cultured in the presence of the virus (Suradhat et al., 2003). We postulated that the induction of IL-10 might be one of the strategies used for interfering with the host’s immune responses to PRRSV. In this study, we have further examined the in vivo effects of PRRSV on cytokine gene expression in both PBMC and bronchoalveolar lavage cells (BALC). In addition, both European and North American PRRSV isolates were compared for their effects on cytokine gene expression.

METHODS

Animal and experimental protocols. Twenty-one 3-week-old crossbred pigs were obtained from a PRRSV-free commercial farm. These pigs had neither serological nor virological evidence of previous exposure to PRRSV prior to virus inoculation (data not shown). Pigs were inoculated with 2 ml (1 ml intranasally and 1 ml intramuscularly) of the North American (01NP1) or European (02SB3) strain of the Thai PRRSV isolates (nine pigs per genotype), at a dose of 10^3.5 TCID_{50} ml^{-1}. Three pigs were used as a negative control group, receiving culture media from uninfected Marc-145 cells. Pigs were examined daily for 15 days after inoculation. Gross and microscopic findings, as well as lung scores, were recorded using a previously described protocol (Halbur et al., 1995; Thanawongnuwech et al., 1998).

Viruses and cells. Thai PRRSV isolates used for experimental infection were recovered from the pooled sera of PRRSV-infected pigs from PRRSV-infected farms and designated as either North American genotype (01NP1) or European genotype (02SB3) strain of the Thai PRRSV isolates (nine pigs per genotype), at a dose of 10^3.5 TCID_{50} ml^{-1}. Three pigs were used as a negative control group, receiving culture media from uninfected Marc-145 cells. Pigs were examined daily for 15 days after inoculation. Gross and microscopic findings, as well as lung scores, were recorded using a previously described protocol (Halbur et al., 1995; Thanawongnuwech et al., 1998). The re-isolated viruses were subjected to testing by multiplex PCR to determine whether they were the same as the inoculated viruses and in order to verify any possible cross-contamination between the groups.

Isolation of porcine PBMC and BALC. Heparinized blood samples were collected on the challenge day (day 0) and on days 5 and 12 post-infection (p.i.). Porcine PBMC were isolated by density centrifugation using Isoprep separation medium (Robbins Scientific Corporation), according to the manufacturer’s protocol. On days 5, 9 and 15 p.i., pigs were euthanized (one pig from the control group and three pigs from both PRRSV-infected groups) and bronchoalveolar lavage (BAL) was performed by lavaging the lung with PBS. The BAL fluid was centrifuged and the pellet of BAL cells (BALC) was washed twice with PBS. The BALC were stained and subjected to a differential count based on cell morphology.

Approximately 3 x 10^6 PBMC or BALC were counted and resuspended in 200 µl RNAlater (Ambion). Cells were kept at -20°C until needed.

RNA extraction and reverse transcription. Total RNA was extracted from approximately 3 x 10^6 cells using the Nucleospin RNA II kit (Macherey-Nagel), according to the manufacturer’s instruction. Contaminating DNA was removed by the DNase I treatment provided in the kit. In the final step, the total RNA from each sample was eluted in 60 µl RNase-free water. Ten µl total RNA from each sample was reverse-transcribed using the Omniscript RT kit (Qiagen) in a total volume of 20 µl. The reverse transcription reaction was carried out in the presence of 0-5 µg random hexamers (Promega) and 40 U ribonuclease inhibitor (RNaseOUT; Invitrogen) at 37°C for 60 min, followed by heat inactivation at 93°C for 5 min and rapid cooling on ice.

Multiplex PCR. The multiplex PCR (MPCR), which allowed simultaneous amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-10, IFN-γ, IL-2 and IL-4 genes, was performed in a 50 µl reaction using the protocol and primers described previously (Suradhat et al., 2003). Following MPCR, 10 µl PCR product was subjected to agarose gel electrophoresis, using 2-5 % agarose (Sigma) in 1 x TBE buffer (Gibco-BRL) in the presence of 0-5 µg ethidium bromide ml^{-1} (Research Organics).

Quantification of the PCR products. Images of the MPCR products resolved in ethidium bromide-stained gels were visualized using UV illuminator and digitally saved using the ‘Photo-print’ photodocumentation system (Vilber Lourmat). The images were further processed for quantification of the band by densitometry using the Scion Image software (Scion Corporation). The expression level of each product was determined by normalizing its expression against GAPDH. The results were expressed as the percentage of cytokine expression/GAPDH expression and referred to as % expression. When the populations were considered as a group, the values were averaged, the standard error of the mean (SEM) was calculated and values were expressed as mean % expression ± SEM.

In our experience, the levels of background cytokine gene expression in PBMC vary among pigs. In addition, unstimulated PBMC also have some background levels of cytokine gene expression. Thus, in order to minimize variability, the background of % gene expression in PBMC of each pig on day 0 was subtracted from the % expression of the PBMC measured during the experiment.

Statistical analysis. Statistical analyses and non-parametric tests were performed using GraphPad Prism (GraphPad Software) or SAS (SAS Institute Inc.). The comparisons between groups were considered as statistically significant when P < 0.05.

RESULTS

Clinical picture and pathology following PRRSV inoculation

Following PRRSV inoculation, all infected pigs showed clinical signs of PRRSV. Respiratory disease signs were characterized by transient dyspnoea and tachypnoea and were apparent upon handling for temperature taking or by pigs being otherwise stressed 5 days p.i. Moderate lethargy, anorexia and rough hair was obvious in the North American strain-infected group from 5 days p.i. to the end of the experiment (15 days p.i.). The pigs infected with the EU strain of PRRSV exhibited similar but milder symptoms. More fever days (50 % of pigs with a rectal temperature higher than 40°C) were observed in the US group (9 days) compared with the EU group (4 days). At necropsy, more severe pathological changes were observed in the lungs from pigs infected with the North American strain (Table 1). Diffused lymphohistiocytic interstitial pneumonia,
characterized by septal thickening by hyperplasia and hypertrophy of pneumocyte type II with lymphocytic infiltration in the alveolar wall, was observed in both infected groups. No evidence of any viral pneumonia was seen in the control pigs. In summary, the pigs infected with the North American strain of PRRSV had more intense clinical signs and pathological changes than the European strain PRRSV.

Viraemia was seen from as early as 5 days p.i. through to 15 days p.i. (data not shown). PRRSV was isolated from the serum of all PRRSV-infected pigs and characterized by the nested multiplex RT-PCR. No cross contamination between groups was found (data not shown), and no PRRSV was isolated from the control pigs.

An in vivo effect of PRRSV on porcine cytokine gene expression in PBMC

PRRSV infection resulted in a significant upregulation of IL-10 gene expression \((P<0.05)\) in PBMC of infected pigs at 5 and 12 days p.i. The level of IL-10 expression induced by the European and North American PRRSV strains was similar \((P>0.05)\) at both tested time-points. The control (uninfected) pigs did not show any increase in cytokine gene expression (Fig. 1). In this experiment, minimal changes in IL-2 and IL-4 gene expression levels were observed (data not shown).

An in vivo effect of PRRSV on porcine cytokine gene expression in BALC

In contrast to PBMC, no changes in cytokine gene expression were observed in the BALC population at 5 days p.i. However, significant upregulation of both IL-10 and IFN-γ gene expression was observed in BALC isolated from PRRSV-infected pigs at 9 and 15 days p.i. (Fig. 2). As in PBMC, minimal changes in IL-2 and IL-4 gene expression levels were observed in BALC (data not shown).

The differential blood count showed that the major leukocyte subpopulation in the BALC of all groups at 5 days p.i. was macrophages. However, at 9 and 15 days p.i., there were significant increases in the percentage of lymphocytes in the BALC from PRRSV-infected pigs. The percentage of macrophages and lymphocytes in the control pigs remained unchanged throughout the experiment. There were minimal changes in the percentage of neutrophils in the BALC among the groups (Table 2).

### DISCUSSION

We have previously shown the effect of PRRSV in upregulating IL-10 gene expression of porcine PBMC in vitro (Suradhat et al., 2003). In this report, we have demonstrated that PRRSV infection results in upregulation of IL-10 gene expression in both PBMC and BALC of infected pigs. IL-10 is known to be a potent immunosuppressive cytokine with

<table>
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<tr>
<th>Time of necropsy (days p.i.)</th>
<th>Control (uninfected)</th>
<th>N. American (01NP1)</th>
<th>European (02SB3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0(^a)</td>
<td>7.67 ± 6.66(^b)</td>
<td>1.5 ± 0.0(^c)</td>
</tr>
<tr>
<td>9</td>
<td>0(^a)</td>
<td>54.00 ± 19.00(^c)</td>
<td>27.67 ± 23.75(^b)</td>
</tr>
<tr>
<td>15</td>
<td>0(^a)</td>
<td>60.67 ± 8.62(^c)</td>
<td>33.00 ± 11.00(^b)</td>
</tr>
</tbody>
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Values in the same row with different letters are statistically different \((P<0.05)\).
the capability of downregulating host immune responses. The exploitation of IL-10 appears to be the common mechanism of immunosuppression of several intracellular pathogens that specifically target macrophages for infection (reviewed in Redpath et al., 2001; Fickenscher et al., 2002). IL-10 profoundly inhibits a broad spectrum of macrophage/monocyte functions and proliferation/differentiation of CD4+ T cells, possibly by downregulating the antigen-presenting cell functions (Moore et al., 2001). Moreover, IL-10 appears to play a key role in the differentiation of regulatory T cells (McGuirk & Mills, 2002). Therefore, induction of IL-10 production at an early stage of infection may enhance virus survival within the host and delay the induction of protective immunity. There is at least one report describing an increase in IL-10 mRNA expression from PBMC of piglets born from infected sows (Feng et al., 2000). In addition, PBMC from piglets infected in utero with PRRSV contain less IFN-γ- and TNF-α-producing cells, suggesting an immunomodulatory effect of PRRSV, possibly through the systemic overproduction of IL-10 (Aasted et al., 2002).

Fig. 2. Mean % expression of the porcine IFN-γ (white bars) and IL-10 (black bars) genes in BALC of pigs infected with the European (EU) or North American (US) PRRSV strain or in uninfected pigs (Control) at 5 (A), 9 (B) and 15 (C) days p.i. At each time-point, the numbers of pigs tested from the EU, US and control groups were three, three and one, respectively.

Table 2. Mean (±SD) of % leukocyte subpopulation in BALC of control (uninfected) pigs and pigs infected with the European (EU) or North American (US) strain of PRRSV at different times post-infection

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>Group (n)</th>
<th>% Leukocyte subpopulation in the BALC</th>
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<tr>
<td></td>
<td></td>
<td>Neutrophil</td>
</tr>
<tr>
<td>5</td>
<td>Control (1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>EU (3)</td>
<td>0.38±0.53</td>
</tr>
<tr>
<td></td>
<td>US (3)</td>
<td>0.17±0.14</td>
</tr>
<tr>
<td>9</td>
<td>Control (1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>EU (3)</td>
<td>4.67±1.04</td>
</tr>
<tr>
<td></td>
<td>US (3)</td>
<td>0.83±1.04</td>
</tr>
<tr>
<td>15</td>
<td>Control (1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>EU (3)</td>
<td>1.92±1.38</td>
</tr>
<tr>
<td></td>
<td>US (3)</td>
<td>1.08±1.28</td>
</tr>
</tbody>
</table>

PRRSV is very infectious and PRRS has been observed in pigs infected by intramuscular inoculation or mechanical routes such as contaminated needles or blood-sucking insects (Lager & Mengeling, 2000; Otake et al., 2002a, b), suggesting that there is a very efficient mechanism of virus transport to the permissive cells. PRRSV infection generally results in viraemia within 6–12 h following infection (Rossow et al., 1995). Furthermore, PRRSV induces productive infection predominantly in the macrophages of the lungs, regardless of the route of entry (Murtaugh et al., 2002). In the present study, we inoculated pigs with PRRSV using both the intranasal and the intramuscular route and demonstrated that the pigs exhibited typical clinical signs of PRRSV infection, despite receiving a low-dosage inoculation. Similar to a previous report (Halbur et al., 1995), the more severe clinical signs were induced by the North American genotype of the Thai isolate compared with those induced by the European genotype. It is well documented that European and US strains of PRRSV are antigenically and genotypically heterologous and differ in their virulence (Meng, 2000). However, we did not observe any significant
difference in the levels of IL-10 expression between the two groups during the experiment. This finding suggests that the ability to induce IL-10 may be conserved between the two genotypes of PRRSV and may explain why both genotypes appear to have a similar disease outcome in relation to their immunomodulatory properties observed in the field.

Despite a significant upregulation of IL-10 gene expression in PBMC starting from 5 days p.i., we did not observe a significant upregulation of the IFN-\(\gamma\) gene in PBMC throughout the experiment (Fig. 1). Our finding supports the previous data that IFN-\(\gamma\) production in response to PRRSV infection is slow and weak (reviewed in Murtaugh et al., 2002). The upregulation of IL-10, but not IFN-\(\gamma\), gene expression that we observed early on in the PRRSV-infected pigs implies that the virus might have a regulatory effect on the host’s immune system. The inhibitory effect of IL-10 on IFN-\(\gamma\) production in T helper (Th) and natural killer (NK) cells has been extensively reviewed elsewhere (Moore et al., 2001; McGuirk & Mills, 2002). It is well established that PRRSV mainly replicates in macrophages of the lymphoid tissues and lungs during the acute phase of infection (Duan et al., 1997). The systemic effect of IL-10, particularly on macrophage/monocyte populations, could contribute to the poor pro-inflammatory cytokine production and cellular functions, which facilitate the establishment of virus infection and replication in the target cells. In addition, systemic and local IL-10 production may increase the chances of a secondary bacterial infection, possibly through inhibitory effects on macrophages and other effector cells.

Our findings on upregulation of IL-10 and IFN-\(\gamma\) gene expression in BALC are in agreement with previous work reporting high levels of IFN-\(\gamma\) and IL-10 gene expression in BALC from pigs infected in utero with PRRSV after birth (Johnsen et al., 2002). In a previous experiment, we also observed upregulation of IL-10 gene expression in BALC isolated from PRRSV-infected pigs. Furthermore, the levels of IL-10 gene expression in the BALC appeared to correlate well with the amount of IL-10 measured by ELISA (Thanawongnuwech & Thacker, 2003). The upregulation of IL-10 and IFN-\(\gamma\) gene expression was first observed at 9 days p.i. in BALC (Fig. 2), which was later than in PBMC. Interestingly, the evidence of IL-10 and IFN-\(\gamma\) gene upregulation in BALC was concurrent with an increased percentage of the lymphoid cell population in the BALC (Table 2). Consistent with the results obtained by RT-PCR, increased numbers of IL-10-positive cells were also observed in the BALC of infected pigs using an immunofluorescent technique. Furthermore, the majority of IL-10-producing cells in the BALC appeared to have lymphocyte-like morphology (S. Suradhat, unpublished observation). This finding suggests a role for lymphoid cells in contributing to cytokine production in local tissues following PRRSV infection.

Although the percentages of the subpopulations obtained from our experiment may not necessarily reflect absolute number of cells, it has been previously reported that there was a significant increase in the number of BALC from day 10 to day 21 of PRRSV infection (Samsom et al., 2000). During this period, the number of lung macrophages remained constant until 14 days after infection and the increased BALC number was mainly due to an influx of lymphocytic cells with cytolytic phenotypes, i.e. cytotoxic T lymphocytes, starting from day 7 of infection. In addition, the same study demonstrated that the increased percentage of the lymphocyte subpopulation was limited to CD8\(^+\) cells and not CD4\(^+\)CD8\(^-\) (Th), CD4\(^+\)CD8\(^+\) (memory Th) or other myeloid cells (Samsom et al., 2000). The effects of IL-10 in promoting growth, differentiation and cytotoxic activity in both CD8\(^+\) and NK cells has been well established (Moore et al., 2001). Moreover, IL-10 is known to be a strong chemotactic factor for CD8\(^+\) T cells (Redpath et al., 2001). Therefore, IL-10 production induced by PRRSV in the lung could be one of the factors, in addition to the presence of PRRSV, involved in recruiting and activating the CD8\(^+\) cell population in the BALC. It is likely that the enhanced IFN-\(\gamma\) gene expression observed in the BALC of infected pigs was due to the upregulation of IFN-\(\gamma\) gene expression in this activated cytotoxic T-cell population. This notion is supported by previous work showing that both lymphocytes and macrophages in the lungs of PRRSV-infected pigs were positively stained for IFN-\(\gamma\) starting from 7 days p.i., suggesting that they are the cytokine producers in the lungs (Thanawongnuwech et al., 2003). At present, it is not clearly known which cellular subpopulation is responsible for the systemic and local IL-10 production following PRRSV infection and which PRRSV component(s) is the IL-10 inducer. This phenomenon is currently under investigation in our laboratory. Nevertheless, our results suggest that the induction of IL-10 production is critical for the establishment of infection, the clinical picture and the pathogenesis of PRRSV.

Taken together, our results show that PRRSV infection results in upregulation of IL-10 gene expression in vivo and that the effect is observed in PBMC earlier than in BALC. In addition, both European and North American strains induced comparable levels of IL-10 gene expression in the infected pigs, regardless of the clinical severity. Our results imply that the induction of IL-10 production may be one of the strategies used by PRRSV to regulate the host immune system, which could contribute to the unique clinical picture observed following PRRSV infection.

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