Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Veterinary Immunology and Immunopathology 102 (2004) 165–178
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

The combination of PRRS virus and bacterial endotoxin as a model for multifactorial respiratory disease in pigs

Steven Van Gucht, Geoffrey Labarque, Kristien Van Reeth*

Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

Abstract

This paper reviews in vivo studies on the interaction between porcine reproductive and respiratory syndrome virus (PRRSV) and LPS performed in the authors' laboratory. The main aim was to develop a reproducible model to study the pathogenesis of PRRSV-induced multifactorial respiratory disease. The central hypothesis was that respiratory disease results from an over-production of proinflammatory cytokines in the lungs. In a first series of studies, PRRSV was shown to be a poor inducer of TNF-α and IFN-α in the lungs, whereas IL-1 and the anti-inflammatory cytokine IL-10 were produced consistently during infection. We then set up a dual inoculation model in which pigs were inoculated intratracheally with PRRSV and 3–14 days later with LPS. PRRSV-infected pigs developed acute respiratory signs for 12–24 h upon intratracheal LPS inoculation, in contrast to pigs inoculated with PRRSV or LPS only. Moreover, peak TNF-α, IL-1 and IL-6 titers were 10–100 times higher in PRRSV–LPS inoculated pigs than in the singly inoculated pigs and the cytokine overproduction was associated with disease. To further prove the role of proinflammatory cytokines, we studied the effect of pentoxifylline, a known inhibitor of TNF-α and IL-1, on PRRSV–LPS induced cytokine production and disease. The clinical effects of two non-steroidal anti-inflammatory drugs (NSAIDs), meloxicam and flunixin meglumine, were also examined. Pentoxifylline, but not the NSAIDs, significantly reduced fever and respiratory signs from 2 to 6 h after LPS. The levels of TNF-α and IL-1 in the lungs of pentoxifylline-treated pigs were moderately reduced, but were still 26 and 3.5-fold higher than in pigs inoculated with PRRSV or LPS only. This indicates that pathways other than inhibition of cytokine production contributed to the clinical improvement. Finally, we studied a mechanism by which PRRSV may sensitize the lungs for LPS. We hypothesized that PRRSV would increase the amount of LPS receptor complex in the lungs leading to LPS sensitisation. Both CD14 and LPS-binding protein, two components of this complex, increased significantly during infection and the amount of CD14 in particular was correlated with LPS sensitisation. The increase of CD14 was mainly due to infiltration of strongly CD14-positive monocytes in the lungs. The PRRSV–LPS combination proved to be a simple and reproducible experimental model for multifactorial respiratory disease in pigs. To what extent the interaction between PRRSV and LPS contributes to the development of complex respiratory disease is still a matter of debate.

Keywords: PRRSV; LPS; Lungs; Synergy; Respiratory signs; Cytokines; Pentoxifylline; CD14; LBP

Abbreviations: BAL, bronchoalveolar lavage; CDCD, caesarean-derived colostrum-deprived; DPI, days post-inoculation; LBP, LPS-binding protein; NSAID, non-steroidal anti-inflammatory drug; PRCV, porcine respiratory coronavirus; PRRSV, porcine reproductive and respiratory syndrome virus; TLR4, Toll-like receptor 4

* Corresponding author. Tel.: +32 9 264 73 69; fax: +32 9 264 74 95.
E-mail address: kristien.vanreeth@ugent.be (K. Van Reeth).

0165-2427/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2004.09.006

1. Introduction

Interactions between viruses and bacteria in the induction of severe respiratory disease have been described since the early thirties (Shope, 1931). However, little remains known about the mechanisms whereby respiratory viruses can predispose for infection and/or disease by secondary agents. This also holds true for the porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus of swine, which is believed to play a key role in the porcine respiratory disease complex (Thacker, 2001; Brockmeier et al., 2002).

PRRSV suddenly emerged during the late eighties. The virus spread quickly and became enzootic in pig populations all over the world. An uncomplicated PRRSV infection, particularly under experimental circumstances and with European virus isolates, fails to induce overt respiratory disease (Van Reeth et al., 1996; Solano et al., 1997; Labarque et al., 2000). Still, PRRSV is considered as one of the most important etiological agents in multifactorial respiratory disease of swine, both in Europe and in the US (Done and Paton, 1995; Thacker, 2001; Brockmeier et al., 2002). Few studies, however, have been able to reproduce clinical respiratory disease by experimental inoculation with PRRSV followed by a secondary virus or bacterium. Dual infections have been performed with PRRSV followed by various bacteria such as *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Salmonella choleraesuis* and *Streptococcus suis* (Galina et al., 1994; Albina et al., 1995; Cooper et al., 1995; Van Alstine et al., 1996; Carvalho et al., 1997; Pol et al., 1997; Solano et al., 1997; Segalés et al., 1999; Thacker et al., 1999; Brockmeier et al., 2000; Halbur et al., 2000; Thanawongnuwech et al., 2000; Wills et al., 2000; Brockmeier et al., 2001; Schmitt et al., 2001). The outcome of these combinations ranged from no interaction (*H. parasuis* and *P. multocida*) to increased lung colonization (*B. bronchiseptica*) or increased mortality (*S. suis*). Most important, many of these studies yielded conflicting results. Using the same PRRSV–bacterium combination no disease was reported by some researchers and severe disease by others (Albina et al., 1995; Cooper et al., 1995; Van Alstine et al., 1996; Thacker et al., 1999; Wills et al., 2000). In the latter case, it was not always clear whether the effects of the combined inoculation were additive or synergistic. One exception was the combination of PRRSV and *S. suis*: a true synergistic interaction between both agents was reproduced by two different groups. However, the mechanisms underlying the interactions between PRRSV and the respective bacteria have remained largely unexplored in these studies.

We have performed dual infections with PRRSV followed by porcine respiratory coronavirus (PRCV) or swine influenza virus (Van Reeth et al., 1996, 2001). The clinical outcome of these dual infections, such as that with PRRSV and influenza virus, varied strongly within and between experiments. Within experiments, the proportion of pigs that developed enhanced respiratory disease varied from 20 to 100%. The severity of respiratory disease and weight loss also differed strongly between experiments. Conventional pigs were affected more severely than caesarean-derived colostrum-deprived (CDCD) pigs, which points towards a role of the sanitary status of pigs. Though our data support that interactions between respiratory viruses can aggravate respiratory disease in the field, experimental inoculations with PRRSV and PRCV or swine influenza virus lack the reproducibility that is required to study the pathogenesis of multifactorial respiratory disease. This is probably due to the fact that even a single experimental infection with respiratory viruses has intrinsic variation in virological, inflammatory and clinical parameters. A second infection will likely enhance this variation, as the outcome of the second infection is in part dependent on that of the first infection. We therefore chose to develop an alternative model consisting of a primary inoculation with PRRSV followed by a second inoculation with a non-replicating agent, namely LPS.

Here, we review the studies on the interaction between PRRSV and LPS that have been performed in the authors' laboratory. This paper compiles data of different primary publications which are referred to in each section. Section 4, which describes the effects of different pharmacological agents on the PRRSV–LPS induced disease, presents new data. The central hypothesis throughout these studies is that PRRSV–LPS induced respiratory disease results from an overproduction of proinflammatory cytokines locally

in the lungs. We will therefore first describe the cytokine profile after infection with PRRSV alone.

2. Cellular pathogenesis and cytokine profile of a PRRSV infection in the lungs

PRRSV causes a persistent infection of the lungs lasting 5–7 weeks (Mengeling et al., 1995; Labarque et al., 2000). In experimental infection studies, peak virus titers are obtained 7–10 days post-inoculation (DPI). PRRSV has a specific tropism for differentiated macrophages (Vanderheijden et al., 2003). Although not proven, several researchers suggest that destruction of lung macrophages by PRRSV is the key event in making the lungs susceptible for secondary invaders such as *B. bronchiseptica* or *S. suis* (Galina et al., 1994; Brockmeier et al., 2000). Indeed, in vitro infection of macrophages results in cell death within 48 h after inoculation (Suaréz et al., 1996; Oleksiewicz and Nielsen, 1999). Additionally, studies on lung tissue demonstrated that part of the macrophages surrounding infected cells undergo apoptosis (Sirinarumitr et al., 1998; Sur et al., 1998; Labarque et al., 2001, 2003; Choi and Chae, 2002). Another study showed that the capacity of the lungs to clear copper particles from the blood was reduced during PRRSV infection, probably because of destruction of intravascular lung macrophages (Thanawongnuwech et al., 2000). All these studies are consistent with the idea that the non-specific host defence may be impaired in PRRSV-infected lungs. In our studies, however, we never found a decrease of the total number of alveolar macrophages in PRRSV-infected lungs (Labarque et al., 2000). On the contrary, the infection caused a 5-fold increase of the number of alveolar macrophages between 5 and 52 DPI. This increase was due to infiltration of blood monocytes, which are known to differentiate into macrophages. Another important finding is that at the most 3% of the bronchoalveolar (BAL) lavage cells are infected during virus replication in the lungs (Mengeling et al., 1995; Duan et al., 1997; Labarque et al., 2000).

The production of proinflammatory cytokines in the lungs during PRRSV infection was studied intensively in our laboratory (Van Reeth et al., 1999; Labarque et al., 2003). IFN-α, IL-1 and TNF-α are important mediators of several infectious and inflammatory lung diseases (Bielefeldt-Ohmann, 1995; Murtaugh et al., 1996). They are among the first cytokines that are produced in the lungs during an infection. IFN-α is a typical antiviral cytokine, which can activate macrophages and natural killer cells (Tizard, 1995). IL-1 and TNF-α cause infiltration and activation of leukocytes in the lungs, increased microvascular permeability and bronchoconstriction (Martin et al., 2001). They also induce a cascade of secondary cytokines, such as IL-6, a potent inducer of acute-phase proteins in the liver (Murtaugh et al., 1996). The production of these cytokines in the lungs has been associated with general signs of disease, such as fever, depression and anorexia. Besides proinflammatory cytokines, we also examined the production of IL-10. IL-10 is a potent anti-inflammatory cytokine capable of reducing the inflammatory response, suppressing among other things the production of IL-1 and TNF-α (Steinhauser et al., 1999; Oberholzer et al., 2002). CDCD pigs were used to determine the profile of the respective cytokines during a PRRSV infection. Pigs were inoculated intranasally with PRRSV ($10^{6.0}$ $TCID_{50}$, Lelystad strain) at the age of 4–5 weeks and control pigs were left non-inoculated. IFN-α, IL-1 and TNF-α were quantified in the BAL fluids by specific bioassays, described in a previous paper (Van Reeth et al., 1999). IL-10 was quantified by an ELISA for porcine IL-10 (Biosource). The evolution of the virus titers and cytokine levels is presented in Fig. 1. The levels of two of the three proinflammatory cytokines were low or undetectable. IFN-α was detected at very low levels (29–168 U/ml) between 1 and 14 DPI and was undetectable thereafter. Bioactive TNF-α was undetectable, except at 14 DPI when low amounts (32–109 U/ml) were found. Only IL-1 was produced consistently from 1 (483 U/ml) to 52 DPI (256 U/ml). IL-1 levels peaked at 9 DPI (1265 U/ml), a time point which corresponds with the peak of virus replication in the lungs. IL-10 was detected from 5 (19 pg/ml) to 25 DPI (17 pg/ml) and the highest levels were also detected at 9 DPI (139 pg/ml). Both IL-1 and IL-10 levels were correlated well with each other and with PRRSV titers. The Spearman rank correlation coefficients ($P < 0.05$) ranged between 0.60 and 0.92. The uninfected control pigs were negative for

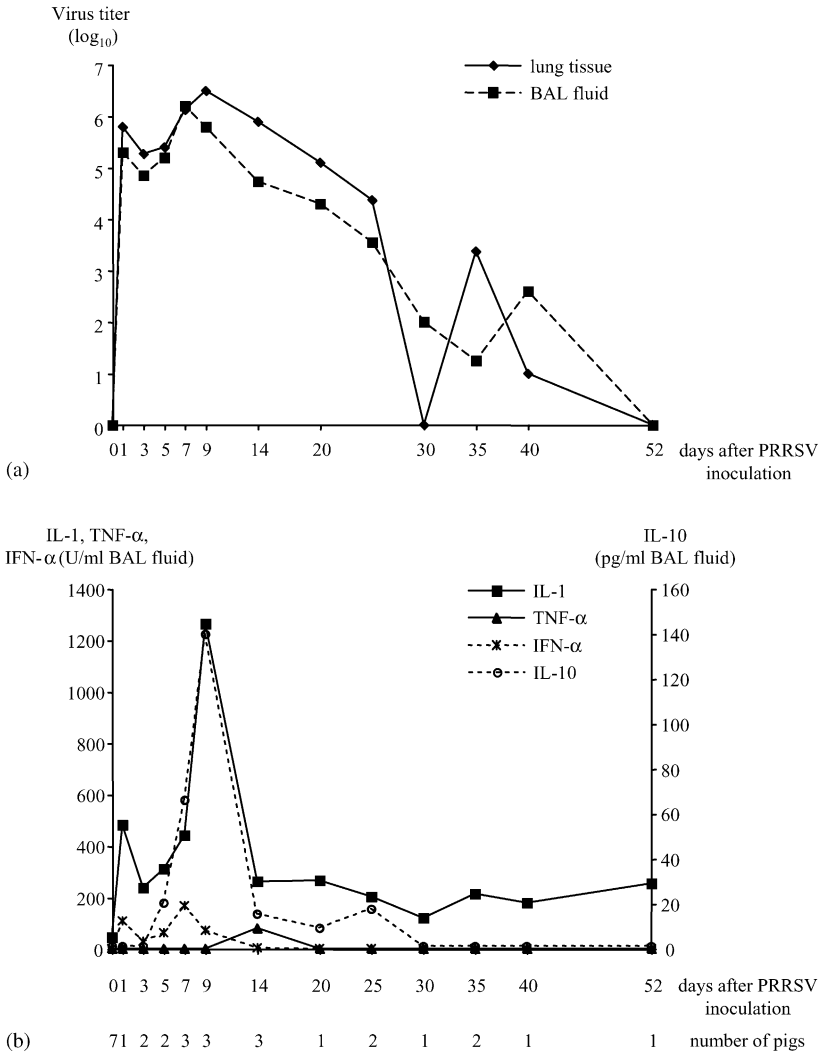

Fig. 1. Evolution of mean titers of PRRSV (a) and the cytokines IFN-α, TNF-α, IL-1 and IL-10 (b) in the lungs of PRRSV-inoculated pigs. Both virus titers in lung tissue (log_{10} $TCID_{50}$/g) and BAL fluids (log_{10} $TCID_{50}$/ml) are presented. IFN-α, TNF-α and IL-1 are expressed as bioactive units and IL-10 as pg/ml BAL fluid.

all cytokines, except one pig which tested positive for IL-1 (71 U/ml).

These studies demonstrate that PRRSV is a poor inducer of the proinflammatory cytokines TNF-α and IFN-α. This is in contrast to other porcine respiratory viruses, such as swine influenza virus, which elicits 10 and 1000-fold higher levels of the respective cytokines in the lungs (Van Reeth et al., 1998). IL-10 concentrations have not been determined in the lungs of pigs infected with other viruses than PRRSV. It is unclear therefore whether the IL-10 production in the lungs is typical of PRRSV. Some authors speculate that PRRSV-induced IL-10 could have an anti-inflammatory or even immunosuppressive effect (Feng et al., 2003; Suradhat and Thanawongnuwech, 2003). However, such an effect of endogenous IL-10 has not yet been proven in vivo in large animal species.

3. Search for a more straightforward virus–bacterium model: the combination of PRRSV and LPS

To study the interaction between PRRSV and bacterial components, we set up a dual inoculation model with PRRSV and LPS (Labarque et al., 2002). LPS was chosen because it is the main endotoxin and a major component of the outer membrane of Gram-negative bacteria. Pigs are likely to be exposed to LPS under farm conditions, as LPS is present in stable dust in concentrations up to several μg/m^3 air (Rask-Andersen et al., 1989; Zhiping et al., 1996). Also, LPS is released at high concentrations in the lungs during pulmonary infections with Gram-negative bacteria (Pugin et al., 1992). Treatment with antibiotics, especially those belonging to the β-lactam family, can induce sudden and massive release of LPS from the bacterial cell wall (Periti and Mazzei, 1999). LPS is one of the primary agents in organic dust that causes airway inflammation and bronchoconstriction (reviewed by Thorn (2001)). Adverse reactions after inhalation of LPS in stable dust, such as headache, coughing and decreased lung function, have been reported numerous times in farmers. Airborne LPS is considered an important factor of various inflammatory lung diseases of humans such as ''farmer's lung disease'' and asthma.

The response to inhaled LPS is strictly dose-dependent. For humans a no effect level of 10 ng LPS/m^3 during an 8 h workday has been proposed (Rylander, 2002). Several data suggest that healthy pigs can cope with the usual levels of LPS in stable dust without developing respiratory disease signs (Jolie, 1998; Urbain et al., 1999). We therefore hypothesized that a PRRSV infection might sensitize the lungs of pigs for respiratory disease upon exposure to a subclinical dose of LPS. To test this hypothesis, we set up a dual inoculation model with PRRSV and LPS.

In all our experiments, we used the Lelystad strain of PRRSV at a dose of $10^{6.0}$ $TCID_{50}$. LPS from *Escherichia coli* O111:B4 (Difco Laboratories) was used at a dose of 20 μg/kg. The clinical effects were studied in 5-week-old conventional pigs. Pigs were inoculated intratracheally with PRRSV, followed by LPS 5 days later. Pigs inoculated with PRRSV only and LPS only were included as controls.

Exposure of pigs to PRRSV or LPS only resulted in a transient fever (40–40.9 °C), but respiratory symptoms were minimal or absent (<45 breaths/min). Exposure of PRRSV-infected pigs to LPS, on the other hand, resulted in severe respiratory disease, characterized by tachypnoea (45–154 breaths/min), abdominal breathing and dyspnoea in 87% of the pigs. These pigs also showed enhanced general signs, such as high fever (≥41.0 °C) and depression. Clinical signs started within 1 h after LPS, reached a climax 2–4 h later and disappeared between 12 and 24 h after the LPS inoculation. There was clearly a strong synergy between PRRSV and LPS in the induction of respiratory disease. Since the initial experiment, 108 pigs in 12 different experiments have been inoculated with both PRRSV and LPS, together with the appropriate control pigs. In all experiments, we were able to reproduce the respiratory disease.

The pathogenesis of PRRSV–LPS induced disease was studied in CDCD pigs (Van Gucht et al., 2003b). Macroscopic and microscopic lesions, number of inflammatory cells and proinflammatory cytokine levels in the lungs were compared between pigs inoculated with PRRSV + LPS, PRRSV only, LPS only and PBS. In this experiment, the interval between PRRSV and LPS inoculations ranged from 3 to 14 days. Pigs were euthanized 6 h after LPS. Again, the PRRSV–LPS inoculated pigs developed severe respiratory disease, in contrast to the singly inoculated controls. PRRSV infection significantly enhanced cytokine production in response to LPS. Peak TNF-α, IL-1 and IL-6 titers were 10–100 times higher in PRRSV–LPS inoculated pigs than in the singly PRRSV or LPS-inoculated pigs and they correlated with respiratory signs. Fig. 2a shows the respiratory scores and bioactive TNF-α levels in the lung lavage fluids of pigs exposed to LPS at the different time intervals, ranging from 3 to 14 days, after PRRSV inoculation. The IL-1 and IL-6 levels are presented in detail in Van Gucht et al. (2003b). The clinical and cytokine synergy occurred at all intervals, but the highest cytokine titers were observed at 5–14 days after PRRSV inoculation. No cytokines were detected in the lungs of PBS-inoculated pigs. However, neutrophil infiltration, macroscopic and microscopic lesions in the lungs of PRRSV–LPS inoculated pigs resembled the combined effects of the single PRRSV and LPS inoculations without synergy. The histological lung lesions of PRRSV-infected pigs were little aggravated by subsequent LPS exposure

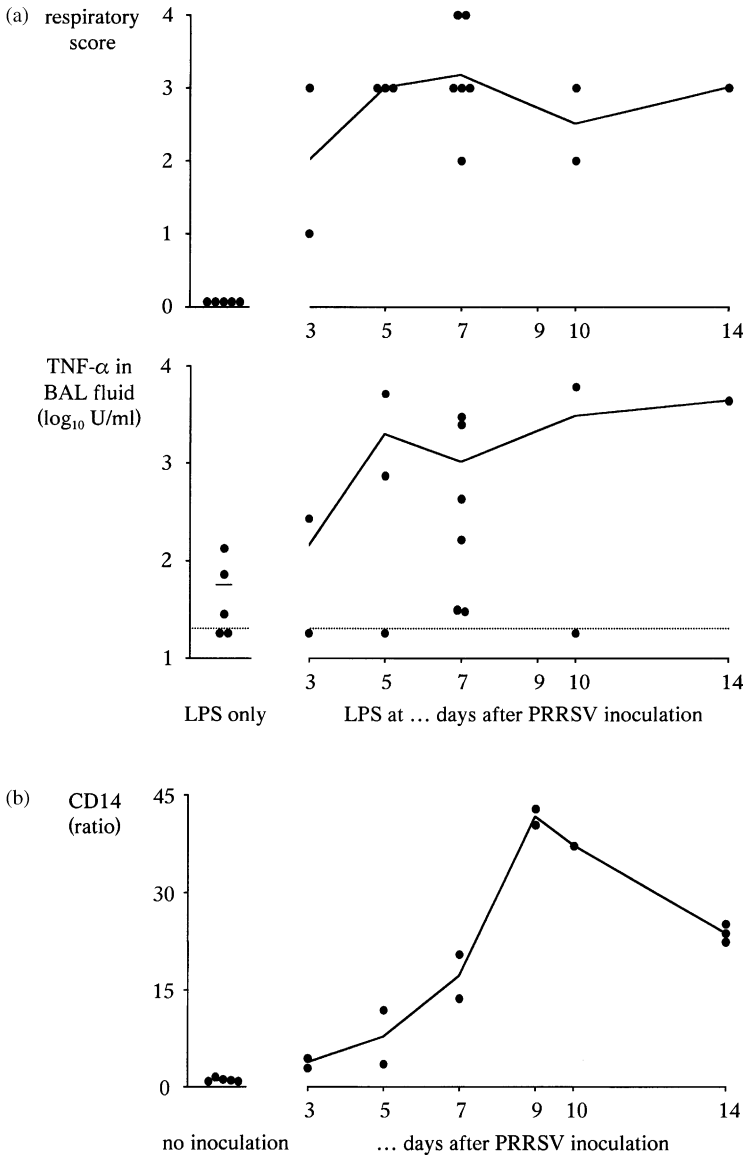

Fig. 2. Evolution of respiratory scores and lung TNF-α titers in pigs exposed to LPS at different days after PRRSV inoculation (a) and evolution of the amount of CD14 in lung tissue sections during PRRSV infection (no LPS exposure) (b). Each dot corresponds to one pig and the solid line represents the mean at each time point. The dotted line represents the detection limit. Respiratory scores range from 0 to 4, whereby 0 = normal; 1 = tachypnoea when stressed; 2 = tachypnoea at rest; 3 = tachypnoea and dyspnoea at rest; 4 = severe tachypnoea and dyspnoea with laboured, jerky breathing. Pigs inoculated with PRRSV only showed no respiratory signs (score 0) and had no detectable TNF-α, except at 14 DPI (32-109 U/ml) (see Fig. 1b).

(described in Van Gucht et al. (2003b)). This suggests that the difficult breathing of PRRSV–LPS inoculated pigs resulted from functional disturbances such as bronchoconstriction, rather than from structural lung damage.

It was clear from this experiment that PRRSV and LPS synergize in the induction of proinflammatory cytokines and that the overproduction of these cytokines is associated with disease. Although PRRSV is a poor inducer of proinflammatory

cytokines on its own, it can strongly enhance the cytokine response to a secondary agent. It is remarkable that IL-10, which is produced in the lungs from 5 up until 25 DPI with PRRSV, did not compromise the innate immune response against LPS.

4. Effect of a phosphodiesterase inhibitor and prostaglandin inhibitors on PRRSV–LPS induced clinical signs

The previous study in CDCD pigs indicated that the PRRSV–LPS induced clinical signs result from an acute overproduction of proinflammatory cytokines in the lungs. To confirm the role of these cytokines, we tested the effect of a known cytokine inhibitor on PRRSV–LPS induced disease. Pentoxifylline (Torental®, Hoechst) is a non-selective phosphodiesterase inhibitor that suppresses the production of proinflammatory cytokines, especially TNF-α and IL-1 (Noel et al., 1990; Neuner et al., 1994). Pentoxifylline has been successfully used to suppress systemic TNF-α levels in pigs (Gibson et al., 1991). Additionally, we have also examined the effects of two non-steroidal anti-inflammatory drugs (NSAIDs), namely meloxicam (Metacam®, Boehringer Ingelheim) and flunixin meglumine (Finadyne®, Schering-Plough). Both NSAIDs are registered to treat inflammation in swine. They inhibit the synthesis of prostaglandins and thromboxanes, which are eicosanoid mediators of inflammation (Odensvik et al., 1989; Schmidt and Banting, 2000; Hirsch et al., 2003).

Five-week-old conventional pigs were inoculated intratracheally with 10^6 $TCID_{50}$ of the Lelystad strain of PRRSV and 5 days later with LPS (20 μg/kg). Ten hours and 1 h before the LPS inoculation, pigs were treated with pentoxifylline (120 mg/kg orally, $n = 15$), meloxicam (1.5 mg/kg i.m., $n = 8$) or flunixin meglumine (5.5 mg/kg i.m., $n = 3$) or they were left untreated ($n = 17$). Untreated PRRSV-inoculated ($n = 7$), LPS-inoculated ($n = 8$) and non-inoculated pigs ($n = 8$) were also included. Clinical signs were monitored every 2 h from 0 to 12 h after LPS inoculation and evaluated using a scoring system. At 4 h after LPS, 14 pigs of the PRRSV–LPS group (7 pentoxifylline-treated and 7 untreated), 3 of the PRRSV group and 4 of the LPS group were euthanized. Bioactive levels of TNF-α, IL-1 and IL-6, and number of inflammatory cells were determined in BAL fluids.

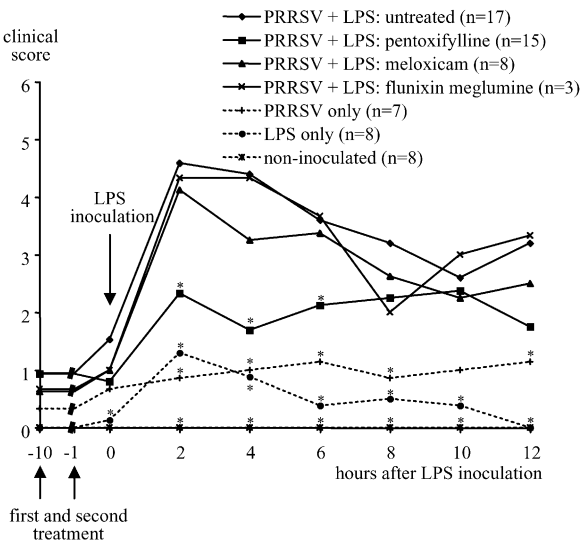

Fig. 3. Effect of pentoxifylline, meloxicam and flunixin meglumine treatment on clinical scores of PRRSV–LPS inoculated pigs. Clinical scores are calculated as shown in Table 1. Values with an asterisk are significantly different from the untreated PRRSV–LPS group (Mann–Whitney test, $P < 0.05$).

The evolution of clinical scores in all groups is presented in Fig. 3. Treatment with pentoxifylline significantly reduced fever and respiratory signs, but side effects like nervousness and tremor were seen in 40% of the pigs. Meloxicam and flunixin meglumine had no significant effect on fever and respiratory signs. The results of cytokine titrations are presented in Table 1. Mean TNF-α, IL-1 and IL-6 levels were 11–126 times higher in PRRSV–LPS inoculated pigs than in pigs inoculated with PRRSV or LPS only. Pentoxifylline treatment of PRRSV–LPS inoculated pigs reduced the mean TNF-α and IL-1 levels 5 and 3-fold respectively, but these levels were still 26 and 3.5-fold higher than those of the singly inoculated pigs. Pentoxifylline treatment had no effect on IL-6 levels.

This study demonstrated that pentoxifylline, a phosphodiesterase inhibitor, was more effective for the treatment of virus-LPS induced disease than classic prostaglandin synthesis inhibitors. Levels of TNF-α and IL-1 in PRRSV–LPS inoculated pigs were reduced by pentoxifylline treatment, but they were still considerably higher than those of the singly inoculated control pigs. This suggests that other

Table 1
Effect of pentoxifylline treatment on proinflammatory cytokines and inflammatory cells in bronchoalveloar (BAL) fluids of PRRSV–LPS inoculated pigs at 4 h after LPS

Group	*n*	Man clinical score (±S.E.M.)[a]	Mean BAL cytokine titers (U/ml, ±S.E.M.)			Mean BAL cells ($\times 10^6$, ±S.E.M.)	
			TNF-α	IL-1	IL-6	Monomorphonuclear cells	Neutrophils
PRRSV–LPS: pentoxifylline	7	1.7 (±0.5)[b]	1369 (±912)[b]	4037 (±1356)[b]	31196 (±23168)[b]	545 (±101)[b]	1316 (±381)[b,c]
PRRSV–LPS: untreated	7	4.7 (±0.4)[c]	6561 (±1789)[c]	12359 (±1947)[c]	32802 (±23683)[b]	669 (±118)[b]	1377 (±209)[b]
PRRSV: untreated	3	0.7 (±0.3)[b]	<40 (±0)[d]	490 (±668)[d]	382 (±625)[c]	772 (±157)[b]	315 (±146)[c]
LPS: untreated	4	0.8 (±0.3)[b]	52 (±39)[d]	1149 (±614)[d]	1443 (±456)[c]	659 (±100)[b]	1039 (±255)[b,c]

[a] Pigs were scored before euthanasia for fever (0: ≤39.9 °C, 1: ≥40 to ≤40.9 °C, 2: ≥41 °C), tachypnoea (0: ≤45, 1: ≥46 to ≤59, 2: ≥60), abdominal thumping (0: absent, 1: present) and dyspnoea (0: absent, 1: present). The total score per pig was obtained by adding the scores for each parameter and ranged from 0 to 6. Mean clinical scores per group are shown in the table.
[b] Values with different superscripts are significantly different (Mann–Whitney test, $P < 0.05$).
[c] Values with different superscripts are significantly different (Mann–Whitney test, $P < 0.05$).
[d] Values with different superscripts are significantly different (Mann–Whitney test, $P < 0.05$).

mechanisms may have contributed to the clinical improvement of pentoxifylline-treated pigs. Pentoxifylline can inhibit neutrophil adhesion and activation, improve blood perfusion and cause bronchodilatation (Tighe et al., 1990; Cortijo et al., 1993). Myers et al. (2002) studied the effect of pentoxifylline on acute pleuropneumonia caused by *A. pleuropneumoniae* in swine. They found that a dose of 20 mg/kg s.c. had no effect on the expression of proinflammatory cytokines in the lungs. Higher doses (200 mg/kg, s.c.) induced side effects such as vomiting, diarrhoea and tremor. Our results agree with those of Myers et al. (2002) in that pentoxifylline is probably not a good tool to study the role of proinflammatory cytokines in lung disease.

Both NSAIDs had little effect on fever and respiratory signs, though they were used at doses 2.5–3 times higher than prescribed by the respective companies. These results indicate that eicosanoid mediators have no direct effect on the acute clinical signs induced by the combination of PRRSV and LPS.

5. Increase of components of the LPS receptor complex in the lungs during PRRSV infection: a potential mechanism for the PRRSV–LPS synergy

Recent research in our laboratory focused on the mechanisms by which PRRSV can sensitize the lungs for LPS (Van Gucht et al., 2003a, 2005). The overproduction of proinflammatory cytokines seems to play an important role in the pathogenesis of PRRSV–LPS induced disease, but it is still unclear how PRRSV enhances cytokine production in response to LPS. A possible mechanism relates to the expression of the LPS receptor complex in the lungs. The biological effects of LPS depend on the binding to this complex. CD14, LPS-binding protein (LBP) and Toll-like receptor 4 (TLR4) are three major components of this complex (Heumann and Roger, 2002). CD14, also called the primary LPS receptor, is expressed on the membranes of monocytes, macrophages and to a lesser extent on neutrophils (Antal-Szalmas et al., 1997; Antal-Szalmas, 2000). CD14 is a so-called ‘‘pattern recognition receptor’’. This is a receptor that recognizes conserved molecules of several pathogens, such as LPS from Gram-negative bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria and chitosan from fungi and

insects, thereby initiating an inflammatory response against these organisms. Binding of LPS to CD14 can be enhanced by LBP, a soluble acute-phase protein produced by liver and lung epithelial cells (Fenton and Golenbock, 1998; Dentener et al., 2000). LBP increases the effect of LPS a 100-fold (Hailman et al., 1994). Finally, the CD14–LPS complex binds to TLR4, which sends a danger signal towards the nucleus followed by transcription of inflammatory genes.

We hypothesized that a PRRSV infection would increase the amount of LPS receptor complex in the lungs, thereby sensitizing the lungs for LPS. We therefore quantified the amount of CD14 and LBP in the lungs of CDCD pigs throughout the course of a PRRSV infection. TLR4 could not be examined, because there are no tools available for detection of this protein in pigs. PBS-inoculated pigs served as negative controls. CD14 was quantified in the lung interstitium by use of immunofluorescence stainings of lung tissue sections and image analysis. The amount of CD14 was expressed as a ratio compared to one of the PBS control lungs. LBP was quantified in BAL fluids by ELISA (Hycult biotechnology).

Fig. 2b shows the evolution of the amount of CD14 in the lungs during PRRSV infection. PRRSV infection caused a clear increase of CD14 expression (40×) and LBP (7×) in the lungs of pigs. CD14 was increased from 3 to 40 DPI and LBP from 7 to 14 DPI. Both parameters peaked at 9–10 DPI and were correlated tightly with virus replication in the lungs.

Double stainings demonstrated that the increase of CD14 was due to a massive infiltration of strongly CD14-positive monocytes in the lung interstitium. Compared to PRRSV-infected lungs, the amount of CD14 in uninfected lungs was low. There was minimal infiltration of monocytes in these lungs and resident macrophages expressed little CD14 on their membranes.

Thus, PRRSV caused a clear increase of CD14 and LBP in the lungs during infection. As both CD14 and LBP are components of the LPS receptor complex, their massive increase in the lungs during a PRRSV infection may explain why PRRSV sensitizes the lungs for the production of proinflammatory cytokines upon exposure to LPS. The PRRSV infection sensitized the lungs for LPS as early as 3 DPI (see Fig. 2a and b). The present data therefore suggest that CD14, which was increased from 3 DPI onwards, may be more important for the synergy with LPS than LBP, which was increased only from 7 DPI onwards.

6. Discussion

The research described in this paper was inspired by the apparent paradox that European strains of PRRSV, which cause minimal respiratory signs under experimental conditions, are a major cause of respiratory disease in the field.

Our first aim was to set up a reproducible model with PRRSV and LPS to study multifactorial respiratory disease in pigs. In spite of its subclinical course, PRRSV synergized with LPS in the induction of respiratory disease. The synergy between PRRSV and LPS proved to be reproducible under experimental circumstances. It is one of the few combined inoculations in pigs that consistently induces respiratory signs.

Our second aim was to unravel the pathogenesis of PRRSV–LPS induced disease. Emphasis was on the production of proinflammatory cytokines. PRRSV in itself was clearly a poor inducer of proinflammatory cytokines, such as IFN-α and TNF-α. We believe that this limited production of proinflammatory cytokines contributes to the lack of severe respiratory signs. Another important factor is that PRRSV does not replicate in or damage pneumocytes and endothelial cells, two cell types which are essential for normal gas exchange (Teifke et al., 2001; Howerth et al., 2002). Though a poor cytokine inducer as such, PRRSV sensitizes the lungs for the production of proinflammatory cytokines upon LPS stimulation. The acute overproduction of these cytokines probably plays a key role in the clinical signs. Treatment of PRRSV–LPS inoculated pigs with pentoxifylline only moderately reduced TNF-α and IL-1 levels and more specific cytokine inhibitors are needed to confirm the role of these cytokines in the acute respiratory disease.

PRRSV causes an increase of the amount of CD14 and LBP, both components of the LPS receptor complex, in the lungs. The increase of CD14 was mainly due to a massive influx of highly CD14-positive monocytes in the lung interstitium. It is likely that the increase of CD14 and, to a lesser extent LBP, sensitizes the lungs for LPS. CD14 not only acts as a

receptor for LPS, but also for conserved molecules of the cell wall of Gram-positive bacteria, such as peptidoglycan and lipoteichoic acid, leading to an activation of the innate immune response (Muhvic et al., 2001; Schroder et al., 2003). It is therefore possible that PRRSV also sensitizes the lungs for cell wall components other than LPS. Moreover, research indicates that LPS, peptidoglycan and lipoteichoic acid synergize in the induction of cytokines (De Kimpe et al., 1995; Wray et al., 2001). Stable dust contains a mixture of these molecules and the effect of LPS may be enhanced by other bacterial cell wall components (Zhiping et al., 1996).

Earlier research in our laboratory demonstrated a similar cytokine and clinical synergy between PRCV and LPS (Van Reeth et al., 2000). PRCV is, like PRRSV, a subclinical respiratory virus of swine and preliminary data suggest that a PRCV infection also increases CD14 expression in the lungs. The synergy with LPS is thus not unique for PRRSV and it may be a common feature of different respiratory viruses. However, PRRSV replicates for 5–7 weeks in the lungs, while PRCV replication lasts only 7–10 days. Therefore, interactions with LPS are more likely for PRRSV than for PRCV. There have been few studies on the interactions between other viruses and LPS in vivo. To our knowledge, PRRSV and PRCV are the first respiratory viruses that were shown to synergize with LPS in the induction of respiratory disease. Recently, it has been described that systemic infection of mice with lymphocytic choriomeningitis virus or vesicular stomatitis virus leads to fatal shock upon intraperitoneal inoculation with a sublethal dose of LPS (Nguyen and Biron, 1999; Nansen and Thomsen, 2001). The shock syndrome appeared to result from an overproduction of TNF-α. Studies with IFN knock-out mice indicated that virus-induced interferons, both IFN-α/β and -γ, were responsible for the increased sensitivity to LPS (Doughty et al., 2001; Nansen and Thomsen, 2001). It is unlikely, however, that IFN-α is involved in the sensitisation of PRRSV-infected pigs for LPS, because IFN-α production is minimal during infection with PRRSV (Albina et al., 1998; Van Reeth et al., 1999).

The significance of the interaction between PRRSV and LPS in respiratory disease in the field is difficult to assess and may depend on several factors, such as the level of virus replication in the lungs and the level of LPS exposure. Exposure to LPS is variable and may depend on several factors, such as concentration of stable dust, load of Gram-negative bacteria in the lungs and use of antibiotics. In our experiments, LPS was administered to pigs at a dose of 20 μg/kg body weight. Assuming an environment with an endotoxin concentration of 5 $\mu g/m^3$ air (Zhiping et al., 1996) and a respiratory volume of 0.3 m^3/h, pigs of the same age as in our experimental model would be exposed to a total dose of airborne endotoxins of approximately 36 μg/day. Thus, one could argue that pigs in the field are exposed to lower doses of airborne LPS than those used experimentally. Unlike for humans, there are almost no epidemiological studies on the role of airborne LPS in respiratory disease of pigs (Jolie, 1998). LPS is not only inhaled with dust but also released locally in the lungs during an infection with Gram-negative bacteria. It was shown that at least part of the lung lesions and clinical signs of an infection with Gram-negative bacteria, such as *A. pleuropneumoniae*, are caused by the release of LPS from the bacterial cell wall (Udeze et al., 1987; Idris et al., 1993). In theory, any infection of the deeper lungs with Gram-negative bacteria has the potential of synergizing with PRRSV, if sufficient amounts of LPS are released. We ourselves did not perform dual inoculations with PRRSV and whole Gram-negative bacteria, but several groups were unable to demonstrate a clinical synergy between PRRSV and bacteria, like *H. parasuis* and *P. multocida* (Cooper et al., 1995; Carvalho et al., 1997; Solano et al., 1997). It should be mentioned, however, that bacteria could not be isolated from the lungs of most dually inoculated pigs in these studies. This means that there was no opportunity for an interaction between PRRSV and locally released LPS. Also, the biological activity of LPS, which is determined by the structure of the lipid A component, depends on the species of bacteria (Erridge et al., 2002). LPSs of *E. coli* or *Salmonella* spp., for example, have a stronger endotoxic activity than those of pseudomonas or *Chlamydia* spp.

We do not know whether prolonged LPS exposure will lead to chronic respiratory disease or, on the contrary, to LPS tolerance. LPS tolerance has been shown in numerous animal models and is characterized by a decreased sensitivity to LPS after repeated LPS exposure (reviewed in Cavaillon et al. (2003)). In unpublished experiments, we have exposed PRRSV-

infected pigs up to three times to LPS at 3, 6 and 9 days after virus inoculation without a decrease of the clinical response to LPS, suggesting that PRRSV-infected pigs did not become refractory to LPS. Also, recent research demonstrated that 5-day and 8-week exposure of mice to an aerosol of LPS led to respectively sustained cytokine production and chronic pneumonia (Brass et al., 2003).

The PRRSV–LPS combination is a simple and reproducible experimental model to study multi-factorial respiratory disease in pigs. It is likely that most pigs undergoing a PRRSV infection in the field are simultaneously exposed to LPS, either by inhalation of stable dust or pulmonary bacterial infections. To what extent the interaction between PRRSV and LPS will contribute to the development of complex respiratory disease is still a matter of debate.

Acknowledgements

Steven Van Gucht and Kristien Van Reeth are Fellows of the Fund for Scientific Research-Flanders (Belgium) (F.W.O.-Vlaanderen). Part of this research was supported by grant 5772A from the Belgian Ministry of Agriculture. The authors would like to thank Chris Bracke, Dieter Defever, Fernand De Backer and Lieve Sys for their excellent technical assistance.

References

Albina, E., Kobisch, M., Cariolet, R., Morvan, P., Kéranflec'h, A., Beaurepaire, B., Hutet, E., Labbé, A., 1995. Le syndrome dysgénésique et respiratoire du porc (SDRP): Etude expérimentale des effets de l'infection sur la réponse immunitaire et la résistance aux infections Aujeszky et *Mycoplasma hyopneumoniae* chez le porc en croissance. Journées Rech. Porcine en France 27, 107–116.

Albina, E., Carrat, C., Charley, B., 1998. Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. J. Interf. Cytok. Res. 18 (7), 485–490.

Antal-Szalmas, P., Strijp, J.A., Weersink, A.J., Verhoef, J., Van Kessel, K.P., 1997. Quantitation of surface CD14 on human monocytes and neutrophils. J. Leukoc. Biol. 61 (6), 721–728.

Antal-Szalmas, P., 2000. Evaluation of CD14 in host defence. Eur. J. Clin. Invest. 30 (2), 167–179.

Bielefeldt-Ohmann, H., 1995. Role of cytokines in the pathogenesis and treatment of respiratory disease. In: Myers, M.J., Murtaugh, M.P. (Eds.), Cytokines in Animal Health and Disease. Marcel Dekker, New York, pp. 291–332.

Brass, D.M., Savov, J.D., Gavett, S.H., Haykal-Coates, N., Schwartz, D.A., 2003. Subchronic endotoxin inhalation causes persistent airway disease. Am. J. Physiol. Lung Cell. Mol. Physiol. 285 (3), L755–L761.

Brockmeier, S.L., Palmer, M.V., Bolin, S.R., 2000. Effects of intranasal inoculation of porcine reproductive and respiratory syndrome virus, *Bordetella bronchiseptica*, or a combination of both organisms in pigs. Am. J. Vet. Res. 61, 892–899.

Brockmeier, S.L., Palmer, M.V., Bolin, S.R., Rimler, R.B., 2001. Effects of intranasal inoculation with *Bordetella bronchiseptica*, porcine reproductive and respiratory syndrome virus, or a combination of both organisms on subsequent infection with *Pasteurella multocida* in pigs. Am. J. Vet. Res. 62, 521–525.

Brockmeier, S.L., Halbur, P.G., Thacker, E.L., 2002. Porcine respiratory disease complex. In: Brogden, K.A., Guthmiller, J.M. (Eds.), Polymicrobial Diseases. ASM Press, Washington, DC, pp. 231–258.

Carvalho, L.F.O.S., Segalés, J., Pijoan, C., 1997. Effect of porcine reproductive and respiratory syndrome virus on subsequent *Pasteurella multocida* challenge in pigs. Vet. Microbiol. 55, 241–246.

Cavaillon, J.M., Adrie, C., Fitting, C., Adib-Conquy, M., 2003. Endotoxin tolerance: is there a clinical relevance? J. Endotoxin Res. 9 (2), 101–107.

Choi, C., Chae, C., 2002. Expression of tumour necrosis factor-α is associated with apoptosis in lungs of pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Res. Vet. Sci. 72, 45–49.

Cooper, V.L., Doster, A.R., Hesse, R.A., Harris, N.B., 1995. Porcine reproductive and respiratory syndrome: NEB-1 PRRSV infection did not potentiate bacterial pathogens. J. Vet. Diagn. Invest. 7, 313–320.

Cortijo, J., Bou, J., Beleta, J., Cardelus, I., Llenas, J., Morcillo, E., Gristwood, R.W., 1993. Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus. Brit. J. Pharmacol. 108 (2), 562–568.

De Kimpe, S.J., Kengatharan, M., Thiemermann, C., Vane, J.R., 1995. The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. Proc. Natl. Acad. Sci. U.S.A. 92 (22), 10359–10363.

Dentener, M.A., Vreugdenhil, A.C., Hoet, P.H., Vernooy, J.H., Nieman, F.H., Heumann, D., Janssen, Y.M., Buurman, W.A., Wouters, E.F., 2000. Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells: implications for local defence to bacterial endotoxins. Am. J. Respir. Cell. Mol. Biol. 23 (2), 146–153.

Done, S.H., Paton, D.J., 1995. Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. Vet. Rec. 136, 32–35.

Doughty, L.A., Nguyen, K.B., Durbin, J.E., Biron, C.A., 2001. A role for IFN-αβ in virus infection-induced sensitization to endotoxin. J. Immunol. 166, 2658–2664.

Duan, X., Nauwynck, H.J., Pensaert, M.B., 1997. Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Microbiol. 56 (1–2), 9–19.

Erridge, C., Bennett-Guerrero, E., Poxton, I.R., 2002. Structure and function of lipopolysaccharides. Microb. Infect. 4 (8), 837–851.

Feng, W.H., Tompkins, M.B., Xu, J.S., Zhang, H.X., McCaw, M.B., 2003. Analysis of constitutive cytokine expression by pigs infected in utero with porcine reproductive and respiratory syndrome virus. Vet. Immunol. Immunopathol. 94 (1–2), 35–45.

Fenton, M.J., Golenbock, D.T., 1998. LPS-binding proteins and receptors. J. Leukoc. Biol. 64 (1), 25–32.

Galina, L., Pijoan, C., Sitjar, M., Christianson, W.T., Rossow, K., Collins, J.E., 1994. Interaction between *Streptococcus suis* serotype 2 and porcine reproductive and respiratory syndrome virus in specific pathogen-free piglets. Vet. Rec. 134, 60–64.

Gibson, R.L., Redding, G.J., Henderson, W.R., Truog, W.E., 1991. Group B streptococcus induces tumor necrosis factor in neonatal piglets. Effect of the tumor necrosis factor inhibitor pentoxifylline on hemodynamics and gas exchange. Am. Rev. Respir. Dis. 143 (3), 598–604.

Hailman, E., Lichenstein, H.S., Wurfel, M.M., Miller, D.S., Johnson, D.A., Kelley, M., Busse, L.A., Zukowski, M.M., Wright, S.D., 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J. Exp. Med. 79 (1), 269–277.

Halbur, P., Thanawongnuwech, R., Brown, G., Kinyon, J., Roth, J., Thacker, E., Thacker, B., 2000. Efficacy of antimicrobial treatments and vaccination regimens for control of porcine reproductive and respiratory syndrome virus and *Streptococcus suis* coinfection of nursery pigs. J. Clin. Microbiol. 38, 1156–1160.

Heumann, D., Roger, T., 2002. Initial responses to endotoxins and Gram-negative bacteria. Clin. Chim. Acta 323, 59–72.

Hirsch, A.C., Philipp, H., Kleemann, R., 2003. Investigation on the efficacy of meloxicam in sows with mastitis-metritis-agalactia syndrome. J. Vet. Pharmacol. Ther. 26 (5), 355–360.

Howerth, E.W., Murphy, M.D., Roberts, A.W., 2002. Failure of porcine reproductive and respiratory syndrome virus to replicate in porcine endothelial cell cultures. J. Vet. Diagn. Invest. 14, 73–76.

Idris, U.E., Harmon, B.G., Udeze, F.A., Kadis, S., 1993. Pulmonary lesions in mice inoculated with *Actinobacillus pleuropneumoniae* hemolysin and lipopolysaccharide. Vet. Pathol. 30 (3), 234–241.

Jolie, R., 1998. Pulmonary inflammatory and hematological changes in pigs after exposure to airborne contaminants in swine farms: field and experimental studies. Ph.D. Thesis. University of Wisconsin-Madison, pp. 176–178.

Labarque, G.G., Nauwynck, H.J., Van Reeth, K., Pensaert, M.B., 2000. Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. J. Gen. Virol. 81, 1327–1334.

Labarque, G.G., Nauwynck, H.J., Van Reeth, K., Pensaert, M.B., 2001. Apoptosis in the lungs of pigs during an infection with a European strain of porcine reproductive and respiratory syndrome virus. Adv. Exp. Med. Biol. 494, 691–697.

Labarque, G., Van Reeth, K., Van Gucht, S., Nauwynck, H., Pensaert, M., 2002. Porcine reproductive–respiratory syndrome virus (PRRSV) infection predisposes pigs for respiratory signs upon exposure to bacterial lipopolysaccharide. Vet. Microbiol. 88 (1), 1–12.

Labarque, G., Van Gucht, S., Nauwynck, H., Van Reeth, K., Pensaert, M., 2003. Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines. Vet. Res. 34 (3), 249–260.

Martin, C., Wohlsen, A., Uhlig, S., 2001. Changes in airway resistance by simultaneous exposure to TNF-α and IL-1β in perfused rat lungs. Am. J. Physiol. Lung Cell. Mol. Physiol. 280 (4), L595–L601.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1995. Diagnosis of porcine reproductive and respiratory syndrome. J. Vet. Diagn. Invest. 7, 3–16.

Muhvic, D., El-Samalouti, V., Flad, H.D., Radosevic-Stasic, B., Rukavina, D., 2001. The involvement of CD14 in the activation of human monocytes by peptidoglycan monomers. Mediat. Inflamm. 10 (3), 155–162.

Murtaugh, M.P., Baarsch, M.J., Zhou, Y., Scamurra, R.W., Lin, G., 1996. Inflammatory cytokines in animal health and disease. Vet. Immunol. Immunopathol. 54, 45–55.

Myers, M.J., Baarsch, M.J., Murtaugh, M.P., 2002. Effects of pentoxifylline on inflammatory cytokine expression and acute pleuropneumonia in swine. Immunobiology 205 (1), 17–34.

Nansen, A., Thomsen, A.R., 2001. Viral infection causes rapid sensitization to lipopolysaccharide: central role of IFN-αβ. J. Immunol. 166, 982–988.

Neuner, P., Klosner, G., Schauer, E., Pourmojib, M., Macheiner, W., Grunwald, C., Knobler, R., Schwarz, A., Luger, T.A., Schwarz, T., 1994. Pentoxifylline in vivo down-regulates the release of IL-1 beta, IL-6, IL-8 and tumour necrosis factor-alpha by human peripheral blood mononuclear cells. Immunology 83 (2), 262–267.

Nguyen, K.B., Biron, C.A., 1999. Synergy for cytokine-mediated disease during concurrent endotoxin and viral challenges: roles for NK and T cell IFN-γ production. J. Immunol. 162, 5238–5246.

Noel, P., Nelson, S., Bokulic, R., Bagby, G., Lippton, H., Lipscomb, G., Summer, W., 1990. Pentoxifylline inhibits lipopolysaccharide-induced serum tumor necrosis factor and mortality. Life Sci. 47 (12), 1023–1029.

Oberholzer, A., Oberholzer, C., Moldawer, L.L., 2002. Interleukin-10: a complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug. Crit. Care Med. 30, S58–S63.

Odensvik, K., Cort, N., Basu, S., Kindahl, H., 1989. Effect of flunixin meglumine on prostaglandin F2 alpha synthesis and metabolism in the pig. J. Vet. Pharmacol. Ther. 12 (3), 307–311.

Oleksiewicz, M.B., Nielsen, J., 1999. Effect of porcine reproductive and respiratory syndrome virus (PRRSV) on alveolar lung macrophage survival and function. Vet. Microbiol. 66, 15–27.

Periti, P., Mazzei, T., 1999. New criteria for selecting the proper antimicrobial chemotherapy for severe sepsis and septic shock. Int. J. Antimicrob. Agents 12 (2), 97–105.

Pol, J.M.A., van Leengoed, L.A.M.G., Stockhofe, N., Kok, G., Wensvoort, G., 1997. Dual infections of PRRSV/influenza or PRRSV/*Actinobacillus pleuropneumoniae* in the respiratory tract. Vet. Microbiol. 55, 259–264.

Pugin, J., Auckenthaler, R., Delaspre, O., Van Gessel, E., Suter, P.M., 1992. Rapid diagnosis of Gram negative pneumonia by assay of endotoxin in bronchoalveolar lavage fluid. Thorax 47 (7), 547–549.

Rask-Andersen, A., Malmberg, P., Lundholm, M., 1989. Endotoxin levels in farming: absence of symptoms despite high exposure levels. Brit. J. Ind. Med. 46 (6), 412–416.

Rylander, R., 2002. Endotoxin in the environment—exposure and effects. J. Endotoxin Res. 8 (4), 241–252.

Schmidt, H., Banting, A., 2000. Meloxicam (Metacam®) for the alleviation of inflammatory conditions in swine—selection of an effective dose. In: Proceedings of the 16th IPVS Congress, Melbourne, Australia, p. 99.

Schmitt, C.S., Halbur, P.G., Roth, J.A., Kinyon, J.M., Kasorndorkbua, C., Thacker, B., 2001. Influence of ampicillin, ceftiofur, attenuated live PRRSV vaccine, and reduced dose *Streptococcus suis* exposure on disease associated with PRRSV and *S. suis* coinfection. Vet. Microbiol. 78, 29–37.

Schroder, N.W., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zahringer, U., Gobel, U.B., Weber, J.R., Schumann, R.R., 2003. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J. Biol. Chem. 278 (18), 15587–15594.

Segalés, J., Domingo, M., Solano, G.I., Pijoan, C., 1999. Porcine reproductive and respiratory syndrome virus and *Haemophilus parasuis* antigen distribution in dually infected pigs. Vet. Microbiol. 64, 287–297.

Shope, R.E., 1931. Swine influenza. III. Filtration experiments and etiology. J. Exp. Med. 54, 373–385.

Sirinarumitr, T., Zhang, Y., Kluge, J.P., Halbur, P.G., Paul, P.S., 1998. A pneumo-virulent United States isolate of porcine reproductive and respiratory syndrome virus induces apoptosis in bystander cells both in vitro and in vivo. J. Gen. Virol. 79, 2989–2995.

Solano, G.I., Segalés, J., Collins, J.E., Molitor, T.W., Pijoan, C., 1997. Porcine reproductive and respiratory syndrome virus (PRRSv) interaction with *Haemophilus parasuis*. Vet. Microbiol. 55, 247–257.

Steinhauser, M.L., Hogaboam, C.M., Kunkel, S.L., Lukacs, N.W., Strieter, R.M., Standiford, T.J., 1999. IL-10 is a major mediator of sepsis-induced impairment in lung antibacterial host defense. J. Immunol. 162, 392–399.

Suaréz, P., Diaz-Guerra, M., Prieto, C., Esteban, M., Castro, J.M., Nieto, A., Ortin, J., 1996. Open reading frame 5 of porcine reproductive and respiratory syndrome virus as a cause of virus-induced apoptosis. J. Virol. 70 (5), 2876–2882.

Sur, J.H., Doster, A.R., Osorio, F.A., 1998. Apoptosis induced *in vivo* during acute infection by porcine reproductive and respiratory syndrome virus. Vet. Pathol. 35, 506–514.

Suradhat, S., Thanawongnuwech, R., 2003. Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 84, 2755–2760.

Teifke, J.P., Dauber, M., Fichtner, D., Lenk, M., Polster, U., Weiland, E., Beyer, J., 2001. Detection of European porcine reproductive and respiratory syndrome virus in porcine alveolar macrophages by two-colour immunofluorescence and in-situ hybridization-immunohistochemistry double labelling. J. Comp. Pathol. 124, 238–245.

Thacker, E.L., Halbur, P.G., Ross, R.F., Thanawongnuwech, R., Thacker, B.J., 1999. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. J. Clin. Microbiol. 37, 620–627.

Thacker, E.L., 2001. Immunology of the porcine respiratory disease complex. Vet. Clin. N. Am.: Food Anim. Pract. 17 (3), 551–565.

Thanawongnuwech, R., Brown, G.B., Halbur, P.G., Roth, J.A., Royer, R.L., Thacker, B.J., 2000. Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to *Streptococcus suis* infection. Vet. Pathol. 37, 143–152.

Thorn, J., 2001. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. Inflamm. Res. 50 (5), 254–261.

Tighe, D., Moss, R., Hynd, J., Boghossian, S., al-Saady, N., Heath, M.F., Bennett, E.D., 1990. Pretreatment with pentoxifylline improves the hemodynamic and histologic changes and decreases neutrophil adhesiveness in a pig fecal peritonitis model. Crit. Care Med. 18 (2), 184–189.

Tizard, I.R., 1995. Interferons. In: Myers, M.J., Murtaugh, M.P. (Eds.), Cytokines in Animal Health and Disease. Marcel Dekker, New York, pp. 1–57.

Udeze, F.A., Latimer, K.S., Kadis, S., 1987. Role of *Haemophilus pleuropneumoniae* lipopolysaccharide endotoxin in the pathogenesis of porcine *Haemophilus pleuropneumonia*. Am. J. Vet. Res. 48 (5), 768–773.

Urbain, B., Mast, J., Beerens, D., N'Guyen, T.Q., Goddeeris, B., Ansay, M., Gustin, P., 1999. Effects of inhalation of dust and endotoxin on respiratory tracts of pigs. Am. J. Vet. Res. 60, 1055–1060.

Van Alstine, W.G., Stevenson, G.W., Kanitz, C.L., 1996. Porcine reproductive and respiratory syndrome virus does not exacerbate *Mycoplasma hyopneumoniae* infection in young pigs. Vet. Microbiol. 49, 297–303.

Van Gucht, S., Van Reeth, K., Labarque, G., Nauwynck, H., Pensaert, M., 2003a. PRRSV infection increases CD14 expression and LPS-binding protein in the lungs: possible mechanism of PRRSV–LPS clinical synergy. In: Proceedings of the Fourth International Symposium on Emerging and Re-emerging Pig Diseases, Rome, Italy, p. 49.

Van Gucht, S., Van Reeth, K., Pensaert, M., 2003b. Interaction between porcine reproductive–respiratory syndrome virus and bacterial endotoxin in the lungs of pigs: potentiation of cytokine production and respiratory disease. J. Clin. Microbiol. 41 (3), 960–966.

Van Gucht, S., Van Reeth, K., Nauwynck, H., Pensaert, M., 2005. Porcine reproductive–respiratory syndrome virus infection increases CD14 expression and lipopolysaccharide-binding protein in the lungs of pigs. Viral Immunol., in press.

Van Reeth, K., Nauwynck, H.J., Pensaert, M.B., 1996. Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study. Vet. Microbiol. 48, 325–335.

Van Reeth, K., Nauwynck, H., Pensaert, M., 1998. Broncho-alveolar interferon-α, tumor necrosis factor-α, interleukin-1 and inflammation during acute influenza in pigs: a possible model for humans? J. Infect. Dis. 177, 1076–1079.

Van Reeth, K., Labarque, G., Nauwynck, H., Pensaert, M., 1999. Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. Res. Vet. Sci. 67, 47–52.

Van Reeth, K., Nauwynck, H., Pensaert, M., 2000. A potential role for tumour necrosis factor-alpha in synergy between porcine respiratory coronavirus and bacterial lipopolysaccharide in the induction of respiratory disease in pigs. J. Med. Microbiol. 49 (7), 613–620.

Van Reeth, K., Nauwynck, H., Pensaert, M., 2001. Clinical effects of experimental dual infections with porcine reproductive and respiratory syndrome virus followed by swine influenza virus in conventional and colostrum-deprived pigs. J. Vet. Med. B 48, 283–292.

Vanderheijden, N., Delputte, P.L., Favoreel, H.W., Vandekerckhove, J., Van Damme, J., van Woensel, P.A., Nauwynck, H.J., 2003. Sialoadhesin can serve as an endocytic receptor mediating porcine arterivirus entry into alveolar macrophages. J. Virol. 77 (15), 8207–8215.

Wills, R.W., Gray, J.T., Fedorka-Cray, P.J., Yoon, K.J., Ladely, S., Zimmerman, J.J., 2000. Synergism between porcine reproductive and respiratory syndrome virus (PRRSV) and *Salmonella choleraesuis* in swine. Vet. Microbiol. 71, 177–192.

Wray, G.M., Foster, S.J., Hinds, C.J., Thiemermann, C., 2001. A cell wall component from pathogenic and non-pathogenic Gram-positive bacteria (peptidoglycan) synergizes with endotoxin to cause the release of tumour necrosis factor-alpha, nitric oxide production, shock, and multiple organ injury/dysfunction in the rat. Shock 15 (2), 135–142.

Zhiping, W., Malmberg, P., Larsson, K., Larsson, L., Saraf, A., 1996. Exposure to bacteria in swine-house dust and acute inflammatory reactions in humans. Am. J. Respir. Care Med. 154 (5), 1261–1266.