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VIRAL PATHOGENICITY

# 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

KRISTIEN VAN REETH, HANS NAUWYNCK and MAURICE PENSAERT

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

**This study examined whether exposure of pigs to both porcine respiratory coronavirus (PRCV) and bacterial lipopolysaccharide (LPS) can potentiate respiratory disease and lung secretion of tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-1 (IL-1). Caesarian-derived colostrum-deprived pigs were inoculated intratracheally with PRCV, with LPS from *Escherichia coli* O111:B4 (20 $\mu$g/kg), or with a combination of the two, and killed at set times after inoculation. Clinical signs, virus replication and (histo)pathological changes in the lungs, percentage of neutrophils and bioactive TNF-$\alpha$ and IL-1 in broncho-alveolar lavage (BAL) fluids were examined. The effects of separate virus or LPS inoculations were subclinical and failed to induce high and sustained cytokine levels. In a preliminary study, pigs were inoculated with PRCV and then with LPS 24 h later and killed sequentially. Severe respiratory disease and significantly enhanced TNF-$\alpha$ titres (208–3601 U/ml *versus* 40–89 U/ml after LPS only) were seen during the first 12 h after LPS inoculation. IL-1 levels (106–1631 U/ml *versus* 28–654 U/ml after LPS only) were also increased, but persisted for longer after clinical recovery than TNF-$\alpha$. In a second study, pigs were inoculated with PRCV and subsequently with LPS at various time intervals ranging from 0 to 24 h, and killed 5 h after inoculation with LPS. A time interval of at least 12 h between inoculations was necessary for prominent respiratory signs to develop. Production of TNF-$\alpha$, but not IL-1, was also dependent on the time interval between inoculations and was tightly correlated with disease. Lung neutrophil infiltration and pathological changes were comparable after combined PRCV-LPS and single LPS inoculations, and were not associated with disease. These data show that exposure to high endotoxin concentrations in swine buildings can precipitate respiratory disease in PRCV-infected pigs, and that TNF-$\alpha$ is probably an important mediator of these effects. This is the first in-vivo demonstration of synergy between respiratory viruses and LPS.**

## Introduction

Coronaviruses cause clinically mild or asymptomatic infections of the upper and lower respiratory tracts in man, cattle and pigs. Porcine respiratory coronavirus (PRCV) is ubiquitous in Europe and appears to have become increasingly prevalent in swine herds in North America [1, 2]. In Belgium, most pigs become infected between 6 and 15 weeks of age, and antibodies to PRCV are prevalent in *c*. 90% of pigs at slaughter. PRCV replicates readily in the epithelial cells of nasal mucosa, tonsils, trachea and lungs. In experimental infection studies, lung virus titres reach up to $10^{7.5}$–$10^{8.3}$ TCID50/g tissue and broncho-interstitial pneumonia is consistently observed, but clinical respiratory disease does not usually develop [3–5]. However, PRCV is increasingly mentioned as a contributor to multifactorial respiratory disease conditions, known as the 'porcine respiratory disease complex'. The respiratory agents or factors that may work in conjunction with the virus to cause disease, and possible disease mechanisms, have not been explored.

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Corresponding author: Dr K. Van Reeth (e-mail:kristien.vanreeth@rug.ac.be).

Endotoxin, or its main component lipopolysaccharide (LPS), is a major constituent of the cell walls of gram-negative bacteria. Airborne endotoxin concentrations in swine confinement units can be especially high, ranging from 0.04 to 1.2 $\mu g/m^3$ [6, 7]. Nebulisation or intratracheal inoculation of purified LPS induces a dramatic infiltration of the lungs with neutrophils and macrophages in pigs [8–10], human volunteers [11] and laboratory animals [12]. Furthermore, endotoxin exposure in swine farmers has been associated with airway inflammation, decreased respiratory function tests and chronic coughing [7, 13, 14]. Many of the biological effects of LPS are due to the release of tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-1 (IL-1) [12, 14]. These cytokines mediate leucocyte recruitment and activation in the lungs, increased lung microvascular permeability and pulmonary dysfunctions [15]. It is important to note that cytokine induction and lung injury by LPS are strictly dose-dependent. For example, if sufficient amounts of LPS are given to animals or man, TNF-$\alpha$ and IL-1 production, pulmonary oedema, focal necrosis and decreased lung function are observed [11, 12]. Slightly smaller LPS doses, on the other hand, will cause a mild lung inflammation only.

There have been few studies on the possible interactions between respiratory viruses and LPS. A few in-vitro studies have documented synergy between viruses and LPS in the induction of cytokines. For example, mouse-adapted influenza virus – which induces minimal TNF-$\alpha$ in leucocyte cultures – was shown to prime cells for massive TNF-$\alpha$ secretion in response to minute amounts of LPS from *Haemophilus influenzae* or *Escherichia coli*, itself also inefficient in inducing TNF-$\alpha$ [16]. Similarly, an enhanced serum TNF-$\alpha$ response was observed when influenza-infected mice were given an intravenous LPS trigger [17]. Previous investigations in pigs have shown that experimental, uncomplicated PRCV infection induces negligible lung levels of both TNF-$\alpha$ and IL-1, which may explain the very mild clinicopathological manifestations observed [18]. The aims of the present study were to examine whether combined exposure of pigs to PRCV and LPS can potentiate respiratory disease and lung production of TNF-$\alpha$ and IL-1, and whether cytokines and disease are correlated.

## Materials and methods

### *Virus and LPS preparations*

The 91V44 isolate of PRCV [19] was at the second passage in swine testis (ST) cells. The virus stock was purified by sucrose density gradient centrifugation and contained $<1.25$ endotoxin units/ml by the gel-clot *Limulus* amoebocyte lysate assay (Pyrogent plus, BioWhittaker, Walkersville, USA). The inoculation dose was $10^{7.0}$ TCID50/pig.

*E. coli* LPS (O111:B4) was obtained from Difco Laboratories and used at a dose of 20 $\mu g$/kg body weight. This dose was based on data from a preliminary experiment, and selected to cause no clinical disease and minimal TNF-$\alpha$ and IL-1 secretion in the lungs.

Virus and LPS were diluted in sterile pyrogen-free phosphate-buffered saline (PBS; Gibco, Merelbeke, Belgium) to obtain a 3-ml inoculum.

### *Pigs and experimental design*

Forty-five Caesarian-derived colostrum-deprived pigs (3–4 weeks old) were used in experiments. They were housed in individual 'Horsefall type' isolation units with positive pressure ventilation and fed with commercial UHT-treated cows' milk. All inoculations were performed intratracheally, with a 14-gauge Teflon catheter which was inserted through the skin cranial to the sternum.

In a first study (Table 1), 25 pigs were allocated to four groups. Three pigs were mock-inoculated with PBS and killed 24 h later. Six pigs were inoculated exclusively with PRCV and killed sequentially at 27, 29, 42, 44, 48 and 72 h after inoculation. Eight pigs were given LPS only; eight pigs were inoculated with PRCV and, 24 h later, with LPS. Pigs from the latter two groups were killed sequentially at 3, 5, 8, 12, 18, 20, 24 and 48 h after inoculation with LPS.

In a second study (Table 2), two experiments were performed on two separate occasions, with a total of 20 pigs. In each experiment, one pig was inoculated with PBS only and killed 24 h later. Three pigs were given PRCV only and killed 6, 12 and 16 (experiment 1) or 24 (experiment 2) h after inoculation. One pig received LPS only, and five pigs were inoculated with PRCV followed by LPS at intervals varying from 0 to 24 h. These time intervals were selected to represent progressing stages of the PRCV infection. All pigs inoculated with LPS or PRCV-$\Delta$t-LPS were killed 5 h after inoculation with LPS.

In both studies, pigs killed at corresponding times after inoculation with virus or LPS, or both, were littermates. Samples from the left diaphragmatic lung were collected for virological, histopathological and standard bacteriological examinations. The right lung was lavaged with 60 ml of cold Dulbecco's PBS without $Ca^{2+}$ and $Mg^{2+}$ (Gibco) *via* an 18-gauge blunt needle inserted through the trachea. Recovered bronchoalveolar lavage (BAL) fluids (45–47 ml) were separated into cells and cell-free fluids by centrifugation (400 *g*, 10 min, 4°C).

### *Clinical, virological and pathological examinations*

A clinical respiratory disease score was given three times per day throughout the experiments, and every

**Table 1.** Evolution of clinical scores, BAL cell counts and percentage of neutrophils after combined PRCV-24 h-LPS inoculation and corresponding single inoculations

| Inoculation | Time after inoculation (h) | Clinical score* | Total BAL cells ($\times 10^6$) | Neutrophils (%) |
|---|---|---|---|---|
| PCRV only | 27 | 0 | 36 | 7 |
| | 29 | 0 | 38 | 1 |
| | 32 | ND | ND | ND |
| | 36 | ND | ND | ND |
| | 42 | 0 | 37 | 2 |
| | 44 | 0 | 48 | 20 |
| | 48 | 0 | 75 | 10 |
| | 72 | 0 | 50 | 8 |
| LPS only | 3 | 0 | 738 | 60 |
| | 5 | 0 | 415 | 66 |
| | 8 | 0 | 457 | 41 |
| | 12 | 0 | 818 | 56 |
| | 18 | 0 | 184 | 44 |
| | 20 | 0 | 103 | 44 |
| | 24 | 0 | 380 | 71 |
| | 48 | 0 | 130 | 31 |
| PRCV-24h-LPS (PRCV/LPS) | 27/3 | 3 | 624 | 75 |
| | 29/5 | 3 | 657 | 74 |
| | 32/8 | 2 | 200 | 61 |
| | 36/12 | 0 | 478 | 67 |
| | 42/18 | 0 | 69 | 54 |
| | 44/20 | 0 | 195 | 48 |
| | 48/24 | 0 | 105 | 56 |
| | 72/48 | 0 | 110 | 9 |

ND, not done.
*Clinical respiratory disease scores at euthanasia: 0 = normal; 1 = tachypnoea and/or dyspnoea when stressed; 2 = tachypnoea and/or dyspnoea when at rest; 3 = severe tachypnoea and dyspnoea with laboured, jerky breathing.

**Table 2.** Clinical scores and BAL cell changes after combined PRCV-LPS inoculations at various time intervals and corresponding single inoculations

| Inoculation | Hours after inoculation with PRCV/LPS | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|---|
| | | Clinical score* | Total BAL cells $\times 10^6$ (% neutrophils) | Clinical score* | Total BAL cells $\times 10^6$ (% neutrophils) |
| PRCV | 6/– | 0 | 107 (0) | 0 | 155 (0) |
| | 12/– | 0 | 114 (0) | 0 | 141 (3) |
| | 16/– | 0 | 91 (6) | ND | ND |
| | 24/– | ND | ND | 0 | 238 (10) |
| LPS | –/5 | 0 | 415 (66) | 0 | 619 (61) |
| PRCV-0h-LPS | 5/5 | 0 | 720 (61) | 0 | 613 (61) |
| PRCV-6h-LPS | 11/5 | 0 | 527 (57) | 0 | 644 (64) |
| PRCV-8h-LPS | 13/5 | 1 | 586 (56) | 0 | 815 (61) |
| PRCV-12h-LPS | 17/5 | 3 | 250 (21) | ND | ND |
| PRCV-16h-LPS | 21/5 | 3 | 162 (37) | 3 | 1122 (82) |
| PRCV-24h-LPS | 29/5 | ND | ND | 3 | 588 (67) |

–, not applicable; ND, not done.
*Clinical respiratory disease scores at death: 0 = normal; 1 = tachypnoea or dyspnoea, or both, when stressed; 2 = tachypnoea or dyspnoea, or both, when at rest; 3 = severe tachypnoea and dyspnoea with laboured, jerky breathing.

hour during the first 12 h (PRCV-24 h-LPS study) or 5 h (PRCV-$\Delta$t-LPS study) after inoculation with LPS. Scores ranged from 0 to 3: 0 = normal; 1 = tachypnoea and dyspnoea, or both, when stressed; 2 = tachypnoea and dyspnoea, or both, at rest; 3 = severe tachy-pnoea and dyspnoea with laboured, jerky breathing.

PRCV isolation and titration was performed in ST cells according to standard procedures [19]. Three pieces of lung from various parts of the diaphragmatic lobe were examined by immunofluorescence (IF). Cryosections were fixed in acetone and stained with fluorescein isothiocyanate-conjugated monospecific porcine antibody to TGEV/PRCV [19].

Gross lung lesions were scored by visual inspection. For histopathological examination, samples were fixed

in neutral buffered formalin 10%, embedded in paraffin, sectioned and stained with haematoxylin and eosin.

BAL cells were counted in a Türk chamber and cytocentrifuge preparations were stained with Diff Quik (Baxter, Düdingen, Switzerland) to determine the percentage of neutrophils.

### *Cytokine bioassays*

Cell-free BAL fluids were concentrated 20 times by dialysis against a 20% w/v solution of polyethylene glycol (mol. wt 20 000), and cleared of residual virus by centrifugation at 100 000 *g* before analysis in cytokine bioassays. TNF-$\alpha$ and IL-1 bioassays have been described in detail elsewhere [18]. TNF-$\alpha$ was assayed as cytotoxic activity in PK(15) subclone 15 cells (a gift from G. Bertoni, Bern, Switzerland) in the presence of actinomycin D. The plates were stained with crystal violet and read spectrophotometrically. The number of biological units/ml of BAL fluid was defined as the dilution that produced 50% cytotoxicity. Specificity was demonstrated by neutralisation of samples with rabbit anti-human TNF-$\alpha$ (Innogenetics, Zwijndrecht, Belgium). IL-1 was measured as proliferative activity in D10(N4)M cells in the presence of concanavalin A (Grade IV, Sigma, Bornem, Belgium) and recombinant human IL-2 (Genzyme, Cambridge, MA, USA). The percentage proliferation was determined by the MTT method, and optical densities were measured. The number of biological units/ml of BAL fluid was determined as the dilution that produced 50% maximal proliferation. To confirm IL-1 specificity, D10 cells were incubated with monoclonal rat anti-mouse IL-1 receptor type I antibodies (Genzyme). Bioassays were done with two-fold dilutions of samples in 96-well microtitration plates. Laboratory standards were run in each bioassay and used for correction of titres of samples. Samples were tested in three (TNF-$\alpha$) or two (IL-1; duplicates of samples) independent bioassays, and geometric mean titres of the corrected values were calculated.

### *Statistical analysis*

Standard two-sample Mann-Whitney tests were used to compare cytokine levels between singly LPS- and PRCV-LPS-inoculated pigs or between pigs given PRCV-LPS at various time intervals. Spearman rank correlation tests were used to compare cytokine titres and clinical scores. p values $<0.05$ were considered significant.

## Results

The lungs of all pigs were free of bacteria by culture. PRCV was isolated from the lungs of all virus-inoculated pigs, but not from pigs inoculated with LPS only or PBS.

### *Inoculations with PRCV followed by LPS 24 h later*

Control pigs inoculated with PBS remained healthy and did not have macroscopic or microscopic lung pathology. BAL cell numbers of the three individual pigs were 65, 70 and 181 ($\times 10^6$) respectively; $<0.5\%$ of cells were neutrophils and $>95\%$ had macrophage morphology. BAL fluids did not contain detectable TNF-$\alpha$ or IL-1.

Table 1 shows the clinical and BAL cell findings after single and combined inoculations with PRCV and LPS. The single PRCV inoculation did not induce any clinical signs. Lung virus titres were between $10^{5.6}$ and $10^{7.7}$ TCID50/g of lung and viral antigen was detected by IF in the alveolar epithelia and septa of all pigs (data not shown). At gross examination, small areas of lung consolidation, involving $<5\%$ of the total lung, were found exclusively in the pigs killed 48 and 72 h after inoculation. The characteristic microscopic lesion was a mild to moderate alveolitis with septal infiltration of macrophages and a few neutrophils. These lesions became more pronounced with increasing times after inoculation. Alveolar spaces and airway lumina were most often devoid of stainable material, and oedema and airway epithelial necrosis were rare. BAL cell numbers, $(36–75) \times 10^6$, were within normal limits and neutrophils ($\leqslant 20\%$ of BAL cells) were only slightly elevated at any time after inoculation. Minimal TNF-$\alpha$ levels (20 U/ml) were detected in only one of six pigs, killed 72 h after inoculation, while IL-1 was undetectable (data not shown).

Pigs given the single LPS inoculation were asymptomatic. Macroscopic lung lesions were characterised by focal areas of atelectasis and interlobular oedema. The most prominent histopathological features were alveolar septal thickening and bronchiolar infiltration with neutrophils and macrophages, intra-alveolar oedema and focal transudation of erythrocytes. Total BAL cell numbers, $(103–818) \times 10^6$, although variable, were greatly increased in comparison with single PBS or PRCV inoculations. This increase in cell numbers was largely due to a dramatic neutrophil infiltration (31–71% of BAL cells). Lung pathological changes were seen between 3 and 48 h after inoculation. Fig. 1 shows cytokine levels at different times after inoculation of LPS. TNF-$\alpha$ secretion was minimal (40–89 U/ml) and was detected exclusively in the pigs killed 3 and 8 h after inoculation. IL-1 (28–654 U/ml) was found at 3, 5 and 8 h after inoculation, but was no longer apparent thereafter.

In striking contrast to the effects of the single inoculations, the combined PRCV-24 h-LPS inoculation induced a marked tachypnoea and dyspnoea – with

laboured, jerky abdominal breathing – along with anorexia and dullness. All pigs were clinically normal before the LPS inoculation and developed clinical signs within 2 h after inoculation of LPS. Pigs killed 3 and 5 h after inoculation of LPS were most severely affected; those killed 12 h after inoculation or later had already recovered. The extent and kinetics of PRCV replication were not affected by the LPS inoculation (data not shown). Lung pathological changes and BAL cell profiles resembled a combination of the effects induced by the single PRCV and LPS inoculations. Macroscopically, there was some lung oedema and, occasionally, minimal lung consolidation. On histopathology, excessive neutrophil infiltration of alveoli and terminal airways was most prominent. Many alveoli were filled with fibrin strands and proteinaceous debris, and accumulations of erythrocytes. However, the extent and severity of these lesions did not differ appreciably from those after the single LPS inoculation. Total BAL cell numbers, $(69–657) \times 10^6$, and percentages of neutrophils (9–75% of BAL cells) were greater than after PBS or PRCV inoculations, but similar to those after single LPS inoculation. Again, lung pathological damage showed little evolution and failed to correlate with clinical disease. As shown in Fig. 1, the cytokine response was significantly enhanced in magnitude and duration when compared with that induced by LPS only ($p < 0.05$ for both TNF-$\alpha$ and IL-1). TNF-$\alpha$ (208–3601 U/ml) and IL-1 (160–1631 U/ml) were detected from 3 to 12 h and from 3 to 24 h after inoculation of LPS, respectively. Both cytokines peaked 3 and 5 h after inoculation of LPS and were positively correlated with clinical signs (rho = 0.782 for TNF-$\alpha$ and 0.668 for IL-1), but IL-1 was detected for longer time periods after clinical recovery than TNF-$\alpha$.

### *Inoculations with PRCV and LPS at various time intervals*

The single inoculations with PBS and LPS produced subclinical effects, and lung pathological and cytokine findings were similar to those in the previous study. BAL cell numbers of PBS-inoculated control animals

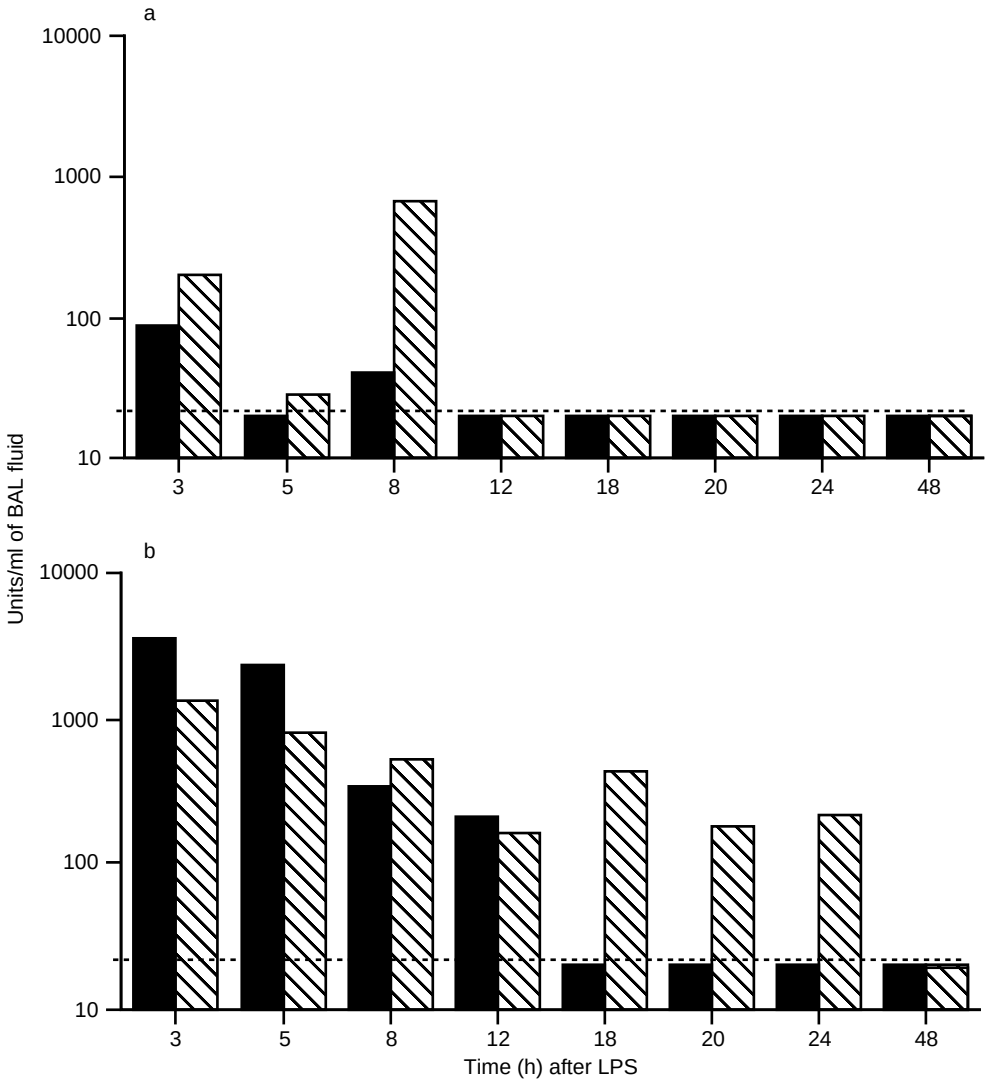


**Fig. 1.** Evolution of TNF-$\alpha$ (■) and IL-1 (▨) titres in BAL fluids after single LPS inoculation and combined PRCV-24 h-LPS inoculation. The dotted line indicates the detection limit of the assays.

were 114 and 156 ($\times 10^6$) in experiments 1 and 2 respectively; $<1\%$ of cells were neutrophils. Table 2 gives clinical scores and BAL cell findings after single PRCV and LPS and combined inoculations.

The single PRCV infection was asymptomatic. Immunofluorescence studies revealed the highest amounts of viral antigen-positive cells between 12 and 24 h after inoculation, when >500 positive cells per section were detectable in most lung tissue pieces, but only a few rare positive cells at 6 h after inoculation. Thus, >6 h were needed for the infection to spread over the entire lung. Gross lung lesions were absent, and minimal alveolitis was seen on histopathology. BAL cell numbers – $(91–114) \times 10^6$ in experiment 1; $(141–238) \times 10^6$ in experiment 2 – were similar to those of PBS-inoculated controls, and neutrophil infiltration was minimal ($\leqslant 6\%$ in experiment 1; $\leqslant 10\%$ in experiment 2). TNF-$\alpha$ was detected at low levels (51 U/ml) in only one of the six PRCV-infected pigs, killed 12 h after inoculation. IL-1 assays were negative in all pigs (data not shown).

In both experiments, the clinical effects of the combined PRCV-LPS inoculation were dependent on the time interval between inoculations. Simultaneous inoculations or those given at 6-h intervals produced subclinical effects, but prominent respiratory disease was seen with 12-, 16- or 24-h intervals. Mild respiratory distress resulted from the PRCV-8 h-LPS inoculation in experiment 1. Virus replication patterns were similar to those of the single PRCV inoculation. Lung (histo)pathological changes, BAL cell numbers and percentage of neutrophils largely resembled those of the single LPS inoculation and were not associated with disease. Cytokine levels were higher in experiment 2 than in experiment 1, but the pattern of secretion was similar in both experiments (Fig. 2). TNF-$\alpha$ titres (20–621 U/ml in experiment 1; 28–3744 U/ml in experiment 2) increased with increasing time intervals between inoculations, and they were significantly higher in clinically affected pigs than in healthy pigs ($p < 0.0005$ in both experiments). IL-1 titres (20–166 U/ml in experiment 1; 146–1794 U/ml in experiment 2), on the other hand, tended to be higher with shorter time intervals. TNF-$\alpha$ (rho = 0.884 in experiment 1 and 0.728 in experiment 2), but not IL-1 (rho = 0.099 in experiment 1 and 0.105 in experiment 2), was correlated with respiratory disease.

## Discussion

This study has shown that PRCV infection can produce respiratory disease in combination with another agent that is subclinical in itself, in this case LPS. To our knowledge, this is the first study in farm animals demonstrating synergy between respiratory viruses and bacterial LPS in the induction of respiratory symptoms and lung cytokines. PRCV was chosen for these studies because this virus induces negligible TNF-$\alpha$ and IL-1 secretion in the pig lung compared with other respiratory viruses [18]. The LPS dose of 20 $\mu$g/kg body weight was also selected to induce minimal bioactive TNF-$\alpha$ and IL-1 by itself. Indeed, a preliminary LPS dose-response experiment had shown that $\geqslant 5$ mg LPS/kg body weight was required for a consistent and substantial release of TNF-$\alpha$ ($\geqslant 150$ U/ml) and IL-1 ($\geqslant 500$ U/ml) in BAL fluids. The clinical and lung inflammatory effects of the single intratracheal LPS inoculation largely agree with those previously reported in pigs [8–10].

Surprisingly, respiratory disease after combined PRCV-LPS inoculation could not be explained by structural or inflammatory changes in the lungs. In fact, lung parenchymal damage was mild and neutrophil and macrophage infiltration appeared qualitatively and quantitatively similar in PRCV-LPS and LPS groups. Also, the clinical symptoms resolved long before the lung pathological changes. On the other hand, functional activities of inflammatory cells were not examined in this study. It is possible that neutrophils in the PRCV-LPS-exposed lung produce more toxic oxygen species and proteolytic enzymes than do cells exposed to LPS only, and somehow complicate respiratory effects. TNF-$\alpha$, for example, which was found at high levels after the PRCV-LPS inoculation, stimulates the release of toxic substances from phagocytes [20–22]. Another, more likely possibility is that functional rather than morphological disturbances – such as airway hyper-responsiveness – contributed to the observed respiratory signs. The specific lung effects promoting clinical respiratory disease in this model are thus still uncertain and need further study.

TNF-$\alpha$ is a crucial mediator in various respiratory tract conditions and it has multiple cellular effects that could contribute to respiratory disease. This cytokine upregulates endothelial cell adhesion molecules which are required for leucocyte emigration [23], and further activates neutrophils and macrophages for enhanced superoxide radical generation as well as enhanced chemotaxis, phagocytosis and degranulation [20–22]. Furthermore, TNF-$\alpha$ directly increases lung vascular permeability [24] and may promote release of other cytokines and mediators, such as platelet-activating factor, leukotrienes, or nitric oxide [15]. Finally, TNF-$\alpha$ has a potent bronchoconstrictor effect and this may be of particular significance in the PRCV-LPS model. In cultured airway epithelial cells, TNF-$\alpha$ induces the production of substances with direct bronchoconstricting properties, such as endothelin [25]. In rats [26] and in normal human subjects [27], aerosolisation of TNF-$\alpha$ causes bronchial hyper-responsiveness. The findings in the present study strongly argue for a role of TNF-$\alpha$ in the pathogenesis of PRCV-LPS-induced respiratory disease. TNF-$\alpha$ levels were not only dramatically enhanced by the combined PRCV-LPS inoculation,

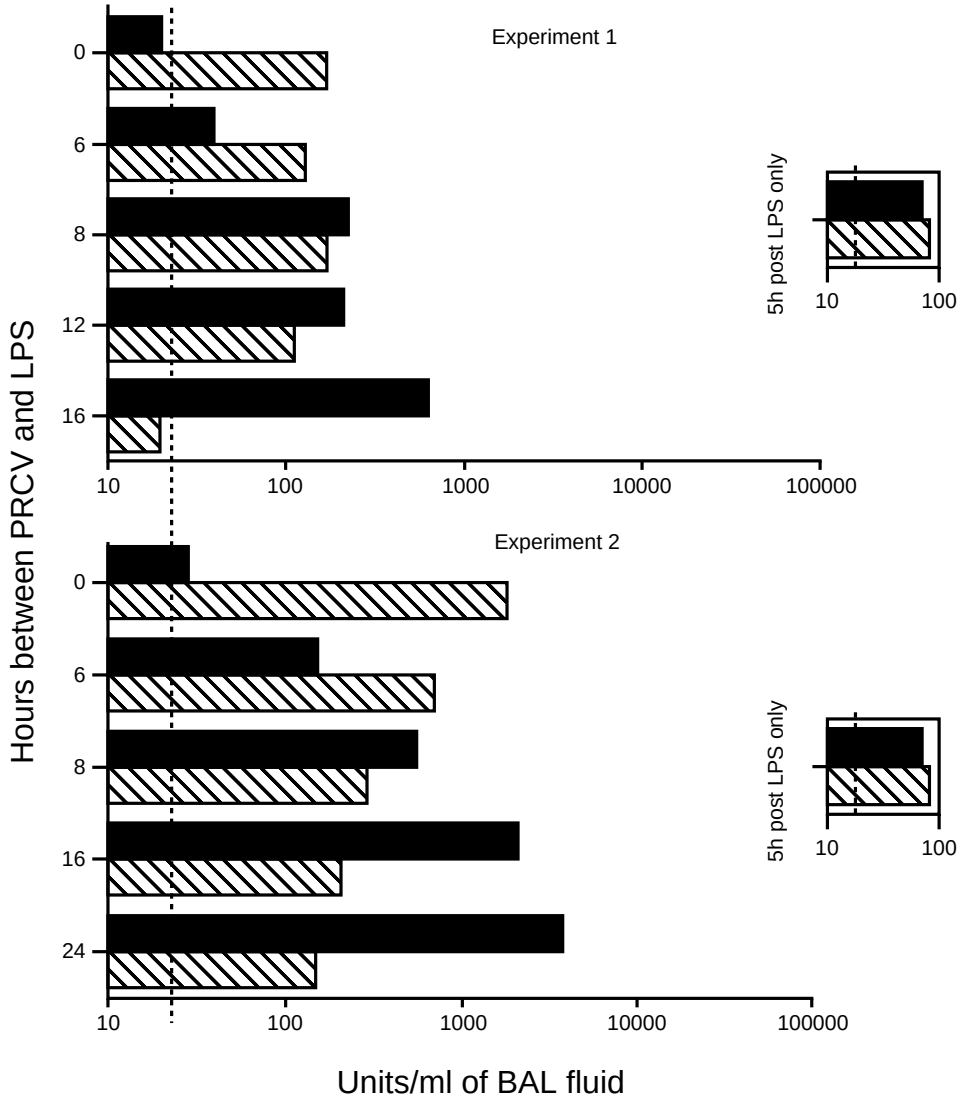


**Fig. 2.** TNF-$\alpha$ (■) and IL-1 (▨) titres in fluids after combined PRCV-LPS inoculations at various time intervals. All pigs were killed 5 h after inoculation of LPS. Data from two separate experiments are shown. The insert shows cytokine titres in a singly LPS-inoculated littermate. The dotted line indicates the detection limit of the assays.

they were also tightly correlated (rho = 0.728–0.884) with clinical disease. Further experiments to confirm and address the specific role of TNF-$\alpha$ are certainly needed, as other cytokines and mediators that were not measured in the present study may be involved.

Clinical and TNF-$\alpha$ responses were dependent on the time interval between PRCV and LPS inoculations. These time intervals were selected on the basis that one replication cycle of coronaviruses lasts for *c.* 6–8 h. Apparently, one virus replication cycle in the lungs must be completed at the time of LPS inoculation for respiratory disease and enhanced TNF-$\alpha$ production to occur. This suggests an initial priming of TNF-$\alpha$ production by the virus infection and subsequent LPS triggering. Similar findings have been made in in-vitro studies with influenza virus and LPS. A maximal TNF-$\alpha$ release in leucocyte cultures was observed when the influenza virus inoculation was followed by LPS at a 4-h interval, whereas prior LPS addition before virus infection was entirely inefficient [28]. The mechanisms whereby viruses can trigger lung cells for an enhanced cytokine secretion remain to be defined. Interestingly, influenza virus infection alone was shown to induce a massive TNF-$\alpha$ mRNA accumulation in leucocytes, but efficient translation into bioactive protein occurred only upon further stimulation by LPS [16, 28].

Unlike TNF-$\alpha$, there are no clear indications for a role of IL-1 in PRCV-LPS-induced respiratory disease. IL-1 levels were potentiated by the combined inoculation, but clearly did not correlate with disease in the second study. However, TNF-$\alpha$ and IL-1 are part of a complex network with potent feedback regulations involved in their synthesis and biological effects. Synergy between TNF-$\alpha$ and IL-1 has been documented at several levels in in-vitro studies [29]. Also, the combination of both cytokines may lead to a secondary cytokine response that is 250-fold greater than that seen with each cytokine alone [30]. Furthermore, potentiating effects between intratracheally administered TNF and IL-1 have been documented in rats [31]. Therefore, the

possibility that IL-1 acts synergically with TNF-$\alpha$ in the PRCV-LPS model cannot be excluded.

The work presented here is of particular significance with regard to respiratory disease in swine and other species in intensive animal husbandry, such as cattle and poultry. All three animal species frequently undergo infections with coronaviruses and other subclinical respiratory viruses, and they are exposed to considerable concentrations of airborne endotoxins [32]. Furthermore, massive amounts of endotoxins are released locally in the lungs during infections with gram-negative bacteria, which often complicate pulmonary virus infections in animals and man. Therefore, the PRCV-LPS inoculation may provide a model system for the study of the pathogenesis of multifactorial respiratory conditions.

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