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Original article

Effect of porcine respiratory coronavirus infection on lipopolysaccharide recognition proteins and haptoglobin levels in the lungs

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Abstract

Porcine respiratory coronavirus (PRCV) potentiates respiratory disease and proinflammatory cytokine production in the lungs upon intratracheal inoculation with lipopolysaccharide (LPS) at 1 day of infection. This study aimed to quantify LPS-binding protein (LBP), CD14 and haptoglobin in the lungs throughout a PRCV infection. LBP and CD14 recognize LPS and enhance its endotoxic activity, whereas haptoglobin dampens it. Gnotobiotic pigs were inoculated intratracheally with PRCV ($n = 34$) or saline ($n = 5$) and euthanized 1–15 days post inoculation (DPI). Virus was detected in the lungs from 1 to 9 DPI. Cell-associated CD14 in lung tissue increased up to 15 times throughout the infection, due to an increase in highly CD14⁺ monocyte-macrophages from 1 to 12 DPI and CD14⁺ type 2 pneumocytes from 7 to 9 DPI. LBP and soluble CD14 levels in bronchoalveolar lavage fluids were elevated from 1–12 DPI, with up to 35- and 4-fold increases, respectively. Haptoglobin levels increased significantly ($\times 4.5$) at 7 DPI. In addition, we found that PRCV could sensitize the lungs to LPS throughout the infection, but the response to LPS appeared less enhanced at the end of infection (7 DPI). The marked increases in LBP, CD14 and haptoglobin were not correlated with the extent of the LPS response.

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1. Introduction

The porcine respiratory coronavirus (PRCV) is highly prevalent in swine populations all over the world. PRCV shares several pathogenetic characteristics with the “severe acute respiratory syndrome” coronavirus (SARS CoV) that recently emerged in humans. Both viruses have a tropism for lung epithelial cells and cause bronchointerstitial pneumonia and necrotizing alveolitis [1,2]. Although PRCV infections often remain mild under experimental conditions, it is generally accepted that PRCV can be involved in respiratory disease in the field, when complicated with secondary (unknown) agents [3].

We have previously shown that PRCV synergizes with lipopolysaccharide (LPS) in the induction of severe respiratory disease [4]. LPS, also called endotoxin, is a major component of the outer membrane of Gram-negative bacteria and a potent inducer of proinflammatory cytokines. In the above-mentioned study, pigs were inoculated intratracheally with PRCV followed by LPS (20 $\mu\text{g}/\text{kg}$) at a time interval of 12–24 h. This led to excessive production of proinflammatory cytokines in the lungs and the simultaneous appearance of high fever, depression and breathing difficulties, which did not occur after inoculation with PRCV or LPS alone.

LPS induces proinflammatory cytokines after binding to its specific receptor complex [5]. LPS-binding protein (LBP) and CD14 are major components of this complex and mediate the early recognition of LPS. LBP is produced by hepatocytes and

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is constitutively present in plasma [6]. Because plasma LBP levels rise several times during systemic acute-phase responses, LBP is considered an acute-phase protein. Recently, type 2 pneumocytes were also shown to produce LBP after *in vitro* stimulation with different proinflammatory cytokines [7]. LBP acts as a lipid transfer molecule, extracting single LPS molecules from bacterial membranes or LPS aggregates and transferring them to CD14. CD14 is a pattern-recognition receptor, which is expressed by monocytes and macrophages and to a lesser extent by neutrophils [8]. CD14 also exists in a soluble form, which is enzymatically cleaved from the membrane or directly secreted from the cytoplasm [9]. Like LBP, soluble CD14 is constitutively produced by hepatocytes, and plasma concentrations can rise significantly during acute-phase responses. Importantly, soluble CD14 facilitates LPS-dependent activation of CD14-negative cells, such as epithelial and endothelial cells [10]. CD14 presents LPS to Toll-like receptor 4, which leads to the activation of different proinflammatory genes. Depending on the amount of inflammatory mediators that are induced, the endotoxic effect of LPS varies from mild inflammation to life-threatening functional disorders.

In contrast to the previous proteins, haptoglobin dampens the endotoxic activity of LPS [11]. This acute-phase protein is produced by hepatocytes and, according to some reports, also by lung epithelial cells [12]. Interestingly, haptoglobin desensitizes monocytes for the effects of LPS, protects mice from lethal endotoxic shock and reduces LPS-induced bronchial hyperreactivity [11].

The main objective of this study was to determine the kinetic profiles of LBP, CD14 and haptoglobin levels in the lungs throughout the PRCV infection. Both cell-associated and soluble CD14 were quantified and CD14-positive cells were identified using different markers. In a previous study, we demonstrated that PRCV potently enhances the response to LPS at 1 day of infection. Additional aims of the present study were therefore to verify whether this also occurs at later time points of infection (namely at 3 and 7 days post inoculation [DPI]) and whether virus-induced sensitization to LPS can be correlated with changes in the bronchoalveolar lavage (BAL) fluid levels of the above-mentioned proteins.

2. Materials and methods

2.1. Virus and LPS preparations

The Belgian 91V44 isolate of PRCV was used at the second passage in swine testis cells. The inoculation dose was 10^7 50% tissue culture infective doses (TCID₅₀) per pig.

LPS of *Escherichia coli* (serotype O111:B4, Sigma—Aldrich, St. Louis, USA) was used at a dose of 20 µg/kg. This dose has been used in previous experiments and causes no respiratory disease signs and minimal production of proinflammatory cytokines in the lungs upon intratracheal inoculation [4,13]. Virus and LPS were diluted in sterile pyrogen-free phosphate-buffered saline (PBS; Gibco, Merelbeke, Belgium) to obtain a 3 ml inoculum.

2.2. Pigs, experimental design and sampling

Forty-eight caesarean-derived colostrum-deprived pigs were used at the age of 3.5 weeks. The pigs originated from six sows and were housed in Horsefall-type isolation units with positive-pressure ventilation and fed with ultrahigh-temperature-treated cow's milk. All inoculations were performed intratracheally with a needle that was inserted through the skin cranial to the sternum.

Thirty-four pigs were inoculated exclusively with PRCV and euthanized at 1 ($n = 5$), 2 ($n = 3$), 3 ($n = 5$), 4 ($n = 3$), 5 ($n = 3$), 7 ($n = 6$), 9 ($n = 4$), 12 ($n = 3$) or 15 ($n = 2$) DPI. Five pigs were mock-inoculated with PBS and euthanized at 1 ($n = 1$), 7 ($n = 2$) or 15 ($n = 2$) DPI. Additionally, six pigs were inoculated with PRCV and 3 ($n = 2$) or 7 ($n = 4$) days later with LPS. They were euthanized 4 h after the LPS inoculation. Previous experiments showed that this time point is optimal for detection of *de novo* synthesis of cytokines upon LPS inoculation [4]. Three control pigs were inoculated exclusively with LPS and euthanized 4 h later.

The right lung was used for lung lavage. The main right bronchus was clamped and a needle was inserted distally. Cold PBS (3×20 ml) was injected, followed by massage of the lung tissue and aspiration. About 75% of the BAL fluid could be aspirated and was kept on ice. BAL fluids were separated into cells and cell-free fluids by centrifugation. Cell-free BAL fluids were concentrated 20 times by dialysis. Tissue samples from the apical, cardiac and diaphragmatic lung lobes of the left lung were collected for virological and bacteriological examinations and immunofluorescence staining. Serum from all pigs was collected at euthanasia.

2.3. Virological and bacteriological examinations

Infectious PRCV in lung tissue homogenates of pooled samples of apical, cardiac and diaphragmatic lung lobes was quantified by virus titration in swine testis cells [4]. Routine bacteriological examination of lung tissue was performed.

2.4. Antibody titration

PRCV-neutralizing antibodies in sera were titrated using a virus-neutralization assay [14].

2.5. BAL cell analysis

Total cell numbers in BAL fluids were counted in a Türk chamber. The percentage of neutrophils was determined using Diff-Quik® (Baxter, Düringen, Switzerland) staining of cytocentrifuge preparations. The percentage of sialoadhesin-, SWC3a-, CD3- and IgM-positive cells was determined using flow cytometric analysis (Becton Dickinson FACSCalibur™, BD Cellquest software) [15]. Sialoadhesin (mAb 41D3) is expressed exclusively on the membrane of macrophages and SWC3a (mAb 74-22-15) is expressed on monocytes, macrophages and neutrophils [16,17]. Resident macrophages of uninfected lungs are sialoadhesin-positive, whereas newly

infiltrated monocyte-macrophages are sialoadhesin-negative [18]. The number of sialoadhesin-negative monocyte-macrophages was determined by subtracting the number of neutrophils and the number of sialoadhesin-positive macrophages from the number of SWC3a-positive cells. CD3 (mAb PPT3) is expressed on T lymphocytes and IgM (mAb 28.4.1) on B lymphocytes [19,20].

2.6. Cell-associated CD14 quantification in lung tissue sections

Cell-associated CD14 in lung tissue sections was quantified using immunofluorescence microscopy and image analysis as described previously [15]. Immunofluorescence staining for CD14 was performed on sections of the apical ($n = 1$), cardiac ($n = 2$) and diaphragmatic ($n = 3$) lobes of each lung using mouse mAb MIL2 [16] and fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse polyclonal antibodies (Molecular Probes, Eugene, OR, USA). Specificity was determined by use of irrelevant mouse mAbs. The average amount of cell-associated CD14 was calculated for each lung (6 sections, 15 pictures/section) and expressed as a ratio compared to a lung tissue section of a PBS control pig.

2.7. Identification of CD14-positive cells in lung tissue sections

CD14-positive cells in lung tissue sections were identified using markers for macrophages (sialoadhesin and SWC3a), monocytes (SWC3a) and epithelial cells (cytokeratin 18 and surfactant protein B). Cytokeratin 18 (mAb CY-90, Sigma–Aldrich) is expressed by bronchiolar epithelial cells and type 2 pneumocytes, and surfactant protein B (rabbit pAb against porcine SP-B, Hycult Biotechnology, Uden, The Netherlands) by type 2 pneumocytes [21,22]. Double immunofluorescence stainings for CD14 and the respective cell markers were performed on sections of the cardiac and diaphragmatic lung lobes [15]. Specificity of the double stainings was determined by use of irrelevant mouse mAbs and rabbit pAbs. Digital images were taken using a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Wetzlar, Germany).

2.8. Soluble CD14 quantification in BAL fluids and sera

The kinetic profiles of soluble CD14 in BAL fluids and sera were determined using a flow cytometric assay, adapted from Antal-Szalmas et al. [23]. This assay is based on the competition between membrane-bound CD14 on macrophages and soluble CD14 in the test sample for binding to anti-CD14 antibodies (mAb MIL2). Briefly, BAL fluids and serum samples were mixed with anti-CD14 antibodies, resulting in an antibody concentration of 1 $\mu\text{g}/\text{ml}$. PBS was used as a negative control. The sample-antibody mixtures were incubated for 30 min at 37 °C. Porcine alveolar macrophages (5×10^6) were then incubated with the sample-antibody mixtures for 1 h at 4 °C. Subsequently, the cells were incubated with

FITC-labeled goat-anti-mouse polyclonal antibodies (4 $\mu\text{g}/\text{ml}$, 10% goat serum) (Molecular Probes) for 1 h at 4 °C. The cells were washed three times with cold PBS after each incubation. The median fluorescence intensity (MFI) of the macrophages was determined using flow cytometric analysis (Becton Dickinson FACSCalibur, BD Cellquest software). Ten thousand cells were analyzed for each sample. Samples that contain soluble CD14 reduce the availability of anti-CD14 antibodies for binding to CD14 on the macrophages, resulting in a decrease in the MFI. The amount of soluble CD14 in the test sample was expressed as a ratio compared to the negative control sample. This ratio was calculated by dividing the MFI of the negative control sample with the MFI of the test sample.

2.9. LBP and haptoglobin quantification in BAL fluids and sera

LBP was quantified using an ELISA (Hycult Biotechnology) and haptoglobin using a colorimetric assay (Phase™ Range Haptoglobin Assay, Tridelta Development Ltd., Maynooth, Ireland).

2.10. Effect of LPS on the lungs at 3 and 7 DPI with PRCV

The response to LPS was assessed by the severity of clinical signs, infiltration of neutrophils and production of proinflammatory cytokines in the lungs. A clinical score for tachypnea (0: <60, 1: 60–90, 2: >90 breaths/min), abdominal thumping (0: absent, 1: present), severe dyspnea (0: absent, 1: present), anorexia (0: absent, 1: present) and depression (0: absent, 1: present) was attributed before euthanasia. The total score per pig was obtained by adding the scores for each parameter and ranged from 0 to 6. The number of neutrophils in the BAL fluids was determined as described above. The proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were quantified in the BAL fluids with bioassays [4,13]. Specificity was demonstrated by neutralization of samples with rabbit anti-human TNF- α antibodies (Innogenetics, Zwijnaarde, Belgium) or goat anti-porcine IL-6 antibodies (R&D systems, Abingdon, UK). Laboratory standards were run in each bioassay. Samples were tested in three individual bioassays, and geometric means were calculated.

2.11. Statistical analysis

Standard two-sample Mann–Whitney tests were used to compare clinical scores, cell numbers, CD14 ratios, LBP and haptoglobin levels. Student's *t*-test was performed to compare mean cytokine titers between groups. Correlation coefficients (ρ) were calculated using the Spearman rank correlation test. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Virus titers in lungs

The kinetic profile of virus titers in the lungs is shown in Fig. 1A. Infectious virus was detected in the lungs of PRCV-inoculated pigs from 1 to 9 DPI. PRCV was isolated from the lungs of all pigs between 1 and 5 DPI. At 7 and 9 DPI, virus was isolated from the lungs of 3 out of 6 pigs and 2 out of 4 pigs, respectively. Mean titers were highest from 1 to 5 DPI

(5.4–7.3 TCID₅₀/g lung tissue) and strongly decreased at 7 DPI (3.3 TCID₅₀/g lung tissue) and 9 DPI (2.4 TCID₅₀/g lung tissue). From 7 DPI onwards, PRCV-specific neutralizing antibodies were found in sera of all PRCV-inoculated pigs. All lungs were free of bacteria.

3.2. Kinetic profile of BAL cells

Mean numbers and percentages of different types of BAL cells are presented in Table 1. PBS control pigs had

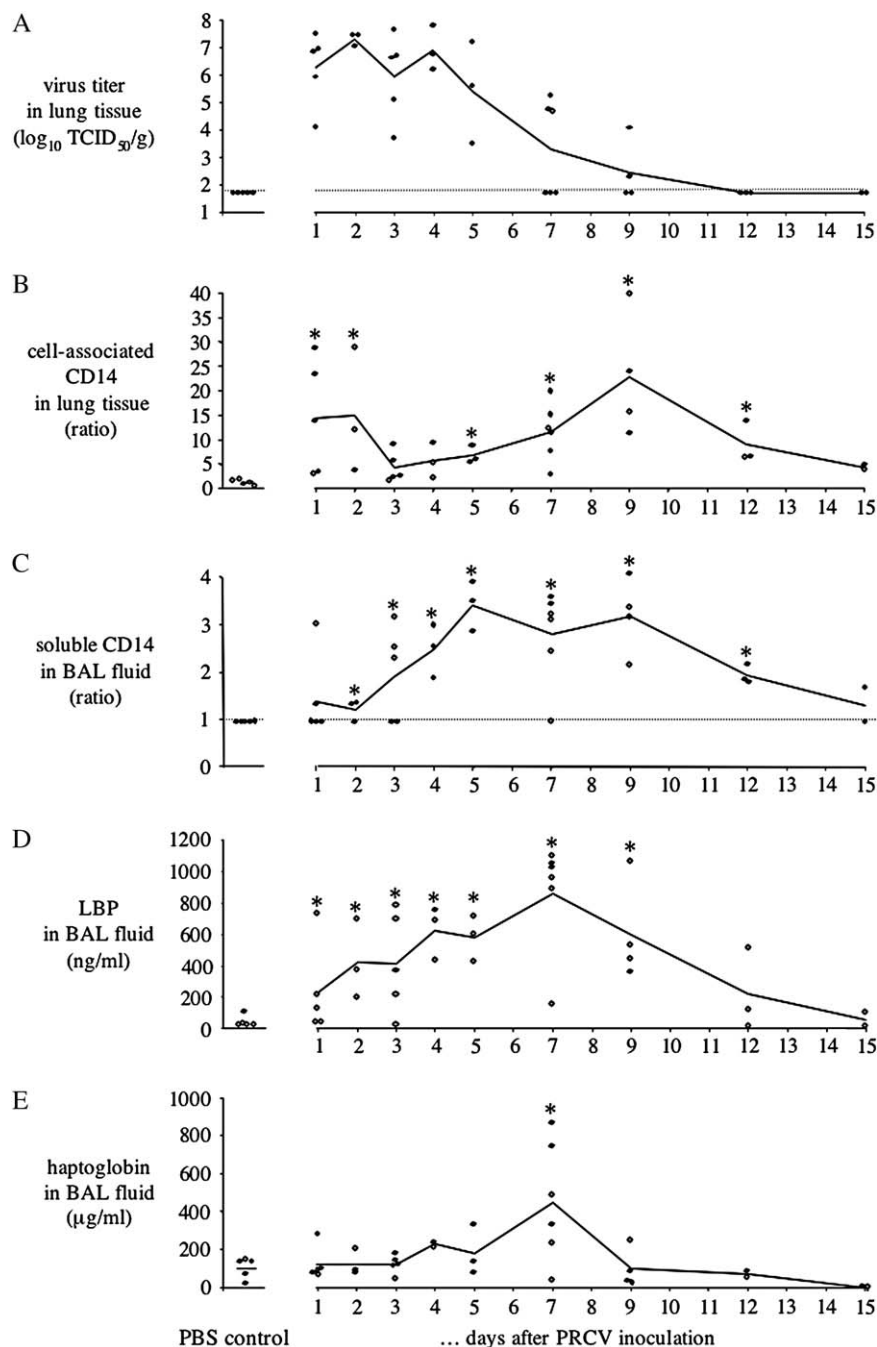


Fig. 1. Kinetic profiles of virus titers (A), cell-associated CD14 in lung tissue (B), soluble CD14 in BAL fluids (C), LBP in BAL fluids (D) and haptoglobin in BAL fluids (E) throughout a PRCV infection. The left part of the graph shows data of PBS control pigs euthanized from 1 to 15 DPI. Each dot corresponds to one pig and the solid line represents the mean. The dotted line represents the detection limit. Means marked with an asterisk differ significantly ($P < 0.05$) from those of the PBS control pigs.

Table 1
Mean numbers and percentages of different types of BAL cells recovered from the right lung half during PRCV infection

Inoculation with	No. of pigs	Euthanasia at...DPI with PRCV	BAL cells \pm standard deviation $\times 10^6$					
			Total	Sial ⁺ macro ^a	Sial ⁻ mono-macro ^b	Neutro ^c	T lympho ^d	B lympho ^e
PBS	5	n.a. ^f	96 \pm 29	79 \pm 0.8 (82% ^g)	3.5 \pm 1.8 (4%)	0.4 \pm 0.1 (0.4%)	1.4 \pm 1.3 (1.5%)	0.7 \pm 0.5 (0.7%)
PRCV	5	1	164 \pm 29*	123 \pm 18 (75%)	20 \pm 8 (12%)	17 \pm 15* (10%)	2.3 \pm 0.3 (1.4%)	2.2 \pm 1.1 (1.3%)
	3	2	142 \pm 19*	74 \pm 9 (52%)	19 \pm 2 (13%)	13 \pm 9* (9%)	1.4 \pm 0.1 (1.0%)	1.6 \pm 0.8 (1.1%)
	5	3	148 \pm 50*	101 \pm 40 (68%)	15 \pm 17 (10%)	10 \pm 13* (7%)	1.9 \pm 1.5 (1.3%)	1.0 \pm 0.7 (0.7%)
	3	4	179 \pm 45*	66 \pm 30 (37%)	53 \pm 35 (30%)	9 \pm 0.7* (5%)	3.7 \pm 0.8 (2.1%)	2.0 \pm 1.0 (1.1%)
	3	5	240 \pm 80*	103 \pm 35 (43%)	101 \pm 51* (42%)	7 \pm 5* (3%)	20 \pm 6* (8%)	3.4 \pm 0.9 (1.4%)
	6	7	304 \pm 83*	102 \pm 50 (34%)	44 \pm 30* (14%)	15 \pm 8* (5%)	51 \pm 32* (17%)	8.1 \pm 5.4* (2.7%)
	4	9	407 \pm 216*	92 \pm 32 (23%)	131 \pm 71* (32%)	79 \pm 108* (19%)	52 \pm 39* (13%)	9.2 \pm 2.9* (2.3%)
	3	12	179 \pm 86	52 \pm 27 (29%)	53 \pm 32 (30%)	25 \pm 19* (14%)	12 \pm 6* (7%)	2.4 \pm 1.3 (1.3%)
2	15	237 \pm 89	148 \pm 106 (62%)	58 \pm 6 (24%)	6 \pm 2 (3%)	15 \pm 3* (6%)	2.9 \pm 0.9 (1.2%)	

*Significantly different ($P < 0.05$) from those of the PBS control pigs.

^a Sialoadhesin-positive macrophages.

^b Sialoadhesin-negative monocyte-macrophages.

^c Neutrophils.

^d CD3-positive T lymphocytes.

^e IgM-positive B lymphocytes.

^f Not applicable.

^g Percentage of total cells.

64–142 $\times 10^6$ BAL cells. Total BAL cells did not increase in control pigs euthanized from 1 to 15 DPI, and the percentage of different cell populations varied little. Eighty-two percent of BAL cells were sialoadhesin-positive macrophages, 4% were sialoadhesin-negative monocyte-macrophages, 1.5% were T lymphocytes and less than 1% were neutrophils or B lymphocytes.

During PRCV infection, the total number of cells increased significantly from 1 to 9 DPI and mean numbers peaked at 9 DPI with a 4-fold increase compared to the PBS control pigs. The number of sialoadhesin-positive macrophages remained rather constant throughout the infection. In contrast, the number of sialoadhesin-negative monocyte-macrophages was increased from 1 to 15 DPI. At 1 DPI, the mean number of these cells was 6 times higher than in the PBS control pigs. Mean numbers peaked at 9 DPI and were 37 times higher than in the PBS control pigs. Neutrophils were significantly increased from 1 to 12 DPI. The mean number of neutrophils was highest at 9 DPI, but this peak was due to an exceptionally high amount of neutrophils in the lungs of one pig (240 $\times 10^6$). Numbers of T lymphocytes and B lymphocytes were significantly increased from 5 to 15 and 7 to 9 DPI, respectively.

3.3. Kinetic profile of cell-associated CD14 in lung tissue sections

The kinetic profile of cell-associated CD14 is presented in Fig. 1B. The amount of cell-associated CD14 in the lung tissue of PBS control pigs showed little variation (ratio of 0.4–1.6), independently of the time point of euthanasia. Mean ratios of PRCV-inoculated pigs were significantly increased at 1 and 2 DPI ($\times 10$ compared to the PBS control pigs). However,

CD14 expression varied strongly between pigs at this stage of infection, and individual CD14 ratios were increased in 3 out of 5 pigs at 1 DPI and in 2 out of 3 pigs at 2 DPI. Mean CD14 ratios at 3 and 4 DPI decreased compared to the first 2 days of infection, but were still higher than in the PBS control pigs ($\times 4$ –6). Mean ratios rose again between 5 and 12 DPI, with a peak at 9 DPI (15 times higher than in the PBS control pigs).

3.4. Identification of CD14-positive cells in lung tissue sections

Fig. 2 shows the results of the double immunofluorescence staining for CD14 and cytokeratin 18 on lung tissue sections of PBS- and PRCV-inoculated pigs. Cells with high CD14 expression were scarce (13 \pm 12 cells/mm²) in the lungs of PBS control pigs. They typically had a monocyte-like phenotype, characterized by round cell morphology, kidney-shaped nucleus and expression of SWC3a, but not of sialoadhesin. Most myeloid cells (>90%) were sialoadhesin-positive, resident macrophages, expressing little CD14 on their membranes. Epithelial cells were CD14-negative.

During infection there was a strong increase in highly CD14-positive monocyte-macrophages in the lung tissue. They stained positive for SWC3a, but negative for sialoadhesin and had a round to kidney-shaped nucleus. The first two days of infection, large clusters of these cells appeared around and inside the bronchiolar wall and to a lesser extent around small bronchi. From 3 DPI onwards, peribronchiolar clusters became rare, but there was a progressive increase in highly CD14-positive monocyte-macrophages in the alveolar tissue. These cells were most abundant at 7 and 9 DPI. At these time points, highly CD14-positive type 2 pneumocytes also

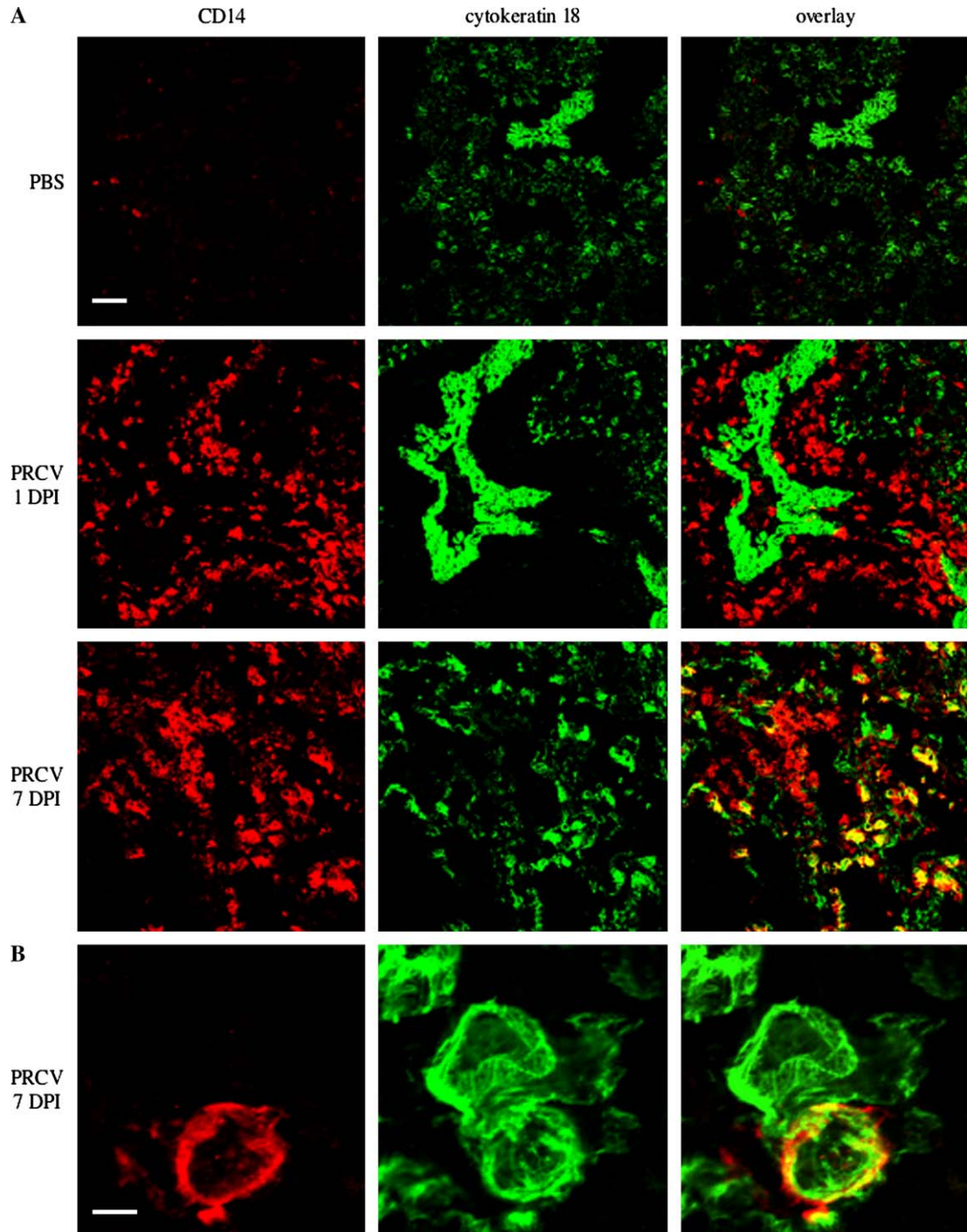


Fig. 2. Double immunofluorescence staining for CD14 and cytokeratin 18 in the lung tissue of PBS- and PRCV-inoculated pigs. Panel A (bar = 50 μ m) demonstrates that PBS-inoculated pigs contain few highly CD14-positive cells in the lung tissue. At 1 DPI, there is a massive accumulation of highly CD14-positive monocyte-like cells near the bronchioli. At 7 DPI, the alveolar tissue contains numerous clusters of highly CD14-positive monocyte-macrophages. At this time point of infection, part of the type 2 pneumocytes also stain positive for CD14, as indicated by the yellow color in the merge picture. Panel B (bar = 10 μ m) illustrates in more detail the CD14 signal on a cytokeratin 18-positive pneumocyte.

appeared in the alveolar tissue. These cells stained positive for cytokeratin 18 and surfactant protein B, but were negative for SWC3a and sialoadhesin. At this stage of infection, there was a strong (regenerative) hyperplasia of type 2 pneumocytes and a small fraction (about 1%) of these cells became positive for CD14.

3.5. Kinetic profiles of LBP, soluble CD14 and haptoglobin in BAL fluids and sera

The kinetic profiles of soluble CD14, LBP and haptoglobin in the BAL fluids of PRCV-infected pigs are presented in Fig. 1C–E. The corresponding serum levels are presented in Fig. 3A–C.

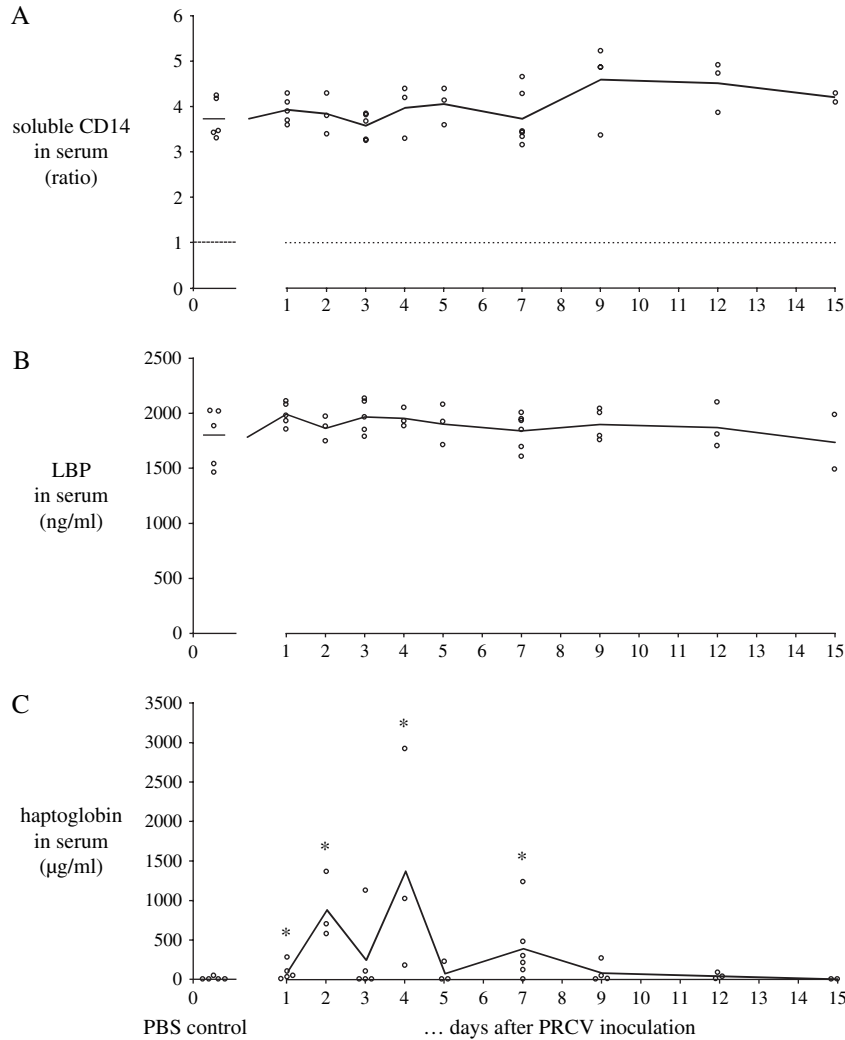


Fig. 3. Kinetic profiles of soluble CD14 (A), LBP (B) and haptoglobin (C) in sera throughout a PRCV infection.

BAL fluids of PBS control pigs contained no detectable soluble CD14 (ratio <1). At 1 DPI, soluble CD14 was detected in 2 out of 5 PRCV-inoculated pigs. At later stages of infection, soluble CD14 was detected in BAL fluids of most pigs. Mean ratios were significantly increased from 2 to 12 DPI, and highest levels were detected between 5 and 9 DPI (3–4 times higher than in PBS control pigs).

PBS control pigs had mean LBP levels of 25 ± 46 ng/ml, and the variation was independent of the time point of euthanasia. Mean levels of PRCV-inoculated pigs were significantly increased from 1 to 9 DPI, with a clear peak of 863 ± 354 ng/ml at 7 DPI. Values returned to normal at 15 DPI.

PBS control pigs had mean haptoglobin levels of 100 ± 55 µg/ml in their BAL fluids, and the variation was independent of the time point of euthanasia. Mean levels did not change significantly during the first 5 days of infection, but rose more than 4-fold at 7 DPI (449 ± 313 µg/ml). At 9–15 DPI, levels were again comparable to those of the PBS control pigs.

The sera of PBS control pigs had mean soluble CD14 ratios of 3.7 ± 0.4 and LBP levels of 1722 ± 271 ng/ml. Serum

levels of soluble CD14 and LBP did not change significantly upon infection. Haptoglobin was not detected in sera of PBS control pigs, except for one pig (48 µg/ml). In contrast, sera of most (>80%) PRCV-inoculated pigs contained detectable levels of haptoglobin between 1 and 12 DPI, ranging from 5 to 2919 µg/ml. Mean haptoglobin levels peaked at 4 DPI (1371 ± 1406 µg/ml). Serum haptoglobin was again undetectable at 15 DPI. Remarkably, soluble CD14, LBP and haptoglobin levels in sera were not correlated ($P > 0.05$) with the levels in the corresponding BAL fluids.

3.6. Effect of LPS on the lungs at 3 and 7 DPI with PRCV

Table 2 compares virus titers and LPS responses (clinical scores, neutrophil numbers and cytokine titers) of PRCV-LPS-inoculated pigs with those of the singly inoculated control pigs. The amount of LPS recognition proteins and haptoglobin in the corresponding BAL fluids is also presented.

The pigs described in the previous sections were used as the PBS and PRCV control pigs. PBS control pigs did not have detectable levels of TNF- α and low levels of IL-6

Table 2

Assessment of the LPS response (clinical signs, neutrophils and cytokines), amount of LPS recognition proteins and haptoglobin in the lungs of PRCV-LPS-inoculated pigs and LPS control pigs at 4 h after the LPS inoculation

Inoculum	Time of euthanasia after inoculation with		Virus titer (log ₁₀ TCID ₅₀ /g)	Clinical score	Neutrophils in BAL fluid		Cytokines in BAL fluid		LPS recognition proteins in BAL fluid		Haptoglobin in BAL fluid (µg/ml)
	PRCV (days)	LPS (h)			×10 ⁶	% of total cells	TNF-α (U/ml)	IL-6 (U/ml)	LBP (ng/ml)	Soluble CD14 (ratio)	
LPS	— ^a	4	<1.7	0	148	54	20	220	0	<1	0
	—	4	<1.7	0	932	71	42	2456	115	1.3	100
	—	4	<1.7	0	420	64	33	715	27	1.5	16
	<i>Mean</i>		<1.7	0	500	63	32	1130	47	1.2	39
PRCV-LPS	3	4	7.7	4 ^b	136	47	1798	95276	511	3.4	117
	3	4	7.0	4 ^b	80	37	2032	47277	619	2.7	146
	<i>Mean</i>		7.4	4*	108	42	1915*	71277*	565	3.1	132
	7	4	2.6	3 ^b	1274	67	756	16890	1199	3.6	26
	7	4	<1.7	4 ^b	403	50	251	6312	847	3.1	265
	7	4	4.2	1	12	4	<20	726	830	2.9	53
	7	4	<1.7	1	38	13	39	1264	852	2.9	189
	<i>Mean</i>		2.5	2.3*	432	34	266	6298	932	3.1	133

*Significantly different ($P < 0.05$) from those of the singly inoculated control pigs.

^a Not applicable.

^b These pigs had no or mild clinical signs (score 0 or 1) immediately before the LPS inoculation, but developed severe respiratory disease within 1 h after the LPS inoculation.

(range: <20–100 U/ml, mean: 67 ± 36 U/ml) in their BAL fluids. PRCV control pigs, euthanized at 3 DPI, had moderate levels of TNF-α (mean: 189 ± 177 U/ml, range: <20–435 U/ml) and of IL-6 (mean: 7290 ± 7914 U/ml, range: 116–20497 U/ml). PRCV control pigs, euthanized at 7 DPI, had no detectable TNF-α and low levels of IL-6 (mean: 367 ± 405 , range: 43–997 U/ml).

Pigs inoculated with LPS only showed no clinical signs before or after the LPS inoculation. LPS induced a massive increase in neutrophils, low levels of TNF-α (20–42 U/ml) and moderate levels of IL-6 (220–2456 U/ml) in the lungs.

The pigs that were inoculated with PRCV and 3 days later with LPS developed a synergistic increase in clinical signs and cytokine titers. Both pigs developed severe respiratory disease, characterized by abdominal thumping, dyspnea, tachypnea (60–90 breaths/min) and depression upon the LPS inoculation. Clinical signs were not observed before the LPS inoculation. Mean TNF-α and IL-6 titers of these pigs were significantly higher than those of the LPS control pigs and of the corresponding PRCV control pigs at 3 DPI. Neutrophil numbers of the PRCV-LPS-inoculated pigs were comparable to those of the pigs inoculated with LPS only.

Two of the four pigs which were inoculated with PRCV and 7 days later with LPS developed a synergistic increase in clinical signs and cytokine titers. Both pigs developed severe respiratory disease, characterized by abdominal thumping, dyspnea, severe tachypnea (>90 breaths/min) and depression after the LPS inoculation. The remaining two pigs were depressed, but showed no respiratory signs after the LPS inoculation. Clinical signs were not observed before the LPS inoculation, except for tachypnea (score 1) in one pig. The TNF-α and IL-6 titers of the pigs with severe respiratory disease were at least 6 times higher than the means of the corresponding PRCV (7 DPI) and LPS control pigs. The titers

were, however, markedly lower than those of pigs inoculated with LPS at 3 days of infection. Cytokine titers of the pigs without respiratory signs were comparable to those of the LPS control pigs, and the neutrophil numbers in their BAL fluids were even markedly lower. Due to the high individual variation, mean cytokine titers did not differ significantly with those of the singly inoculated control pigs.

There were no significant correlations between LBP, soluble CD14 or haptoglobin levels in the BAL fluids and the extent of the LPS response in PRCV-LPS-inoculated pigs. LBP and soluble CD14 levels of PRCV-LPS-inoculated pigs were higher than those of PBS and LPS control pigs, but similar to those of PRCV control pigs. Haptoglobin levels were not or only mildly increased.

4. Discussion

Previous research from our laboratory demonstrates that PRCV synergizes with LPS in the induction of respiratory disease and proinflammatory cytokines in the lungs [4]. The mechanism by which PRCV sensitizes the lungs to LPS is unknown. Several authors suggest that the inflammatory activity of LPS in the lungs depends on the local balance between factors that enhance its endotoxicity (such as LBP and CD14) [5,24] and factors that suppress it (such as haptoglobin) [11]. Our main aim was to determine the kinetics of these proteins in the lungs throughout a PRCV infection. We found significant increases in LBP and CD14 in the lungs throughout the entire infection (1–12 DPI), whereas haptoglobin levels only rose significantly at the end of infection (7 DPI).

The cell types expressing CD14 varied throughout infection. In the beginning of infection, highly CD14-positive monocyte-like cells accumulated near the bronchioli (see Fig. 1). Up to 20% of the epithelial cells in these bronchioli

were infected, as revealed by immunofluorescence staining for PRCV antigens (data not shown). At later stages, infected bronchioli became rare, whereas infected pneumocytes were found throughout the entire infection. This is in line with the observation that we seldom observed peribronchiolar clusters of highly CD14-positive monocytes at stages later than 2 DPI. Instead, there was a progressive increase in highly CD14-positive monocyte-macrophages in the alveolar tissue. It is likely that these highly CD14-positive cells were derived from blood monocytes, which migrated towards the infected cells. Monocyte infiltration starts early after the onset of infection, as indicated by the fulminant increase in monocytes in the BAL fluids at 1 DPI.

Surprisingly, highly CD14-positive pneumocytes appeared in the lung tissue at 7 and 9 DPI (see Fig. 2). At this late stage of infection, there was pronounced hyperplasia of type 2 pneumocytes and a minority of these cells expressed high levels of CD14. This was unexpected, as CD14 is considered a specific marker for myeloid cells [16]. Few researchers reported CD14 expression by other cell types. Fearn et al. [25] describe that lung epithelial cells of mice can be induced to express CD14 upon stimulation with IL-1 or TNF- α . In our study, the corresponding BAL fluids did not, however, contain TNF- α or IL-1 (data not shown), but did contain high amounts of soluble CD14. The positive signal on these pneumocytes might have been due to binding of soluble CD14 on the cell membrane and not to endogenous expression. The biological significance of these CD14-positive pneumocytes remains unclear.

Two soluble components of the LPS receptor complex, namely soluble CD14 and LBP, increased significantly in the BAL fluids during PRCV infection. Bronchial and bronchiolar epithelial cells are continuously exposed to LPS-contaminated dust, but do not express CD14 on their membranes. The presence of soluble CD14 in the bronchoalveolar fluid could thus be crucial for their interaction with LPS. It is unclear whether the increases in LBP, soluble CD14 and haptoglobin in the BAL fluids are due to plasma leakage or local secretion. Interestingly, the 2-fold increase in soluble CD14 on day 3 coincides with a relative decrease in cell-associated CD14 in the lung tissue. So, the increase in soluble CD14 might be due to local shedding of membrane-bound CD14. Monocytes, more than macrophages, are an important source of soluble CD14, which is cleaved from their membranes in response to cytokines such as interferon- γ [26]. Indeed, the amount of soluble CD14 (Fig. 1) correlated positively ($\rho = 0.68$, $P < 0.01$) with the amount of monocytes in the BAL fluids (Table 1). LBP and soluble CD14 levels in sera did not rise, but haptoglobin levels rose dramatically as early as one day after virus inoculation. Importantly, we found no correlation between BAL fluid and blood levels for any of these proteins, emphasizing the compartmentalization of the inflammatory response in the lungs during respiratory virus infections. This agrees with previous studies in which we detected high levels of proinflammatory cytokines, such as TNF- α , in the BAL fluids of virus-infected lungs, whereas blood levels remained low or undetectable (unpublished data).

Previous [4] and present findings demonstrate that PRCV infection can enhance the response to LPS throughout the infection from 1 to 7 DPI. The combination of PRCV and LPS induced severe respiratory disease in 4 out of 6 pigs, whereas the pigs inoculated with PRCV or LPS alone showed no or only mild disease signs. Severe signs coincided with a synergistic increase in TNF- α and IL-6 levels in the lungs. This agrees with a previous study, in which we found that the PRCV-LPS induced respiratory disease was highly correlated with increased levels of TNF- α in the lungs [4]. TNF- α and IL-6 are important disease mediators and increased production in the lungs might contribute significantly to the exacerbation of clinical disease. IL-6 is often considered a secondary pro-inflammatory cytokine, and it is therefore unclear whether it is induced directly by LPS or indirectly by TNF- α . In future studies, we will dissect the role of different pro-inflammatory cytokines in PRCV-LPS induced respiratory disease by use of neutralizing antibodies.

The enhancement of the LPS response was strong at 1 and 3 DPI, but tended to be milder at the end of infection (7 DPI). Several authors describe a correlation between the amount of LBP and CD14 in the lungs and the sensitivity to LPS [27,28]. In a previous study, we found that infection with porcine reproductive and respiratory syndrome virus (PRRSV), another respiratory virus of swine, also increases LBP and CD14 in the lungs, which coincides with enhanced LPS sensitivity [13,15]. Importantly, we found no correlations between LBP, CD14 or haptoglobin levels and the extent of the LPS response in the present study. Indeed, LBP and CD14 levels were highly variable at the first day of infection, whereas most pigs showed a similar increase in the LPS response at the same time point of infection in the study of Van Reeth et al. [4]. Despite exceedingly high levels of LBP and CD14 at 7 DPI, the LPS response tended to be milder than in the beginning of infection. Possibly, the activity of LBP and CD14 is counteracted by the induction of anti-inflammatory mechanisms or LPS scavenger molecules at the end of infection. Indeed, we found a significant increase in the anti-inflammatory haptoglobin in the BAL fluids at 7DPI. However, the pigs that responded poorly to LPS at this time point of infection did not have higher haptoglobin levels than the high responder pigs. The pronounced differences in LPS sensitivity at the end of infection remain unexplained.

Despite the fact that PRCV and PRRSV are both classified in the order Nidovirales and show structural resemblances, the pathogenesis of both virus infections differs strikingly as outlined by Van Reeth et al. [29]. Still, both virus infections increase the amount of LPS recognition proteins in the lungs and dramatically enhance the response to LPS. It is tempting to speculate that both processes represent non-specific responses of the lungs to viral infection and possibly also occur during infection with other respiratory viruses. Interestingly, SARS CoV-infected lungs are typically loaded with “activated” macrophages, and SARS is associated with excessive production of cytokines [30]. LPS recognition proteins have not been quantified in SARS CoV-infected lungs, but an exaggerated response to LPS or other bacterial compounds might also contribute to the development of SARS.

In conclusion, we found that PRCV infection causes a strong increase in the LPS recognition proteins LBP and CD14 and the anti-inflammatory protein haptoglobin in the lungs. Moreover, PRCV can sensitize the lungs to LPS throughout the infection, but the LPS response tended to decrease toward the end of infection. There was, however, no straightforward correlation between the extent of the LPS response and levels of any of the examined proteins. The mechanism of the synergy between PRCV infection and LPS remains, therefore, poorly understood and requires further study.

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