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Petra Reinhold, Nathalie Kirschvink, Dirk Theegarten, Angela Berndt. An experimentally induced *Chlamydia suis* infection in pigs results in severe lung function disorders and pulmonary inflammation. *Veterinary Research*, 2008, 39 (3), pp.1. 10.1051/vetres:2008012 . hal-00902933

HAL Id: hal-00902933

<https://hal.science/hal-00902933>

Submitted on 11 May 2020

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An experimentally induced *Chlamydia suis* infection in pigs results in severe lung function disorders and pulmonary inflammation

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(Received 31 October 2007; accepted 22 February 2008)

Abstract – This study was aimed at evaluating the pathophysiology of pulmonary dysfunctions and inflammatory consequences of an acute respiratory chlamydial infection induced experimentally in conventionally raised pigs (aged 39–44 days). Eight animals were exposed to *Chlamydia suis* (*C. suis*) and four non-infected animals served as controls. The total observation period was from seven days before challenge to seven days post exposure. While non-infected control pigs did not exhibit any clinical symptoms, animals exposed to *C. suis* developed fever and were severely respiratory distressed within the first week after exposure. After *C. suis* infection, pulmonary dysfunctions were characterised by a significant decrease in the diffusion capacity of the lung (i.e. transfer factor of the lung for carbon monoxide; TL CO), a significant increase in the functional residual capacity (FRC), and significant changes in the pattern of ventilation (respiratory rate increased while the tidal volume decreased). In exhaled breath condensate (EBC), leukotriene B₄ (LTB₄) and interleukin 6 (IL-6) showed a tendency to increase after infection. In the broncho-alveolar lavage fluid (BALF) of *C. suis* infected pigs, the activity of matrix metalloprotease 9 (MMP-9) was found to be increased compared to controls. BALF cytology was characterised by increased numbers of granulocytes and activated lymphocytes. Pulmonary inflammation in infected pigs was confirmed by post mortem histology. A prominent dissemination of chlamydial bodies in the lung was accompanied by an influx of macrophages, granulocytes and activated T-cells. Data obtained in this study provide new insight into the pathogenesis of acute respiratory chlamydial infections in pigs.

***Chlamydia suis* model / pulmonary inflammation / lung functions / exhaled breath condensate (EBC) / BALF**

1. INTRODUCTION

The impact of Chlamydiaceae on animal health on pig farms is controversial because an inconsistency seems to exist between the obviously high prevalence of chlamydiae in

clinically normal swine herds [4, 5, 10, 41] and relatively few reports of acute clinical illness. Chlamydial infections in sows have been associated with reproductive disorders, the occurrence of MMA-syndrome (mastitis, metritis, agalactia), or perinatal mortality in piglets, and detection of chlamydiae in semen of boars suggests a potential for venereal

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transmission [5, 15, 16]. In addition, there is some data that chlamydioses in swine can be associated with enteritis, pneumonia, conjunctivitis, and pericarditis [22]. The knowledge about the involvement of Chlamydiaceae in the porcine respiratory disease complex is still limited and reports in the literature are very inconsistent. While lung function was not affected in symptom-free pigs with a naturally acquired presence of chlamydiae in the respiratory system [32], a clear pathogenic potential of *Chlamydia* species for the porcine respiratory system has been proven experimentally [32, 35].

According to current taxonomy, *Chlamydia suis* (*C. suis*), i.e. the former porcine serovar of *C. trachomatis*, has been identified as the major agent causing chlamydioses in swine [22]. However, there appears to be a high prevalence of mixed infections with *C. abortus* making it difficult to attribute clinical pathology to *C. suis* alone [10]. In order to evaluate the aetiological importance of *C. suis*, an experimental challenge model of aerosol infection was established by our group [38]. In contrast to the model described by Rogers et al. [35] where three day old gnotobiotic piglets were found to be susceptible to respiratory chlamydial infection, the pigs challenged in our model were conventionally raised, older (approximately six weeks), and had even a positive carrier status for chlamydiae [38]. Despite lower infection dosages compared to the model described by Rogers et al. [35], they responded to the aerosol challenge of *C. suis* with acute phase reactions, severe clinical signs and significant increases of chlamydia-specific antibody titres [38]. Additionally, the present study was undertaken in order to clarify pathophysiological consequences of *C. suis* challenge particularly for the porcine respiratory system. Thus, (i) variables of lung function, (ii) markers of pulmonary inflammation measurable in exhaled breath, broncho-alveolar lavage fluid and on the cellular level, and (iii) immunohistochemical findings, as well as results of flow cytometric analysis, were evaluated in vivo and post mortem.

2. MATERIALS AND METHODS

2.1. Animals

Twelve colostrum-fed Deutsche Landrasse female pigs, conventionally raised on a farm with no animals being bought from outside, were provided by Charles River (Sulzfeld, Germany). At 3–4 weeks of age, they were brought into the animal house of the institute. They were included in the study after a quarantine period of at least ten days and confirmation of their clinically healthy status. Throughout the entire study, they were housed in conformity with the guidelines for animal welfare. Feeding was twice a day with a commercially available nutrition they received on the breeding farm. Water was supplied ad libitum. None of the feed contained antibiotics. The animals were kept in three groups, each consisting of four animals and housed separately.

2.2. Study design

At the age of 39–44 days, eight animals were exposed to *C. suis* and four non-infected animals served as controls. Each pig inhaled 35–40 L of aerosol produced either from 1.0 mL of chlamydia cell culture containing 10^9 IFU per mL (strain DC6) or from non-infected culture. Aerosol was administered to each pig individually over a period of 10–15 min. Details about aerosol production, aerosol administration and origin of the *C. suis* strain DC6 have been described previously [38].

Daily clinical observation focussed on feed intake, rectal temperature, respiratory rate and symptoms of diarrhoea or respiratory disorders, i.e. cough, nasal discharge or ocular secretions. Within a period ranging from seven days before challenge up to seven days after challenge, i.e. days post infection (dpi), pulmonary function tests, collection of blood samples and collection of exhaled breath condensate were performed as described below. A number was assigned to each pig. The tests each animal underwent are shown in Table I. Body weight was measured individually prior to each lung function test. The pigs were euthanised for necropsy at different time points (Tab. I). The study had ethical approval by the Commission for the Protection of Animals of the state of Thuringia.

2.3. Protocol of pulmonary function testing

Pulmonary functions were evaluated using MasterScreen Diffusion (Viasys Healthcare,

Table I. Study design.

Animal number	-7 d before	-2 d before	+3 dpi	+4 dpi	+5 dpi	+6 dpi	+7 dpi	+8 dpi	+10 dpi	+11 dpi	+17 dpi	+24 dpi	+25 dpi	
1			Necropsy*											
2				Necropsy										
3					Necropsy*									
4							Necropsy*							
5	EBC	PFT	PFT, EBC		PFT, EBC		PFT, EBC		Necropsy*					
6	EBC	PFT	PFT, EBC		PFT, EBC		PFT, EBC				Necropsy			
7	EBC	PFT	PFT, EBC		PFT, EBC		PFT, EBC					Necropsy*		
8	EBC	PFT	PFT, EBC		PFT, EBC		PFT, EBC					Necropsy*		
C9	EBC	PFT			PFT, EBC		Necropsy*							
C10	EBC	PFT			PFT, EBC		PFT, EBC		Necropsy*					
C11	EBC	PFT			PFT, EBC		PFT, EBC			Necropsy*				
C12	EBC	PFT			PFT, EBC		PFT, EBC						Necropsy*	

Animal numbers 1–8: challenged with *C. suis*. Animal numbers C9–C12: non-infected controls. dpi: day post infection. EBC: exhaled breath condensate. PFT: pulmonary function test. Necropsy*: Necropsy included venous blood sampling (in vivo) and broncho-alveolar lavage (ex vivo).

Hoechberg, Germany). Using different test gases, this system allows the simultaneous measure of static and dynamic lung volumes and variables describing the diffusion capacity of the lung. In order to evaluate the diffusion capacity of the lung, the transfer factor of the lung for carbon monoxide (TL CO) was determined according to the rebreathing (rb) method that is also known as the multiple breath or steady state method. The functional residual capacity (FRC) of the lung was measured by the multiple breath helium (He) dilution technique (wash-in). In addition, airflow was measured during spontaneous breathing using a Lilly-type pneumotachograph (mesh resistance: 36 Pa/(L/s)) and was used to calculate spirometric variables (i.e. tidal volume and respiratory rate).

All pulmonary function measurements were performed in sedated pigs (diazepam, 1.5–2.0 mg/kg body weight, intramuscularly) that were restrained using a canvas sling with openings for the limbs and were acclimated to the system individually using a tightly fitting facemask of appropriate size depending on the animal's head [18]. After an adaptation period of approximately 5 min, the pig inhaled the test gas mixture (7–10% helium (He), approximately 0.2–0.3% carbon monoxide (CO), 35% oxygen (O₂), rest nitrogen) from a 5 L reservoir bag. The rebreathing time varied between pigs and time points within the range 117–220 s. All parameters were calculated automatically using the software included in the system. For further analysis, the following variables of pulmonary function were recorded:

- respiratory rate (RR),
- tidal volume (Vt),
- volume of minute ventilation ($V_{min} = V_t \times RR$),
- transfer factor of the lung for carbon monoxide (TL CO rb),
- functional residual capacity of the lung (FRC).

2.4. Collection of exhaled breath condensate samples

The pig was sedated and restrained as for pulmonary function testing, and exhaled breath condensate (EBC) collection was performed immediately after pulmonary function when scheduled together. An EBC sampling system (ECoScreen, Viasys Healthcare) was connected to the facemask as previously described [30]. The animal was allowed to inhale only filtered ambient air (PALL breathing system filter, Pall Europe Ltd,

Portsmouth, UK) in order to avoid contamination of EBC by inhaled ambient particles. The total exhaled breath fraction of tidal breathing was condensed while passing through a non-re-breathing valve in the cooled collection system for at least 30 min. EBC samples were removed from the collection system immediately after collection. Aliquots of the samples were prepared and were stored at -80°C , and analyses were performed within 12 weeks after collection.

2.5. Necropsy, collection of broncho-alveolar lavage fluid (BALF) and lung tissue samples

Immediately before euthanasia, venous blood was collected in 7.5 mL-syringes (S-Monovette[®], Sarstedt AG & Co, Nuembrecht, Germany) for serum production from *Vena jugularis* in the non-fed animal. The trachea was exposed by dissection under conditions of deep anaesthesia (thiamylal-sodium, 1 g per 50 kg body weight, intravenously). Large arterial forceps were applied to clamp the trachea and thus prevent contamination of the airways by aspiration of blood or gastric contents. Subsequently, the animal was exsanguinated via the *Arteria axillaris*, and the lung was removed. A broncho-alveolar lavage (BAL) was performed in the right basal lobe of the lung using a small catheter (that was instilled through the trachea) and glass syringes. Three consecutive washes using 10 mL for each installation of cold cell buffer (140 mM NaCl, 2.8 mM KCl, 10 mM Na₂HPO₄ × 12H₂O, 1.5 mM KH₂PO₄; stored at 4 °C), were performed per individual. After immediate aspiration, the recovered BAL-fluid was about 60%. Immediately after BAL, lung tissue samples were taken from lesions and from macroscopically unchanged areas of the left caudal lung lobe for histopathological examinations.

2.6. Analyses in EBC, BALF, and serum

2.6.1. LTB₄, IL-6, IL-8 in EBC

In order to analyse the concentration of leukotrieneB₄ (LTB₄), a competitive enzyme immuno-assay (Cayman Chemical, Ann Arbor, USA) was used. Validated with LC/MS, the standard stem solution included in the commercial assay was dissolved in Milli-Q water for a concentration of 250 pg/mL LTB₄. This external standard was given on each microtiter plate on four different positions for control of validity of the plate. In

addition, a calibration curve in the expected range of LTB₄ concentration was performed by diluted standard solutions. The calibration curve was linear in the range of measurement. The analysis was performed using a reader for 96 well plates (Millennium Kinetik Analyzer™, DPC Biermann GmbH, Bad Nauheim, Germany) with a filter at $\lambda = 405$ nm and a PC with software for photometers.

For measurement of interleukin 6 (IL-6), EBC samples were concentrated by lyophilisation (500 μ L of each sample were dried and re-suspended in 200 μ L diluents). Because no commercial test kit for porcine IL-6 was available, a special microtiter plate sandwich assay was created using the following commercial components: biotinylated anti-porcine IL-6 antibody (R&D Systems, Minneapolis, USA, lot: CET01), anti-porcine IL-6 antibody (R&D-Systems, lot: CBA01), recombinant porcine IL-6 (R&D-Systems), and the colour reagent system "peroxidase hydrogen peroxide tetramethylbenzidine" for detection (R&D-Systems). The reaction time was 30 min at room temperature. The reaction was stopped by sulphuric acid. The IL-6 standard dissolved in Milli-Q water was used for calibration. The detection limit of this assay was 10 pg/mL. The calibration curve was linear.

The concentration of interleukin 8 (IL-8) was measured using an enzyme immune assay for porcine IL-8 (Trinova Biochem GmbH, Giessen, Germany, Lot: LO 80903) with a detection limit of 10 pg/mL. The IL-8 standard dissolved in Milli-Q water was used for calibration. All measurements were performed in duplicate.

2.6.2. Total protein and 8-iso-prostane in BALF

The concentration of total protein in BALF was analysed colorimetrically using a Pierce Micro BCA™ Reagent Kit (Pierce, Rockford, USA). The reaction was measured by UV/VIS Spectrometer UNICAM UV2 and Software VISION V1.00 (Unicam Chromatography GmbH, Kassel, Germany). The sensitivity of this method was ≥ 0.5 μ g/mL, and the calibration curve was linear.

The concentration of 8-iso-prostane (8-IP) was analysed by EIA enzyme-immunoassay (Cayman Chemical Company), and the results were verified by means of LC/MS using an external 8-IP standard solution of 50 ng/mL (SIGMA-ALDRICH Chemie GmbH, Taufkirchen, Germany). The detection limit was ≥ 3.9 pg/mL. All measurements were

performed in duplicate, and the means were used for further mathematical analysis. The ratio 8-IP/protein was calculated: 8-IP (pg/mL) \times 1000/total protein (μ g/mL).

2.6.3. Matrix metalloprotease activity in BALF and serum

The activity of MMP-2 and MMP-9 was determined in serum and in the supernatant of BALF samples using zymography as previously described [14]. Electrophoretic migration and an incubation time of 18 h (serum) or 72 h (BALF) at 37 °C after migration were used. After colouration, the gels were scanned and spots of lyses, expressed as arbitrary units of lysis, were measured.

2.6.4. BALF cytology and cytometry

Changes of immune cell composition of BALF were analysed by means of flow cytometry at five and seven days after *C. suis* infection. Briefly, BALF of two infected and two non-infected animals were centrifuged at 4 °C (300 \times g, 20 min) and the sediment was washed with cold PBS. After determination of viability and cell number by trypan blue dye exclusion, 100 μ L (2 \times 10⁶ cells) of the respective cell suspensions were incubated with 50 μ L hybridoma culture supernatant of the monoclonal antibodies CD4a (clone 74-12-4; T-helper cells), CD8b (clone 11/295/33; cytotoxic T-cells), CD25 (clone K 231-3B2, activated T-cells), wSWC1a (clone 11/8/1; granulocytes, resting T-cells, monocytes, macrophages; all clones are kind gifts from Prof. Saalmüller, University of Veterinary Medicine, Vienna) or 2G6 (macrophages [2]) for 30 min. After two washing steps, 20 μ L of FITC-conjugated goat anti-mouse immunoglobulin (dilution 1:10, Dako, Germany) were added and incubated for 30 min prior to analysis. Flow cytometry was performed with a FACScalibur flow cytometer (BD Bioscience, Heidelberg, Germany) equipped with a 15 mW, 488 nm argon laser. The parameters "forward light scattering" (FSC), "sideward light scattering" (SSC) and fluorescence (FL1) were stored and processed using the CellQuest research software (BD Bioscience, Heidelberg, Germany). Cell debris was excluded and BALF cells were selected by their cell size (defined by FSC value) and granularity (defined by SSC value) using the FSC-SSC dot-plot diagram.

2.7. Morphology and histology of lung tissue

Lungs were viewed macroscopically; representative specimens were taken and fixed in buffered 3.5% formaldehyde. Fixed lung tissue was embedded in paraffin wax (Tissuewax™; Medite GmbH, Burgdorf, Germany), slides of 3–7 µm thickness were cut using a rotatory microtome (Microm GmbH, Walldorf, Germany) and stained by hematoxylin and eosin (HE). Between four and nine representative microphotographs were taken of the slides of all animals using a Zeiss Axiophot with Axiocam and Axiovision 2 software (Carl Zeiss, Oberkochen, Germany) and critically reviewed.

Immediately after BAL, tissue samples of affected areas of the left caudal lung lobe as well as of the lung lymph node, tonsil and spleen were taken and frozen in liquid nitrogen until use.

To study the dissemination of chlamydial bodies in the lung, lung lymph node, tonsil and spleen as well as changes of the immune cell composition in the lung, frozen sections of tissue samples (two per animal and organ, 7 µm in thickness) were prepared and subsequently stained immunohistochemically. They were fixed with acetone and incubated with the appropriate monoclonal antibodies against chlamydial lipopolysaccharide (LPS) (Chemicon, Hofheim, Germany), wSWC1a, CD2a (clone MSA4; T-cells), CD4a, CD8b, CD25 or 2G6. For visualisation of bound antibodies, a staining kit (PAP, ChemMate Detection Kit, peroxidase anti-peroxidase, rabbit/mouse, DakoCytomation, Hamburg, Germany) was used according to the manufacturer's instructions. Negative control slides were incubated with pre-immune mouse serum (dilution 1:500) instead of the primary antibody. Sections were counterstained with haematoxylin and mounted with Canada balsam (Riedel de Haen AG, Seelze-Hannover, Germany).

2.8. Statistical analysis

Normally distributed data are given as mean \pm standard deviation (SD) whereas non-normally distributed data are always presented as median, minimum and maximum. For analysis of multiple data with normal distribution, multifactorial analysis of variance (ANOVA) was used, i.e. multiple range test based on least significant difference (LSD). To compare two unpaired samples, i.e. differences between two groups at one time point, the unpaired *t*-test was used for normally distributed data (comparison of means) while the Mann

Whitney Wilcoxon test (W-test) was used for data with unknown or non-normal distribution (comparison of medians). Since the given *P* values are ≤ 0.05 , there is a statistically significant difference at the 95.0% confidence level in both tests. All confidence levels are given with the data.

3. RESULTS

3.1. Clinical signs and body weight

Non-infected control pigs did not exhibit any clinical signs during the study, but all animals exposed to *C. suis* showed clinical signs of an acute infection characterised by significantly elevated rectal temperatures (data not shown). Furthermore, severe dyspnoea, dry cough and serous nasal discharge were present. Dyspnoea in challenged pigs was accompanied by a spastic noise of breathing (wheezing), and by short breath breathing or breathlessness. However, neither ocular secretions nor diarrhoea was observed. Duration of respiratory symptoms was one week after exposure.

Body weight increased significantly in the control group from two days before infection to 7 dpi (8.2 ± 1.0 kg to 8.9 ± 0.7 kg; means \pm SD; $P \leq 0.05$). In contrast, there was no significant increase in body weight in pigs exposed to *C. suis* for the same time period (8.6 ± 0.9 kg to 8.8 ± 1.0 kg; means \pm SD; $P > 0.05$).

3.2. Pulmonary functions

Experimental exposure to *C. suis* significantly affected the pattern of ventilation (Fig. 1). Three days after exposure to *C. suis*, the mean tidal volume decreased by 50% to its minimum in the course of the study. Compared to baseline values, *V_t* remained significantly reduced until 7 dpi in pigs challenged with *C. suis*, while it increased physiologically in control pigs. Due to a significant increase in respiratory rate (up to approximately 100 breathing cycles/min after *C. suis* exposure compared to a maximal respiratory rate of 32 breathing cycles/min in control animals at 3 dpi), the volume of minute ventilation was significantly increased and reached its

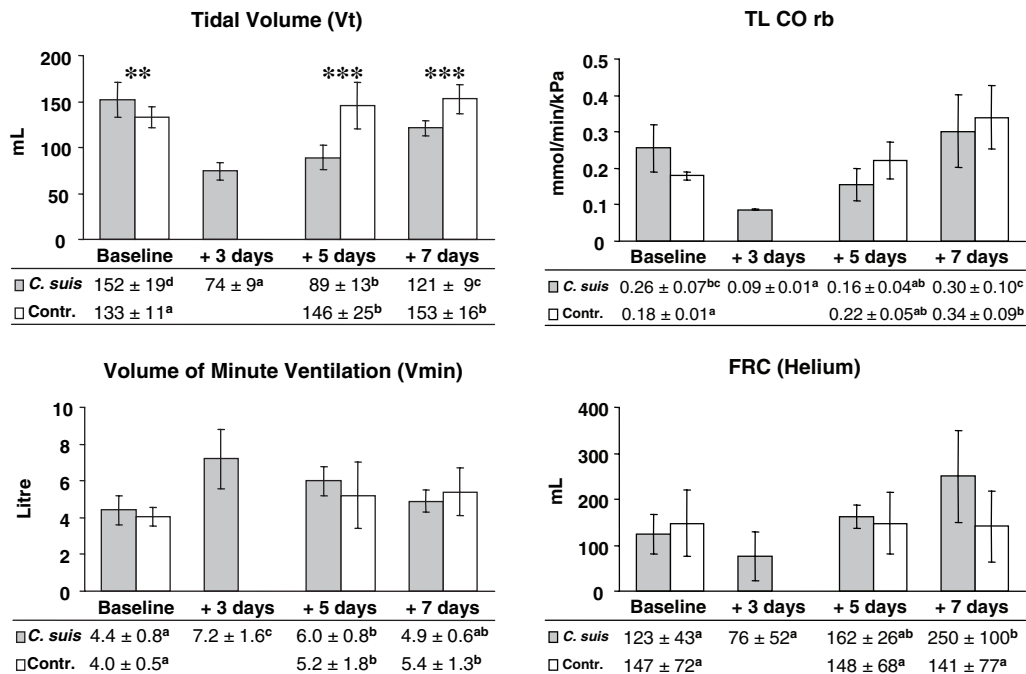


Figure 1. Means ± standard deviations of tidal volume and minute volume in pigs experimentally exposed to *C. suis* and in non-infected controls. Different letters (a, b, c, d) indicate significant differences between time points within one group (ANOVA, LSD, $P \leq 0.01$). **Indicates significant differences between groups at one time point (t -test, $P \leq 0.01$); ***indicates significant differences between groups at one time point (t -test, $P \leq 0.001$).

maximum three days after *C. suis* exposure (Fig. 1).

The CO transfer factor of the lung increased continuously in control pigs. In contrast, it decreased significantly in pigs challenged with *C. suis*, showing its minimum 3 dpi (Fig. 2). Seven days after challenge, the values of TL CO rb were comparable to baseline values. As also shown in Figure 2, the functional residual capacity (FRC) did not change in control pigs. In pigs exposed to *C. suis*, however, it started to increase 5 dpi. Seven days after *C. suis* exposure, FRC was significantly increased to approximately 200% compared to baseline values.

Figure 2. Means ± standard deviations of CO-transfer factor of the lung (TL CO rb) and functional residual capacity (FRC) in pigs experimentally exposed to *C. suis* and in non-infected controls. Different letters (a, b, c, d) indicate significant differences between time points within one group (ANOVA, LSD, $P \leq 0.01$).

3.3. Markers of inflammation

Figure 3 illustrates the concentrations of IL-6, IL-8, and LTB₄ as measured in the EBC of pigs exposed to *C. suis* and non-infected controls. While IL-6 and LTB₄ showed a tendency to increase at least in some of the challenged pigs 5–7 dpi, no change was seen in IL-8.

Serum activity of MMP-2 and MMP-9 was similar in both groups prior to challenge and remained unchanged by either placebo or *C. suis* inhalation challenges. In BALF supernatant, MMP-9 was found to be increased in *C. suis* infected pigs compared to controls (Tab. II). Furthermore, the ratio between the concentration of 8-IP and the concentration of total protein in BALF (8-IP/protein)

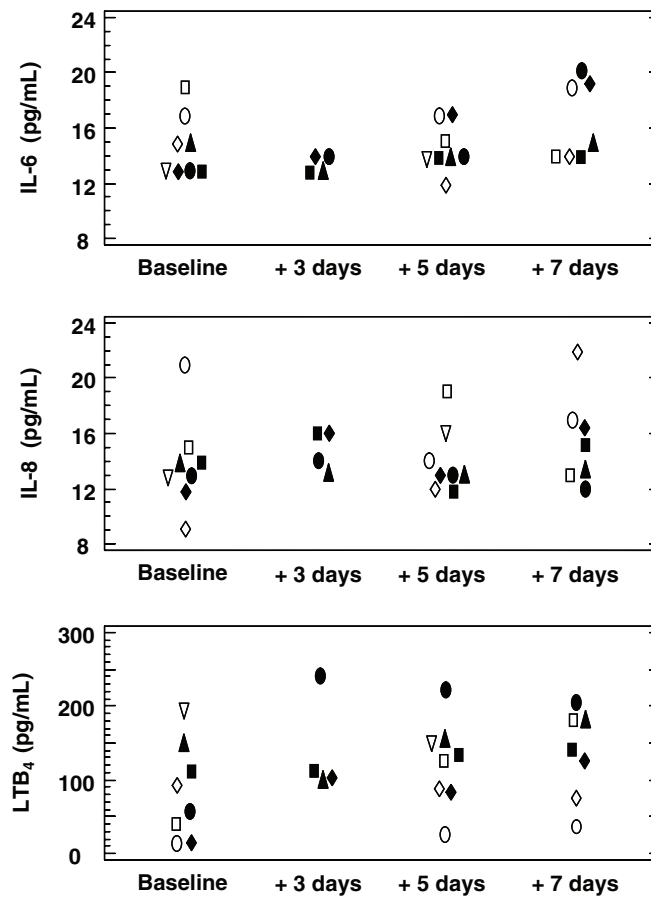


Figure 3. Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8), and leukotrien B₄ (LTB₄) in exhaled breath condensate (EBC) samples collected from four pigs experimentally exposed to *C. suis* (filled symbols) and from four non-infected control pigs (empty symbols).

Table II. Activity of MMP-2 and MMP-9 (arbitrary units) in BALF and serum samples of pigs experimentally exposed to *C. suis* and non-infected controls.

	Animals exposed to <i>C. suis</i>			Controls			<i>t</i> -test
	n	Mean	SD	n	Mean	SD	
Blood (serum)							
MMP-2	6	2 923	1 125	4	2 418	1 082	n.s.
MMP-9	6	3 135	2 393	4	2 964	1 734	n.s.
BALF							
MMP-2	6	6 399	2 395	4	4 927	3 385	n.s.
MMP-9	6	6 378	2 767	4	1 018	776	<i>P</i> < 0.01

n.s.: No significant difference between groups.

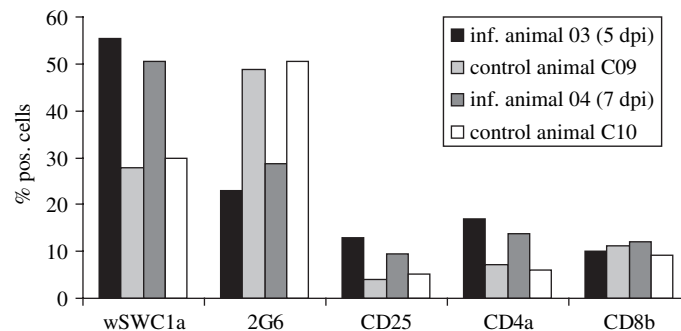


Figure 4. Cellular composition in broncho-alveolar lavage fluid (BALF) of two representative pigs euthanised 5 days and 7 days after challenge (animals 03 and 04: exposed to *C. suis*; animals C09 and C10: non-infected control). After exclusion of cellular debris, values are presented as % positive cells in BALF.

was found to be significantly lower in pigs challenged with *C. suis* compared to controls (130 ± 71 vs 280 ± 103 , means \pm SD, *t*-test, $P = 0.03$).

Taking all animals into account, neither the concentration of 8-IP nor the concentration of total protein in BALF differed significantly between groups. However, the following interesting time trend was seen for both markers: among all data, the highest absolute concentration of 8-IP (77.3 pg/mL) and an extraordinary high amount of total protein (1181 μ g/mL) were measured in the BALF of the single pig sacrificed three days after *C. suis* challenge. In the subsequent period (5–25 dpi), concentrations of both 8-IP and total protein were lower and differed not significantly from control values (ranges for 8-IP: 30–38 pg/mL in controls, 27–69 pg/mL in *C. suis*; ranges for total protein: 96–414 μ g/mL in controls; 110–526 μ g/mL in *C. suis*).

3.4. BALF cytology

For characterisation of the immune cell composition of BALF upon chlamydial infection, different monoclonal antibodies against specific porcine antigens and flow cytometry were used. The results comparing two non-infected and two *C. suis* infected animals at five and seven days after challenge (time points of predominant pathological changes) are shown in Figure 4. The flow cytometric analysis revealed an increased number of wSWC1a⁺ cells as well as of CD4⁺

and CD25⁺ lymphocytes in the *C. suis* exposed animals, while the percentage of 2G6⁺ macrophages declined. These results indicate the recruitment of granulocytes and activated T-cells after infection in BALF. Furthermore, the absolute cell number in BALF was about 2.5 times higher in pigs exposed to *C. suis* compared to control animals, and this finding indicates an absolute increase of all cell subsets including macrophages (data not shown).

3.5. Histology of lung tissue

3.5.1. Histological pulmonary lesions

Morphologic changes in lung tissue could be seen 3–10 dpi and were most conspicuous 4–6 dpi. The lungs of all infected animals showed scattered consolidations with focal hyperinflation. Histologically, a bronchiolitis with lumens filled with inflammatory cells was seen on the third day (Fig. 5A). A severe focal pneumonia with interstitial infiltrates of lymphocytes, neutrophils, and eosinophils, intra-alveolar aggregates of macrophages and neutrophils, as well as dystelettases, was found. Infiltrates were accompanied by a slight to moderate focal oedema, which was not involving the intra-alveolar spaces totally. Signs of diffuse alveolar damage with hyaline membranes were not seen. Inflammatory changes were progredient until the fifth day. After seven days (Fig. 5B), alterations were regressing. Ten days after infection (Fig. 5C)

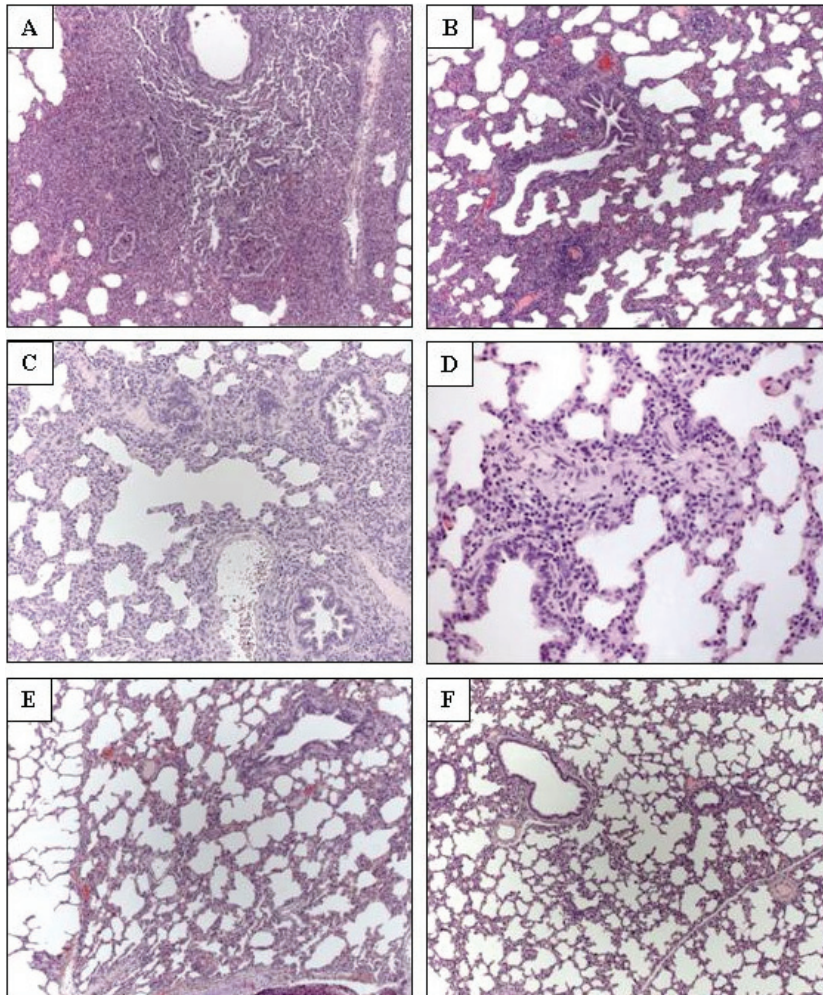


Figure 5. Pathohistology of porcine lung after *C. suis* infection (HE). (A) 3 dpi: florid bronchiolitis and severe focal pneumonia (animal 01; original magnification 40 \times). (B) 7 dpi: clear regression of inflammation (animal 04; original magnification 40 \times). (C) 10 dpi: interstitial infiltrates are still severe (animal 05; original magnification 100 \times). (D) 17 dpi: minimal oedema and slight perivascular infiltrates (animal 06; original magnification 200 \times). (E) 24 dpi: only focal minimal infiltrates but some hyperinflation (animal 07; original magnification 100 \times). (F) Controls revealed only some focal infiltrates without relevance (animal C10; original magnification 12.5 \times).

interstitial infiltrates were still severe. Intramural infiltrates of the bronchioli were moderate, compressing the lumen and inducing dystelectases. On day 16 (Fig. 5D), only minimal oedema, slight perivascular infiltrates of lymphocytes, neutrophiles, eosinophiles,

and some intra-alveolar aggregates of macrophages were found. After 23 days (Fig. 5E), only focal minimal infiltrates but some dystelectases could be detected. Controls revealed only some focal infiltrates as were seen 23 dpi (day 8 post inhalation, Fig. 5F)

Table III. Occurrence of chlamydiae in organs of pigs experimentally exposed to *C. suis* and non-infected controls.

dpi	Animal number	Tonsil	Lung	Lung lymph node	Spleen
3	01	n.d.	+	n.d.	n.d.
4	02	+	+	–	–
5	03	+	+	+	–
7	04	+	+	+	–
10	05	+	+	+	–
17	06	+	+	+	–
24	07	–	+	–	–
24	08	–	+	–	–
6	C09	–	–	–	–
8	C10	–	–	–	–
11	C11	–	–	–	–
25	C12	–	–	–	–

+: *Chlamydia*-positive cells detectable; –: no detectable *Chlamydia*-positive cells, dpi: days post infection; n.d.: not done.

3.5.2. Dissemination of *Chlamydia suis*

To follow the dissemination of *C. suis* infection, cryostat sections of lung, lung lymph node, tonsil and spleen were immunohistochemically stained with an anti-chlamydia-LPS antibody and analysed by

light microscopy concerning the occurrence and localisation of chlamydial bodies. As shown in Tables III and IV, chlamydiae were found in the lungs of all infected animals as well as in tonsils and lung lymph nodes of pigs 4–17 dpi. While single infected cells and a few sporadic detections in sinusoids and germinal

Table IV. Occurrence of BALT and predominance of chlamydiae in lung tissue of pigs experimentally exposed to *C. suis* and non-infected controls.

dpi	Animal number	BALT	Bronchial epithelium	Cells of alveoli and bronchi	Interstitialium	Histiocytes (peribronchial, perivascular)	Septal fibroblasts
3	01	–	+	++	++	–	–
4	02	–	+	+++	+++	–	+
5	03	××	–	++	++	–	+
7	04	×	–	+	+	–	+
10	05	×	–	+	+	+	+
17	06	–	–	+	+	+	+
24	07	–	–	+	+	–	–
24	08	×	–	+	+	+	+
6	C09	–	–	–	–	–	–
8	C10	–	–	–	–	–	–
11	C11	–	–	–	–	–	–
25	C12	–	–	–	–	–	–

×: BALT present; ××: BALT more frequently present; +++: high number of *Chlamydia*-positive cells; ++: moderate number of chlamydia-positive cells; +: low number of *Chlamydia*-positive cells; –: no detectable *Chlamydia*-positive cells/BALT.

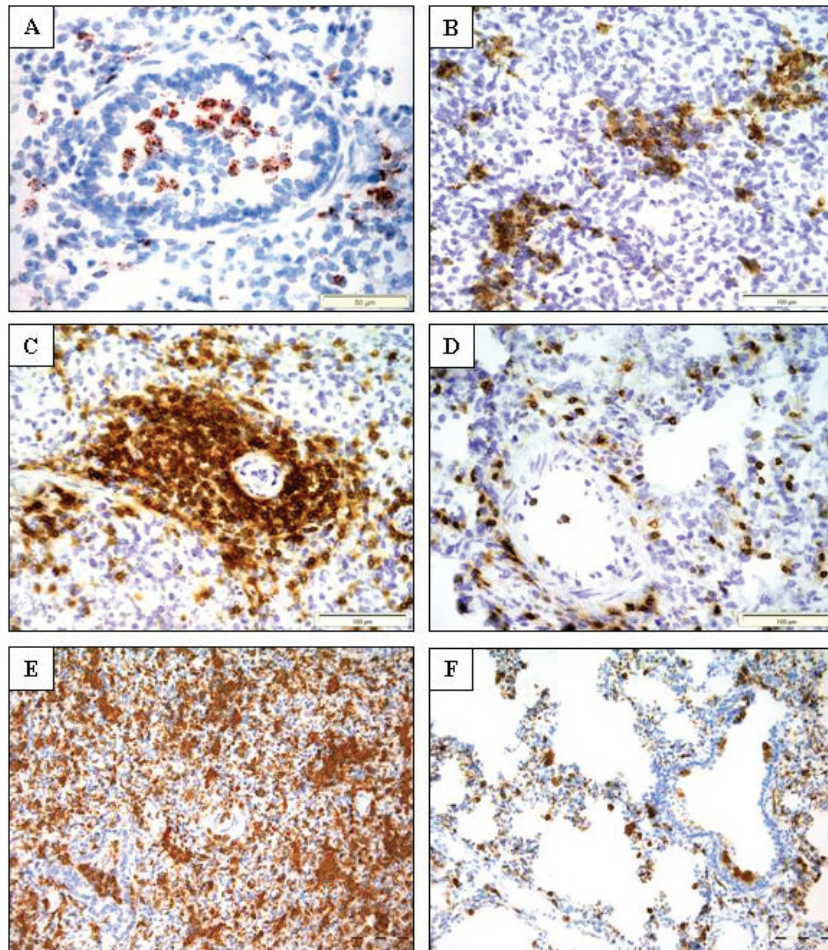


Figure 6. Lung tissue sections of infected (A, B, C, E) and control (D, F) animals immunohistochemically stained (brown) for chlamydiae (A, B), CD2a⁺ T-cells (C, D) and 2G6⁺ macrophages (E, F). (A) Cells fulfilled with chlamydial bodies localised in a bronchioli ten days after *C. suis* exposure (animal 05). (B) Chlamydia infected areas localised in the interstitium three days after *C. suis* exposure (animal 01). (C) T-cells localised perivascularly four days after *C. suis* exposure (animal 02). (D) T-cells in lung tissue of a non-treated control animal (animal C12). (E) Lung tissue closely packed, alveoli filled with macrophages and compressed bronchioli in the lung obtained three days after *C. suis* exposure (animal 01). (F) Wide open alveoli in the lung of a non-infected control animal (animal C12).

centres dominated in lung lymph nodes and tonsils, chlamydiae were highly prominent and visible as larger focal infections in the respiratory tract. Especially pronounced 3–5 dpi, chlamydial bodies were detected in the interstitium and alveolar as well as bronchial spaces of the lung (Tab. IV; Figs. 6A and 6B). Cells of bronchial epithelium were sparsely

chlamydia-positive at 3 dpi and 4 dpi. Later on, chlamydiae were additionally found in histiocytes of peribronchial and perivascular regions (10–24 dpi) as well as in septal fibroblasts (4–24 dpi; Tab. IV). In control pigs, chlamydiae were never observed.

Notably, BALT was seen in the lungs of some *C. suis* infected animals, but not in

Table V. Occurrence of different immune cell subsets in lung tissue of pigs experimentally exposed to *C. suis* and non-infected controls.

dpi	Animal number	wSWC1a	2G6	CD2a	CD4a	CD8b	CD25
3	01	++++	+++	++	+	++	+
4	02	+++	++++	+++	+++	++	++
5	03	++++	++++	++++	++++	+++	+++
7	04	++	+++	++++	+++	+++	++
10	05	++	+++	++++	++++	+++	++
17	06	++	+++	+++	++	+++	++
24	07	++	++	++	+	++	+
24	08	++	++	++	+	++	+
6	C09	++	++	++	+	++	(+)
8	C10	++	++	+++	++	++	(+)
11	C11	++	++	++	+	++	(+)
25	C12	++	++	++	+	++	(+)

++++: Very high number of positive cells; +++: high number of positive cells; ++: moderate number of positive cells; +: low number of positive cells; (+): very low number of positive cells.

control animals (Tab. IV). Especially, one animal (No. 3; 5 dpi) showed very developed BALT with distinct follicle centres and T-cell areas. BALT was preferentially located around bronchioli and small bronchi.

3.5.3. Emergence of immune cells in lung

To study the immune response of the lung, cryostat sections of all animals were immunohistochemically stained with different monoclonal antibodies against immune-relevant cells and evaluated by an individual scoring system. The results are summarised in Table V and representative pictures are shown in Figures 6C–6F.

Compared to control animals, a clear increase in all investigated immune cell subsets was seen upon *C. suis* exposure. While the highest number of wSWC1a⁺ cells was observed 3–5 dpi, the number of other immune cell subsets was initially elevated at 4 dpi and continued up to 10 dpi or 17 dpi in dependence of the detected antigen.

Quantitative changes of 2G6⁺ macrophages were especially prominent and impressive 4–5 dpi in lung tissue. Large numbers of macrophages were filling the alveoli and

widening the alveolar septa leading to a closely packed appearance of lung tissue.

Elevated numbers of T-cells were predominantly localised in peribronchial and perivascular regions but also in the interstitium of chlamydia-affected lung areas. Notably, the number of CD4⁺ and CD25⁺ T-cells increased in the lung tissue of exposed pigs.

4. DISCUSSION

4.1. Evaluation of pulmonary dysfunctions

In control pigs, neither FRC nor the pattern of respiration changed, and continuous increases of TL CO_{rb}, V_t and V_{min} must be considered as physiological due to the growth-related increase in body weight in the course of the study (see Section 3.1.). In contrast, aerosol *C. suis* infection resulted in significant deterioration in pulmonary functions at the same time as clinical signs reached their maxima (3–5 dpi). Diminution of the O₂ diffusion capacity of the lung (i.e. reduced TL CO_{rb}) indicates severe impairment of the O₂ transport from the lung into the blood that might be caused by (i) alteration in the lung fluid balance leading to pulmonary

oedema, (ii) alveolar hypoventilation, or (iii) a mismatching between ventilation and perfusion.

4.1.1. Pulmonary oedema

In the early acute phase (3 dpi), a higher permeability of the lung capillary barrier contributed most likely to the reduced diffusion capacity of the lung as indicated by an extraordinary high concentration of total protein in BALF ($>1000 \mu\text{g/mL}$) and confirmed histologically by focal oedema. In animals sacrificed later (until 24 days after challenge), there was no more evidence for protein leakage into alveoli showing a rapid reversibility of lung capillary barrier disorders.

4.1.2. Alveolar hypoventilation and inhomogeneities between ventilation (V) and perfusion (Q)

The enormous decrease in tidal volume 3–5 dpi indicates that the volume in- and expired per breath was reduced to up to 50%, leading to the consequence of reduced alveolar ventilation and alveolar hypoxia. Pathogenetically, this was most likely caused by both peripheral airway obstructions (bronchospasms) and reduced pulmonary compliance as observed in an earlier study using the same model [32]. Because the porcine lung lacks collateral airways to ventilate the lung periphery of obstructive regions through pathways for collateral airflow [24], atelectases develop frequently in any obstructive conditions leading to ventilatory asynchronisms, i.e. regional inhomogeneities in alveolar ventilation [34]. In addition, inhomogeneities in alveolar ventilation are regularly involved in V-Q inhomogeneities due to the high degree of compartmentalisation of the porcine lung.

All pathogenetic features discussed above were very likely involved in the diminution of O_2 diffusion capacity and contributed to reduced lung gas exchange, and consequently to a reduced oxygen supply to the arterial blood (arterial blood gas analysis was not performed in this study). To compensate for arterial oxygen deficiency, the respiratory

frequency increased significantly leading to an increase in minute ventilation. Due to the reduced tidal volume and the increased respiratory frequency, the pattern of respiration changed to rapid and shallow breaths in experimentally infected pigs. Within one week after exposure, infected pigs tended toward a clinical recovery and both diffusion capacity of the lung and minute ventilation were returned to baseline values indicating that gas exchange was normalised at 7 dpi.

4.1.3. Emphysema

One week after challenge, FRC (i.e. the volume of gas remaining in the lung at the end of expiration) was doubled in comparison to the baseline in pigs challenged with *C. suis*, and the pattern of breathing was still characterised by a significantly decreased tidal volume and a significantly increased respiratory rate. The continuous increase of FRC as observed between day 5 and day 7 after challenge might be suggestive of trapped air or the development of pulmonary emphysema. Pathogenetically, this phenomenon is supported by earlier observations showing that airflow limitations due to peripheral airway obstructions do affect expiration much more than inspiration in *C. suis* infected pigs [32]. Whether peripheral airway obstruction and the developing obstructive emphysema as observed one week after infection are reversible, or whether these phenomena represent an early stage that tends towards chronic airway obstruction, deserves further examination in a longer study period.

4.2. Biochemical evaluation of pulmonary inflammation

Biochemical markers to evaluate pulmonary inflammation were measured in EBC (collected in vivo within the first week after challenge) and BALF (collected ex vivo until 24–25 dpi). Independent of the different time courses, EBC and BALF are not directly comparable diagnostic media [13]. The collection of EBC has the advantage of being completely non-invasive, repeatable and does not require patient cooperation. Although a variety of

mediators measurable in EBC has been described as being influenced by several diseases of airways or lung parenchyma [17, 31] its diagnostic potentials and limitations have yet to be defined in both human and veterinary medicine [11]. BAL, a well accepted diagnostic tool in humans as well as in animals, has the disadvantage when performed *in vivo* to cause an influx of neutrophils and alterations in lung surfactant that may last for three weeks [23]. Consequently, BAL was only performed *ex vivo* in this study in order to avoid any interference with pulmonary function measurements.

4.2.1. Exhaled breath condensate

LTB₄ and IL-6 showed a tendency to increase in some pigs challenged with *C. suis*, while no change was seen in the concentration of IL-8 within the first week after infection. The increase of LTB₄, an inflammatory mediator with chemotactic activity for neutrophils and other cell types, was in agreement with previous findings in calves demonstrating that elevated LTB₄ concentrations in EBC due to respiratory infections were correlated with deteriorations of pulmonary functions [33]. IL-6 is a cytokine that is involved in acute phase, and acute phase response has been evaluated previously [38]. Information about mediators or markers of inflammation as measured in EBC in this study are interesting but should be considered as preliminary results.

4.2.2. Broncho-alveolar lavage fluid

While 8-IP, a stable peroxidative product deriving from arachidonic acid, was measured in BALF to reflect oxidative stress, the concentration of protein was used to evaluate the permeability of the lung capillary barrier. Three days after *C. suis* challenge, the highest concentration of total protein as well as 8-IP was measured indicating that both (i) leakages in the alveolo-capillary barrier and (ii) a certain amount of oxidative stress were present at the same time. These processes were reversible because concentrations of 8-IP and total protein were not significantly different from those measured in BALF of control pigs

in the period 5–25 dpi. Interestingly, the ratio between the concentration of 8-IP and the concentration of total protein in BALF (8-IP/protein) was found to be sensitive to reflect pulmonary inflammation, a finding that has not been described before.

Data for metalloproteinases suggest that acute respiratory *C. suis* infection induced activation of pulmonary MMP-9 but not MMP-2 in pigs. Systemic repercussions on serum MMP-2 and MMP-9 activity were not detected. The absence of a systemic effect parallels findings made in calves with chronic chlamydial infections [14]. Interestingly, chlamydia-infected calves showed a significant increase of MMP-2 activity in BALF, which paralleled subclinical and chronic inflammatory obstructive changes of airways [14]. In the present model of acute pulmonary infection, however, BALF MMP-2 activity remained unchanged in comparison to the control animals, whilst MMP-9 activity increased significantly. These differences might be attributed to the acute character of this infectious model, where large numbers of inflammatory cells, including neutrophils and macrophages, were detected cytologically and histologically. Both neutrophils and macrophages were reported to produce and secrete MMP-9 upon an acute stimulation, whilst MMP-2 is considered as an indicator of fibroblastic activity suggestive of remodelling [40].

In an earlier publication of our group, a significant increase of heat shock protein 60 (Hsp60) was described in the serum of *C. suis* infected pigs [38]. Although no data about Hsp60 within the respiratory tract are available for this earlier study, the increase of Hsp60 in response to *C. suis* infection merits attention. Indeed, it has been shown in murine macrophages that MMP-9 expression and MMP-9 activity increase in response to chlamydial infection as well as in response to chlamydia-induced Hsp60. Inactivation of chlamydial Hsp60 by heat prevented MMP-9 expression, suggesting that Hsp60 might mediate the expression of this proteolytic enzyme [19]. Another study reports that monocyte MMP-9 expression can be upregulated

by Hsp70 overexpression, which increases the activity of transcription factor nuclear factor κ B (NF- κ B) activating factor 1 (AP-1) [21]. These in vitro data support observations made in humans, where the inflammatory responses initiated by chlamydia-specific Hsp appear to play a role in the pathogenesis of asthma [9, 12, 39, 43]. Accordingly, long-term investigations would be interesting in order to describe the relationship between chlamydial infection, Hsp, MMP expression and activities and pulmonary inflammation in vivo.

4.3. Dissemination of *C. suis*, histology and immune response

In former studies using nested PCR, DNA of *C. suis* was detected in the lung as well as in tonsils, lung lymph nodes and spleen [38]. Using immunohistochemistry, only a few chlamydia-positive cells were found in lung lymph nodes and tonsils while the lung presented highly impressive infection foci. This, together with the absence of chlamydiae in the spleen, argues for a rather local infection process mainly restricted to the lung. However, the number of chlamydial bodies occurring seems to be very different in the organs as shown in the present study. The ability of chlamydiae to leave the respiratory tract and cause infections of non-respiratory sites has been demonstrated by other authors for *C. pneumoniae* [8]. As specified, alveolar macrophages transport chlamydiae to the peribronchiolar lymphatic tissue and subsequently the pathogen enters organs via dissemination by peripheral blood monocytes [8]. A predominance of *C. suis* in porcine alveolar macrophages and histiocytes was demonstrated in the present study as well as by confocal laser-scanning microscopy [38].

Unexpectedly, chlamydia-stained bronchial epithelial cells were hardly found in our study. This result indicates an acute respiratory disturbance and is in line with other reports on acute chlamydial infections [37]. In contrast, the bronchiolar epithelium was chlamydia-positive for up to eight weeks in chronic chlamydial infection [8]. Whether there are really different target cells in acute and

chronic pulmonary *C. suis* infection, and how a chronic infection can result from an acute one, has to be further investigated. However, chlamydiae may enter the body rather by the alveoli than through the bronchial epithelium in acute infection, which might be facilitated by the hyperinflated and/or damaged squamous cell lining of alveoli in the course of disease.

The histologically assessed infiltration of granulocytes, macrophages and lymphocytes, as well as the dynamic of inflammatory changes, were confirmed and specified. Granulocytes and macrophages (cells of innate immunity) appeared early after infection. In vitro experiments demonstrated that macrophages interact with chlamydiae [20]. On the one hand, the obligate intracellular chlamydiae can survive and proliferate inside of these cells. On the other hand, macrophages are equipped with the capability to eliminate intracellular pathogens. However, chlamydiae may benefit from a rapid invasion of macrophages for their own multiplication and perhaps transportation into extra-inflammatory sites in vivo. In this way, the elicited immune response does not only play a role in eradication but also in the pathogenesis of that intracellular agent.

The detailed immunohistochemical analysis and flow cytometry proved the involvement of T-cells, especially CD4⁺, CD8⁺ and CD25⁺ T-cells, in the pulmonary host defence against *C. suis*. Altogether, the results of this acute aerogenous *C. suis* infection indicate a more Th1-related immune response in swine. That cell-mediated immune mechanisms are of special importance in chlamydial infections of the lung has been shown by other authors [28, 29, 42].

4.4. Involvement of lipopolysaccharide (LPS) in the pathogenesis

Since LPS produced by gram-negative bacteria may play a significant role in acute respiratory failure in vivo and because endotoxin associated changes in the lung share some features with observations of this study, the possible contribution of chlamydial LPS to the

pathogenetic complexity of this study needs to be discussed. In porcine models of endotoxemia or sepsis, a significantly increased concentration of total protein in BALF was one of the most reproducible findings [3, 26, 27, 44], and increased vascular permeability leading to extra-vascular lung fluid, pulmonary oedema and protein leakage into alveoli has been described as a typical response [3, 25]. With respect to lung functions, a reduced pulmonary compliance was found to be a useful early assessment of evolving lung injury after onset of sepsis [3]. Furthermore, the porcine lung responses to endotoxin primarily by increased perfusion heterogeneity and redistributed perfusion between lung regions [6, 7] and marked pulmonary vasoconstriction leads to pulmonary hypertension and increased pulmonary vascular resistance [26, 27, 44]. The increase of IL-6, an endogenous mediator of LPS-induced fever, might also be related to the presence of chlamydial LPS in the infected pigs, and the lack of IL-8 response corresponds to data reported from humans, where IL-8 did not show a significant change in a human model of inhaled LPS exposure [36]. Last but not least, a significant increase of the lipopolysaccharide binding protein (LBP) in the peripheral blood of *C. suis* infected pigs was observed 3–5 dpi using the same model [38]. At this stage of research, it would be speculative to distinguish to what extent inflammation and lung function disorders were predominantly caused by chlamydial LPS versus the establishment of a replicative infection. Interactions between LPS and the lung have not been fully understood and signalling pathways remain to be clarified even in human medicine [1]. Further studies with the application of heat-inactivated chlamydiae at the same dose will allow the differentiation of direct LPS and infection-related effects.

5. CONCLUSIONS

Data of this study demonstrate that consequences of an experimentally induced *C. suis* infection in pigs comprise complex interactions between cellular responses within the lung, pulmonary inflammation, and pulmonary

function disorders leading pathophysiologically to gas exchange disturbances and clinically to respiratory distress and losses in body weight increase. Data obtained in this study provide new insight into the pathogenesis of acute respiratory chlamydial infections. This large animal model appears suitable for further investigations of chlamydial respiratory infections and can be recommended for studies of chlamydia-associated infections of the human lung.

Acknowledgements. The authors thank Dr Ernst Grossmann and Dr Konrad Sachse for collaboration. They are very thankful to Annelie Langenberg, Sylke Stahlberg, Gerhard Födisch and to all colleagues working in the team of the animal house (FLI Jena, Germany) for their skilful assistance while performing the numerous pulmonary function tests during the study. In addition, they are grateful to Dr Michael Rothe and Dr Gunter Becher (FILT GmbH, Berlin, Germany) for analysing LTB₄, IL-6, and IL-8 in EBC samples as well as total protein and 8-IP in BALF samples. They also thank Katrin Schlehahn (FLI Jena, Germany), Laetitia Wiggers (University of Namur, Belgium), Carina Kruij and Nellia Schatz (Ruhr-University Bochum, Germany) for excellent technical assistance and support in the different laboratories. Help and assistance given by Heike Friedrich (FLI, Jena) in preparing the manuscript is very much appreciated. Furthermore, the authors wish to express their gratitude to Dr Michael Scott Davis (Oklahoma State University, USA) for his critical reading of the manuscript.

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