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Research paper

Anti-TNF- α therapy does not ameliorate disease in a model of acute virus-endotoxin mediated respiratory disease in pigs

Kalina Atanasova, Steven Van Gucht¹, Kristien Van Reeth*

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

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ABSTRACT

Tumour necrosis factor- α (TNF- α) has been shown to play a role in many inflammatory conditions. Currently anti-TNF- α drugs (e.g. etanercept) are used in humans for treatment of autoimmune diseases. In this study we aimed to elucidate the role of TNF- α in the development of virus-endotoxin-induced respiratory disease. Twenty-two caesarean derived colostrum deprived pigs were used. Initially, the availability in the lungs and circulation, and possible clinical and inflammatory effects of etanercept alone were assessed in 4 pigs after intratracheal and intraperitoneal administration of 0.5 mg/per route/per pig. High anti-TNF- α activity was detected in bronchoalveolar lavage (BAL) fluids, peritoneal lavage fluids and serum of all animals for at least 8 h post-inoculation (HPI). No clinical symptoms, lung lesions, lung cell infiltration or induction of IFN- α , IL-1, IL-6, IL-12 and TNF- α in BAL were detected. Subsequently, the ability of etanercept to block porcine TNF- α and its effect on the above mentioned parameters and on lung virus titres were assessed in 8 pigs. They were inoculated intratracheally with porcine respiratory coronavirus (PRCV) followed by lipopolysaccharide (LPS) 24 h later. Etanercept was administered at the time of LPS inoculation via the same routes and dose as in the initial experiment. The parameters were compared with a control group ($n=8$), receiving only PRCV-LPS. Half of the animals from each group were euthanized at 4 and the rest at 8 h after LPS inoculation. TNF- α was completely neutralized in 3 of the 4 animals euthanized at 4 HPI and significantly lower than in the PRCV-LPS group at all times. No significant differences in disease severity, lung lesions, virus replication, lung cell infiltration or levels of IFN- α , IL-1, IL-6 and IL-12/IL-23 were observed between the two groups. Blocking of TNF- α alone was not sufficient to ameliorate disease in the PRCV-LPS model of respiratory disease, possibly due to the redundancy in the proinflammatory cytokine cascade, or the involvement of other unidentified disease mediators.

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1. Introduction

Tumor necrosis factor- α (TNF- α) is a proinflammatory cytokine, which is secreted by many cell types in the earliest stages of inflammation. It is an essential fac-

tor mediating the immune responses against infections. However, it also plays an important role in the induction of septic shock, autoimmune diseases, rheumatoid arthritis and diabetes. TNF- α has become a major therapeutic target in the treatment of many cutaneous and systemic inflammatory diseases, such as moderate and severe psoriasis, psoriatic arthritis, rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, juvenile rheumatoid arthritis and ulcerative colitis (reviewed by Jackson, 2007). The currently existing drugs that are used to inhibit TNF- α -mediated pathology in humans are based on 2 dif-

* Corresponding author. Tel.: +32 9 264 73 69; fax: +32 9 264 74 95.

E-mail address: kristien.vanreeth@ugent.be (K. Van Reeth).

¹ Current address: Communicable and Infectious Diseases, Scientific Institute of Public Health, Engelandstraat 642, B-1180 Brussels, Belgium.

ferent mechanisms of action: (1) the naturally existing TNF-receptor, which binds TNF and mediates its further biological activities (etanercept) or (2) recombinant anti-TNF- α monoclonal antibodies (infliximab, adalimumab) which bind and neutralize specifically human TNF- α . Etanercept represents a fusion protein which consists of the extracellular ligand-binding portion of the human 75 kDa TNF-receptor (TNFR) linked to the Fc portion of human IgG1 antibody (see <http://www.enbrel.com/prescribing-information.jsp> – accessed on 11 August 2009). Thus, it binds to soluble (active form), but not membrane bound (inactive form) TNF- α , and renders it biologically inactive. Adalimumab and infliximab, on the other hand, bind specifically to both soluble and membrane bound TNF- α (reviewed by Jackson, 2007).

In humans TNF- α has been suspected to play also a significant role in different complex lung inflammatory conditions such as asthma, acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD) (reviewed in Mukhopadhyay et al., 2006). However its role is not clear yet. The aetiology of the above mentioned respiratory conditions is very complex and not well understood.

We have previously developed a reproducible model for severe acute respiratory disease in pigs by inoculating them intratracheally with porcine respiratory coronavirus (PRCV) and 24 h later with bacterial lipopolysaccharide (LPS) (Van Reeth et al., 2000). In the study of Van Reeth et al. (2000) TNF- α was assumed to be a potential culprit for the observed clinical synergy since a temporal association was observed between the peak of clinical symptoms (high fever, depression and breathing difficulties) and production of proinflammatory cytokines, TNF- α in particular, in the lungs of the dually inoculated animals. In contrast, pigs inoculated with PRCV or LPS only did not exhibit prominent respiratory symptoms and had minimal or no cytokine production in the lungs. In the current study we aimed to elucidate the role of TNF- α in the PRCV-LPS model of respiratory disease, by blocking TNF- α in the lungs of PRCV-LPS inoculated pigs.

2. Materials and methods

2.1. Virus, LPS and Enbrel[®] preparations

The Belgian 91V44 isolate of PRCV was used at the second passage in swine testis (ST) cells (Van Reeth and Pensaert, 1994). The inoculation dose was 10^7 50% tissue culture infective doses (TCID₅₀) per pig.

Escherichia coli LPS (O111:B4) (Difco Laboratories; Sigma) was used at a dose of 20 μ g/kg body weight. This dose was based on data from previous studies and selected to cause mild or no respiratory signs (Van Reeth et al., 2000; Labarque et al., 2002).

Enbrel[®] was purchased from Wyeth Pharmaceuticals as a lyophilized product containing 25 mg etanercept per package and dissolved according to the manufacturer's instructions in 2 ml pyrogen-free ultra pure water. This product was then dissolved in phosphate buffered saline (PBS) (nonpyrogenic) so that each inoculation syringe contained 0.5 mg etanercept in a total volume of 3 ml. This dose

of Enbrel[®] was determined on the basis of data obtained from previous experiments using the PRCV and LPS model of respiratory disease in pigs and preliminary *in vitro* experiments on the ability of Enbrel[®] to neutralise porcine TNF- α induced cytotoxicity in a bioassay using PK-15 (subclone 15) cells. The dose was calculated so as to be able to completely neutralize TNF- α activity in the lungs within the first 4 h after LPS inoculation.

2.2. Pigs, experimental design and sampling

In this experiment 22 caesarean derived colostrum deprived piglets, originating from 3 sows were used. The pigs were housed individually under sterile conditions in Horsefall-type isolation units with positive-pressure ventilation and fed with commercial ultrahigh-temperature-treated cow's milk. The animals were used at the age of 3.5 weeks. In an initial experiment 4 pigs received 0.5 mg/pig of Enbrel[®] intratracheally and 0.5 mg/pig intraperitoneally and served as controls for availability and side effects of Enbrel[®] alone on the examined parameters ("Enbrel-only" group). Two additional pigs were mock-inoculated with pyrogen-free PBS, both intratracheally and intraperitoneally, and served as negative controls ("mock-inoculated control" group). The remaining 16 pigs were randomly allocated to 2 groups of 8 animals. Both groups were inoculated intratracheally with 10^7 TCID₅₀ of PRCV per pig and 24 h later with LPS at a dose of 20 μ g/kg body weight. One of the groups received 0.5 mg/pig of Enbrel[®] intratracheally (in the same syringe with LPS) and 0.5 mg/pig intraperitoneally at the time of LPS inoculation ("PRCV-LPS-Enbrel" group). The other group did not receive Enbrel[®], and served as positive control ("PRCV-LPS only" group). Half of the animals from all above mentioned groups were euthanized at 4 h after last inoculation (HPI) and the rest at 8 HPI.

All intratracheal inoculations were performed as described previously (Van Gucht et al., 2006). Intraperitoneal injections were performed in the lower part of the abdomen, laterally from the midline in a shallow angle with concurrent aspiration to confirm that the needle is in the peritoneal cavity and not in the urinary bladder or the intestinal lumen. Both intratracheal and intraperitoneal injections were done with a volume of 3 ml inoculum/pig/route.

At euthanasia the lungs were removed from the thoracic cavity and the right lung was used for lung lavage. Collection, concentration of bronchoalveolar lavage (BAL) fluids and BAL-cell analysis was performed as described previously (Van Gucht et al., 2006). Tissue samples from the apical, cardiac and diaphragmatic lobes of the left lung were collected for virological, bacteriological and histopathological examinations. Serum from all pigs was also collected at euthanasia. The peritoneal cavities of the animals in the mock-inoculated control group and the Enbrel-only group were flushed with 5 ml of PBS and the peritoneal fluids were collected for cytokine examination and detection of residual anti-TNF activity.

The animal experiments described in this study were authorized and supervised by the Ethical and Animal Wel-

fare Committee of the Faculty of Veterinary Medicine of Ghent University (EC2007/044, EC2008/109).

2.3. Clinical monitoring and scoring system

All animals were monitored at least 4 times per day before- and every 2 h after the last inoculation. A score was given for the respiratory rate (0: <60 per minute, 1: 60–90 mild tachypnoea, 2: >90 severe tachypnoea), abdominal thumping (0: absent, 1: present), dyspnoea (0: absent, 1: present), anorexia (0: absent, 1: present) and depression (0: absent, 1: present). Occasionally anorexia was accompanied by vomiting which was not scored separately. The total score per pig was obtained by adding the scores for each parameter and ranged from 0 to 6.

2.4. Assessment of gross lung lesions

The gross lung lesions were drawn as accurately as possible onto a lung diagram and the percentage of affected dorsal and ventral lung surface areas was arbitrarily assessed. The percentage of pulmonary lesions was calculated as an average of the approximate percentages of the dorsal and ventral lung surface areas exhibiting evidence of tissue consolidation.

2.5. Quantification of proinflammatory cytokines in BAL fluids

IFN- α , IL-1, IL-6 and TNF- α were quantified by bioassays as described elsewhere (Helle et al., 1988; Van Reeth et al., 1999). Laboratory standards were run in each bioassay. Samples were tested in 3 individual bioassays and geometric means were calculated. IL-12 was measured in a DuoSet ELISA kit detecting the common p40 subunit of both IL-12 and IL-23 (R&D Systems, Abingdon, UK). Since IL-12 and IL-23 bind also to a common receptor chain and have similar proinflammatory activities (Novelli and Casanova, 2004; Watford et al., 2004 and reviewed in Bastos et al., 2004) we did not discriminate between them. Detection limit for all cytokine bioassays was 40 U/ml. Detection limits for the IL-12/IL-23 ELISA was 18 pg/ml. The ELISA was performed according to the instructions of the manufacturer.

2.6. Quantification of residual anti-TNF activity in BAL fluids, lung suspensions, peritoneal lavage fluids and serum

To quantify the residual anti-TNF activity in the collected samples we developed a bioassay based on the TNF- α cytotoxicity assay described earlier (Van Reeth et al., 1999). Briefly, PK 15 (subclone 15) cells were seeded in 96-well culture plates and cultured until 80% confluence. Before the start of the assay the cells were pre-treated for 2 h at 40 °C with the transcriptional inhibitor Actinomycin D to become more sensitive to the effects of TNF- α . Meanwhile two-fold dilutions of the examined samples were mixed with 10 U of a laboratory standard solution of recombinant porcine TNF- α for 1 h at 37 °C allowing the residual etanercept in the samples to bind to the standard and

neutralize its activity. After the cell-sensitisation the pre-treated sample dilutions were brought on the PK15 cells and were further incubated for 18 h at 40 °C. The degree of cytotoxicity was measured via staining with crystal violet solution, washing and dissolving in 33% acetic acid. The optical density of the sample dilutions was measured at 550 nm wave length, using an ELISA reader. The titre of Enbrel[®] in neutralizing units (NU) was determined as the reverse of the dilution at which the effect of the TNF- α standard was neutralized at 50%. The detection limit of the assay was 80 NU/ml. Each sample was tested at least 3 times and geometric means were calculated. Internal controls were run in each test.

2.7. Virological and bacteriological examinations

PRCV was quantified in lung tissue homogenates of pooled samples of apical, cardiac and diaphragmatic lung lobes. Virus titres were determined by titration on ST cells according to standard procedures (Van Reeth and Pensaert, 1994). The detection limit of the assay was $10^{1.7}$ TCID₅₀/g tissue. Virus titres were calculated using the method of Reed and Muench (Reed and Muench, 1938). Routine bacteriological examination of lung tissue was performed as described earlier (Van Gucht et al., 2003).

2.8. Histopathological examination

For histopathological examination, samples of the left cardiac and diaphragmatic lung lobes were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Sections were evaluated visually for three criteria: (1) alveolar damage; (2) apparent thickening of the alveolar septa (as compared to the negative controls); (3) infiltration with inflammatory cells in alveoli and in alveolar septa.

2.9. Statistical analysis

Standard two-sample Mann–Whitney tests were used to compare values of all examined parameters between the different groups of pigs. *P* values <0.05 were considered significant. GraphPad Prism 5 software was used for statistical analysis.

3. Results

3.1. Residual anti-TNF- α activity in BAL fluids, peritoneal lavage fluids and serum of mock-inoculated and Enbrel-only inoculated pigs

All Enbrel-only inoculated animals showed strong anti-TNF- α activity in all examined samples with highest levels at 4 HPI in BAL fluids and serum, and at 8 HPI in peritoneal fluids respectively (see Table 1). Anti-TNF- α activity was not detected in any of the examined samples of the mock-inoculated pigs.

Table 1

Bronchoalveolar lavage cell parameters, anti-TNF- α activity in different body-compartments and levels of IL-1, IL-6, IL-12 and IFN- α in bronchoalveolar lavage fluids of pigs from the control groups (mock-inoculated and Enbrel-only inoculated).

Inoculum	Euthanasia at... HPI ^a	BAL cells		Anti-TNF activity in... (NU/ml)			Cytokines in BAL fluid				
		Total ^b	%NF ^c	BAL fluid	Peritoneal fluid	Serum	TNF- α (U/ml)	IFN- α (U/ml)	IL-1 (U/ml)	IL-6 (U/ml)	IL-12/IL-23 (pg/ml)
PBS	4	34	0	<80	<80	<80	<40	<40	69	<40	<18
	8	63	0	<80	<80	<80	<40	<40	160	<40	<18
Enbrel	4	58	0	268,369	746	10,169	<40	<40	133	<40	<18
	4	72	0	98,487	399	7358	<40	<40	98	<40	<18
	8	102	1	25,833	1491	6571	<40	<40	112	<40	<18
	8	137	2	40,134	1056	5003	<40	<40	129	<40	<18

^a Hours post-inoculation.

^b Total cells in BAL fluid are expressed as 10^6 cells for 5 ml.

^c Percent neutrophils from total amount of BAL cells.

Table 2

Comparison of macroscopic lung lesions and inflammatory cell-infiltration parameters between PRCV-LPS and PRCV-LPS-Enbrel inoculated pigs.

Parameters	Inoculation	Values at... hour post-LPS inoculation ^b							
		4				8			
Macroscopic lung lesions ^a (%)	PRCV-LPS	0.75	0.35	2.75	0	0	0	0.10	0
	PRCV-LPS-Enbrel	0.75	0.35	0.75	3.50	0	70.00	0.75	5.50
Total BAL cells ($\times 10^6$)	PRCV-LPS	490	300	148	255	346	482	468	532
	PRCV-LPS-Enbrel	359	541	440	142	220	173	325	786
Neutrophils (%)	PRCV-LPS	76	62	12	55	56	80	63	76
	PRCV-LPS-Enbrel	74	68	69	55	31	16	53	60

^a Macroscopic lung lesions were calculated as a mean of the percentage of affected dorsal and ventral lung surface area, no lesions were observed in the mock-inoculated controls.

^b Results of two groups of 4 pigs are shown.

3.2. TNF- α titres in BAL fluids, peritoneal lavage fluids and serum

TNF- α was not detected in any of the mock-inoculated animals or Enbrel-only inoculated animals in any of the examined compartments.

TNF- α titres in the BAL fluids of PRCV-LPS inoculated pigs ranged from 126 to 9018 U/ml and no significant difference was observed between pigs euthanized at 4 or 8 HPI ($p > 0.05$). In contrast, the TNF- α levels in the PRCV-LPS-Enbrel group were significantly lower than in the PRCV-LPS only group ($p < 0.05$) and the titres were generally lower at 4 than at 8 HPI, though the difference was not significant ($p > 0.05$) (see Fig. 1).

In general, Enbrel was able to completely neutralize TNF- α in the lungs in 3 out of 4 animals at 4 HPI. By 8 HPI TNF- α was detectable in all pigs but the average titre was still lower than in the PRCV-LPS only group.

3.3. Macroscopic lung lesions

Macroscopic lung lesions were not observed in any of the mock-inoculated or Enbrel-only inoculated animals.

Macroscopic lung-lesion scores for PRCV-LPS and PRCV-LPS-Enbrel inoculated pigs are given in Table 2.

Only 3 of the PRCV-LPS only inoculated pigs had small lesions covering between 0.35 and 2.75% of the total lung surface area.

In the PRCV-LPS-Enbrel group most animals had lesions similar to the PRCV-LPS group covering between 0 and

5.5% of the lung surface ($p > 0.05$). In general Enbrel did not seem to affect the development of gross lung lesions in PRCV-LPS-inoculated pigs, during the first 8 h after administration.

3.4. Inflammatory cells in BAL fluid

Total BAL-cell counts in the mock-inoculated control animals ranged between 34 and 63×10^6 and neutrophils comprised less than 0.1% of these cells. In the Enbrel-only treated group total BAL cells ranged from 58 to 137×10^6

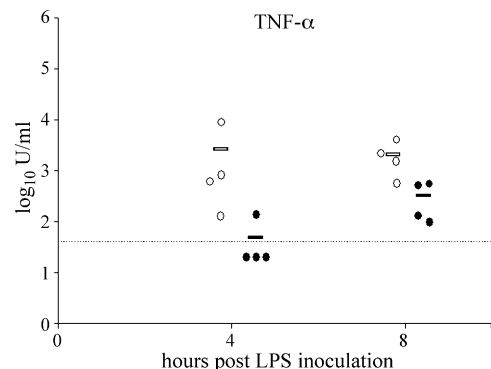


Fig. 1. TNF- α levels in bronchoalveolar lavage fluids of PRCV-LPS (open circles) and PRCV-LPS-Enbrel (filled circles) inoculated pigs. Mean TNF- α titres are shown by dashes. The dotted line represents the detection limit of the assay (40 U/ml).

and low levels (max. 2%) of neutrophils were detected only in the 2 animals euthanized at 8 HPI (see Table 1). Total BAL cells and percent neutrophils in the lungs of PRCV-LPS-Enbrel and PRCV-LPS groups were higher than in the mock and Enbrel-only inoculated groups ($p < 0.05$) (see Table 2), but there were no significant differences in the cell-infiltration parameters between these two inoculation groups ($p > 0.05$).

3.5. Clinical symptoms

Disease symptoms were not observed at any time post-inoculation with PBS or Enbrel[®] alone.

The evolution of the clinical scores in the PRCV-LPS and the PRCV-LPS-Enbrel group are shown in Fig. 2. The PRCV-LPS only treated pigs showed similar symptoms as in our previous experiments (Van Reeth et al., 2000; Van Gucht et al., 2006). At the time of LPS inoculation all animals were still asymptomatic, with the exception of two animals displaying mild depression or tachypnoea. At 2 h, 5 of the 8 pigs had developed marked tachypnoea accompanied by abdominal thumping or dyspnoea, and depression. Anorexia and vomiting were also observed. The remaining 3 pigs developed milder and short lasting symptoms consisting of depression, anorexia or mild tachypnoea. By 8 HPI the symptoms had resolved completely in 3 of the 4 remaining pigs. In the PRCV-LPS-Enbrel treated group only one animal showed mild depression at the time of LPS inoculation. At 2 HPI, four of the 8 animals had developed similar symptoms as in the PRCV-LPS group (tachypnoea, dyspnoea and/or abdominal thumping, depression and/or anorexia with vomiting). Another pig started to show symptoms at 4 HPI. There were no significant differences in the number of affected animals and the severity of clinical symptoms between the PRCV-LPS and PRCV-LPS-Enbrel groups ($p > 0.05$).

3.6. IFN- α , IL-1, IL-6 and IL-12/IL-23 titres in BAL fluids

Fig. 3 compares the levels of IFN- α , IL-1, IL-6 and IL-12/IL-23 between the PRCV-LPS and PRCV-LPS-Enbrel groups. The mock-inoculated and Enbrel-only inoculated animals did not have detectable levels of IFN- α , IL-6 and IL-12/IL-23 and only low levels of IL-1 (69–160 and 98–133 U/ml respectively). In contrast, high levels of all 4 cytokines were detected in both PRCV-LPS and PRCV-LPS-Enbrel groups, and there were no significant differences between the two groups or between the animals euthanized at 4 or 8 HPI in each group ($p > 0.05$).

3.7. Virus titres in lungs

Virus was not detected in the mock-inoculated pigs or the Enbrel-only treated pigs.

Virus titres in the lungs of the PRCV-LPS-Enbrel and the PRCV-LPS only groups were similar ($p > 0.05$) and ranged from 5.0 to 6.7 log₁₀ TCID₅₀/g tissue and from 4.0 to 6.3 log₁₀ TCID₅₀/g tissue respectively.

3.8. Histopathological findings

Fig. 4 compares the most common histopathological findings observed in the 4 inoculation groups. No lesions were observed in either mock- or Enbrel-only inoculated control groups. Typical PRCV-lesions manifested by massive cell infiltration, thickening of the interalveolar septa and collapse of the alveoli, were observed in the lesion-affected areas of the lungs in both PRCV-LPS and PRCV-LPS-Enbrel inoculated animals. The histopathological findings were similar in the PRCV-LPS and PRCV-LPS-Enbrel groups.

4. Discussion

In this study we aimed to elucidate the role of TNF- α in the development of clinical symptoms in virus-endotoxin-induced respiratory disease. A well established and reproducible model of such disease is the PRCV-LPS inoculation in pigs. When administered intratracheally at the time of LPS inoculation, Enbrel[®] was able to successfully block porcine TNF- α produced in the lungs. To our knowledge this is the first *in vivo* study which shows direct blocking of TNF- α in the lungs of pigs by etanercept (Enbrel[®]). Another anti-TNF- α agent (infliximab, Remicade[®]) has been shown to effectively attenuate the decrease in myocardial function after cardiac arrest (simulated by coronary occlusion) in pigs, though plasma levels of TNF- α between infliximab-treated and mock-inoculated animals did not differ significantly (as measured by means of ELISA) (Niemann et al., 2009). This difference in results may partially be explained by the difference in the mechanisms of function and the biochemical nature of infliximab (see <http://www.rxlist.com/remicade-drug.htm#cp> – accessed on 23 August 2009) and etanercept (see <http://www.enbrel.com/prescribing-information.jsp> – accessed on 11 August 2009). Etanercept has previously been shown to reduce endotoxin-induced pulmonary hypertension in pigs (Mutschler et al., 2006), but TNF- α levels in the lungs were not determined in that study. Moreover, clinical symptoms were also not determined since the animals were under inhalation anaesthesia during the experiment. Successful TNF- α blocking by etanercept has so far only been documented in laboratory animals like rats (Di Paola et al., 2007; Zhang et al., 2008) and mice (Fries et al., 2008), though biological activity was not determined in any of the above mentioned studies. Comparison of our data with the results of these studies, however, is difficult not only because of the use of different TNF- α measurement techniques such as Western blot-analysis and immunohistochemistry on tissues (Di Paola et al., 2007) or anti-TNF- α ELISA (Fries et al., 2008; Zhang et al., 2008), but also because of the difference in animal models, disease models and routes of administration of etanercept. In the study of Zhang et al. TNF- α levels in BAL fluids did not differ between etanercept-treated and sham-treated groups, although TNF- α in serum was blocked. This difference can be partially explained by the fact that subcutaneous administration of etanercept may fail to result in sufficient concentrations of the drug in the lungs. During pneumonia, cytokines are produced

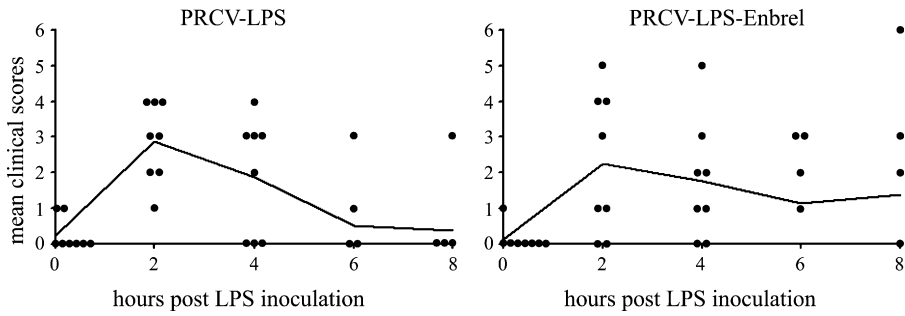


Fig. 2. Comparison of the evolution of clinical symptoms between PRCV-LPS and PRCV-LPS-Enbrel inoculated pigs. Half of the animals in each group were euthanized at 4 h after monitoring.

locally, at the site of infection (reviewed in Schultz et al., 1999). In previous studies in our laboratory we have also noticed that TNF- α production during PRCV-LPS-induced lung inflammation remains very local and no or minimal detection of TNF- α in serum was observed (unpublished data). However, to make sure that systemic TNF- α does not interfere with the observed clinical symptoms we administered etanercept also intraperitoneally. High residual anti-TNF- α activity was detected in the serum of the Enbrel-only inoculated group in comparison with the mock-inoculated group, which confirms the availability of Enbrel[®] in the circulation.

Our data suggests that blocking of TNF- α does not ameliorate clinical symptoms in the PRCV-LPS model, since we did not observe a significant difference between the PRCV-

LPS and the PRCV-LPS-Enbrel inoculated groups. There are many contradicting reports of the effect of anti-TNF- α therapy on disease severity and outcome. In humans anti-TNF- α therapy has shown also no or very little beneficial effect for pulmonary diseases, though in animal models it has shown various levels of success (reviewed in Mukhopadhyay et al., 2006). The criteria for clinical examination, however, are very different in the different studies, and therefore a correct comparison is usually not possible. The physiology of pigs is more similar to that of humans than that of other animal models, such as mice and rats. Therefore pig research is becoming increasingly important.

Virus replication, histopathological findings and macroscopic lung lesions also did not differ notably between the PRCV-LPS and PRCV-LPS-Enbrel groups, apart from

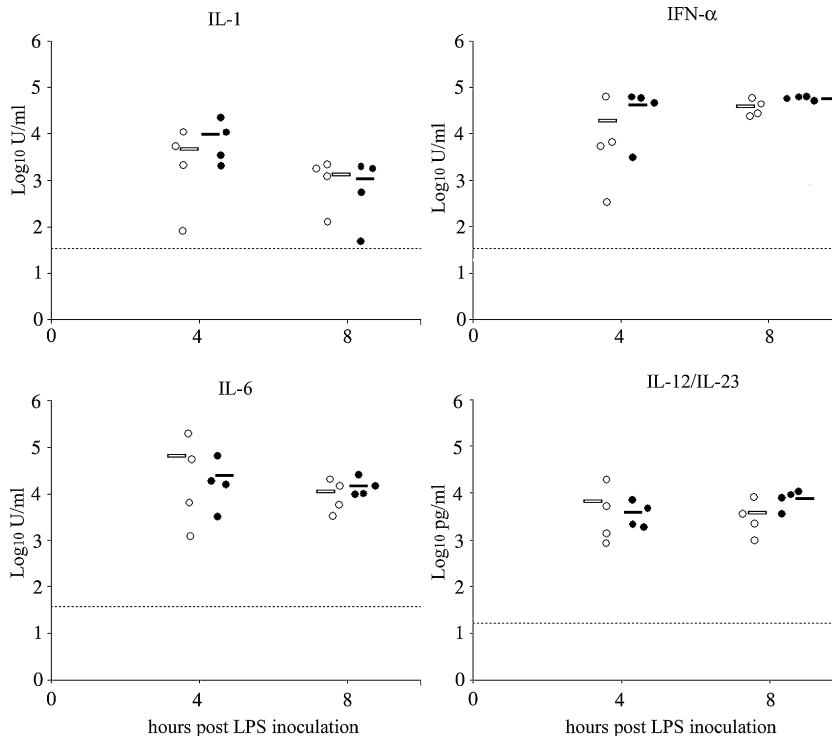


Fig. 3. Comparison of IL-1, IL-6, IL-12/IL-23 and IFN- α levels in bronchoalveolar lavage fluids of PRCV-LPS (open circles) and PRCV-LPS-Enbrel (filled circles) inoculated pigs. Average values are shown by dashes. The dotted line represents the detection limit of each assay (40 U/ml for IFN- α , IL-1 and IL-6, and 18 pg/ml for IL-12/23).

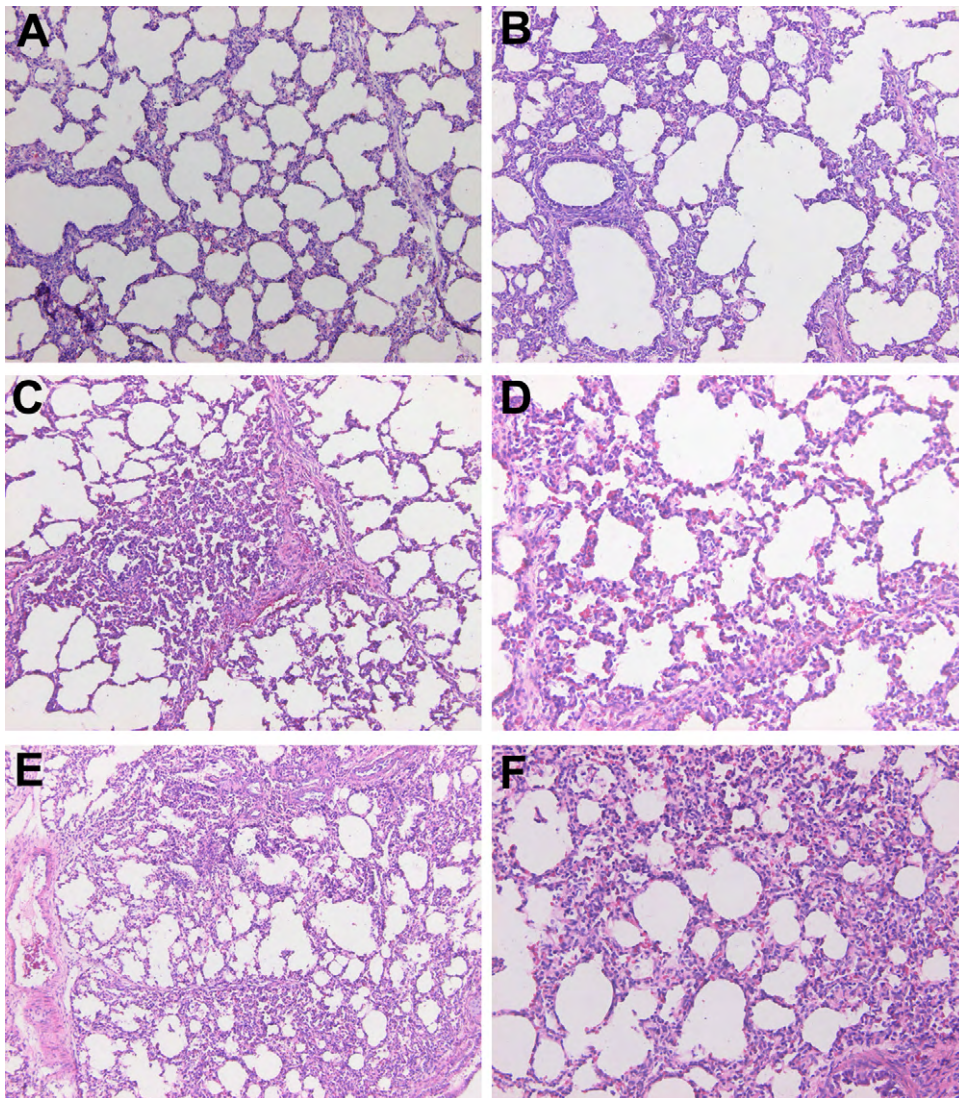


Fig. 4. Haematoxylin–eosin staining of the lungs of pigs inoculated with PBS-only (A) (100 \times), Enbrel-only (B) (100 \times), PRCV-LPS (C) (100 \times) and (D) (200 \times), and PRCV-LPS-Enbrel (E) (100 \times) and (F) (200 \times).

the single pig with high microscopic lung-lesion score. This is partially in accordance with the study of Peper and Van Campen (1995), where they administered rabbit anti-mouse TNF- α -specific antibodies intraperitoneally in influenza A infected mice and saw no effect on virus replication, but gross and histological lung lesions were ameliorated. In the above mentioned study with rats (Zhang et al., 2008) also a reduction in the severity of lung histopathological lesions was observed after etanercept treatment. Our model, however, examines a very early stage after infection and possible effects on lesion development at later stages cannot be excluded.

In our study we observed no significant effects of etanercept administration on neutrophil infiltration or levels of IL-1, IL-6, IL-12/IL-23 or IFN- α in BAL fluids of PRCV-LPS inoculated pigs. This is in contrast with most recent studies in other animal models. For example, in a study of the effect of intraperitoneal administration of etaner-

cept on ventilator-induced lung injury in mice, Wolthuis et al. (2009) observed attenuated neutrophil influx and significant reduction in pulmonary levels of IL-6, MIP-2 and keratinocyte chemoattractant in etanercept-treated animals versus placebo, though blocking of TNF- α was not measured by this group. In the above mentioned study of Zhang et al. (2008) reduced neutrophil infiltration after etanercept administration in passive smoking rats was also observed. Marked reduction of local mucosal neutrophil infiltration in mice with ulcerative colitis (Popivanova et al., 2008) and rats with periodontitis (Di Paola et al., 2007) has also been shown. These differences with our results, may be attributed to the differences in the experimental designs, disease- and animal models used in these studies, as well as to possible secretion or inhibition of other chemotactic cytokines, which have not been examined here. Furthermore, though the PRCV-LPS model of respiratory disease is quite reproducible and has long been used in our lab-

oratory, it represents a complex model of virus-bacterial disease, in which many aspects of the pathogenic mechanisms even of the single agents are not yet well elucidated, and the mechanism of the combination of these pathogenic agents is even less well understood.

In conclusion, this study demonstrates that after intratracheal administration etanercept is able to successfully block TNF- α activity *in vivo* in the lungs of PRCV-LPS inoculated pigs during the first 4–8 HPI. TNF- α reduction, however, was not associated with decrease in disease severity, bronchoalveolar neutrophil infiltration, or altered virus replication and induction of IL-1, IL-6, IL-12/IL-23 or IFN- α in the lungs. There was no obvious difference in macroscopic lung lesions and histopathological findings in the lungs. This data confirms the generally accepted belief that TNF- α , though very important, is not the sole culprit in development of respiratory disease and pathology, and possibly other, yet unidentified, components and mechanisms of the immune system are involved. Complication of respiratory viral infections with secondary bacterial infections and vice versa is very commonly found in field conditions in animals and in humans. Yet, experimental studies of complex diseases are still rare and relatively difficult to reproduce. Therefore, our study is an important addition to the general knowledge about complex virus-bacterial infections and their pathogenic mechanisms, though the exact mechanisms remain still obscure.

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