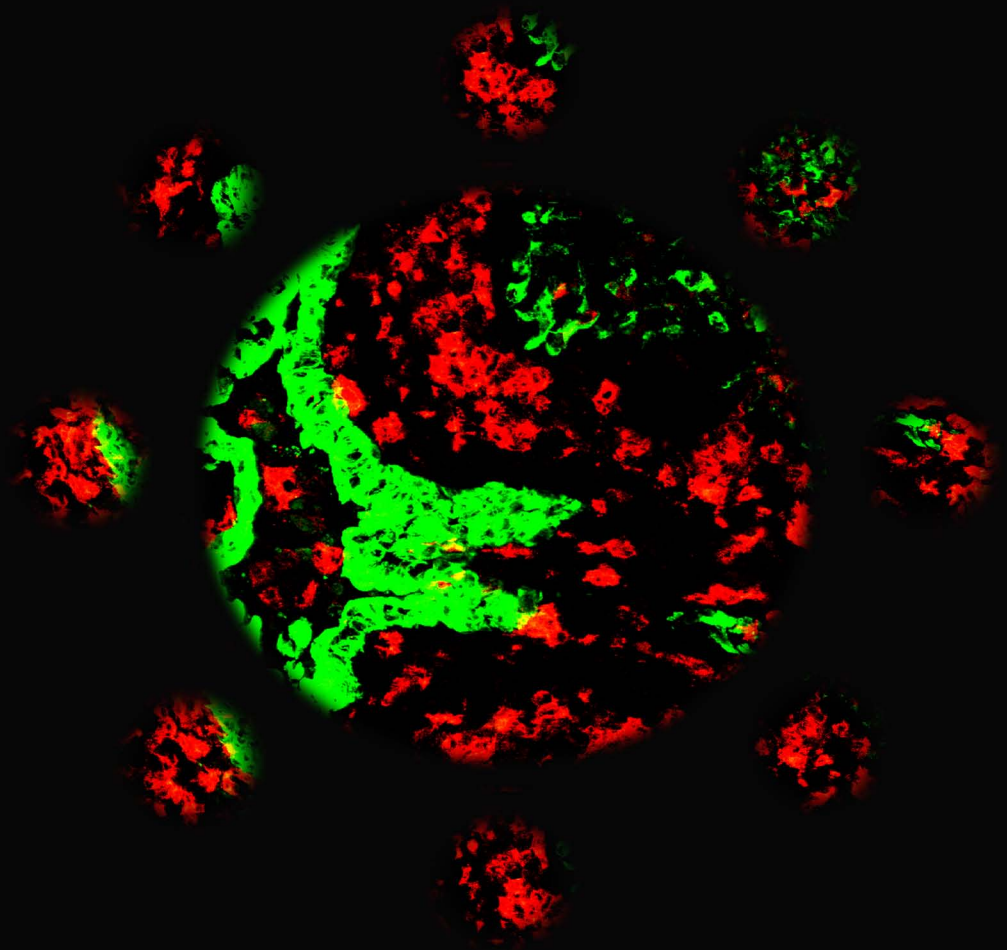
Pathogenesis of the clinical synergy between respiratory viruses and bacterial endotoxin in the lungs of pigs



Steven Van Gucht

"Living organisms seem more than the sum of their parts, and this very paradox might be taken to suggest that there are limits to what we can know about them. The new school of systems biology rests on the premise that complex phenomena can best be understood by observing many events at once. How else can one hope to understand consciousness, development, or immunity? In each example, many separate events contribute to the whole phenomenon and do so simultaneously and in many instances, synergistically."

Bruce Beutler (head of the research team that discovered the function of Toll-like receptor 4)
In: Journal of Leukocyte Biology 2003, 74 (4), p. 479

PATHOGENESIS OF THE CLINICAL SYNERGY BETWEEN RESPIRATORY VIRUSES AND BACTERIAL ENDOTOXIN IN THE LUNGS OF PIGS

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Cover photo: Monocytes, which carry high amounts of the lipopolysaccharide receptor CD14 (red colour), accumulate near a coronavirus-infected bronchiolus (green colour). Details are outlined in chapter 4.2.

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List of abbreviations

ANOVA	analysis of variance
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
CD14	cluster of differentiation 14
CFU	colony forming units
DABCO	1,4-diazabicyclo(2.2.2)octane
DPI	days post inoculation
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
FEV1	forced expiratory volume in one second
FGZ	fosfaat-gebufferde zoutoplossing
FITC	fluorescein isothiocyanate
GP5	glycoprotein 5
GPI	glycosylphosphatidylinositol
IFN	interferon
Ig	immunoglobulin
IL	interleukin
im	intramuscular
kDa	kilodalton
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharide
LSD	least significant difference
M	matrix
MAb	monoclonal antibody
MD-2	myeloid differentiation protein-2
MFI	median fluorescence intensity
mRNA	messenger RNA
MTT	3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MW	molecular weight
N	nucleocapsid
NSAID	non-steroidal anti-inflammatory drug
PAM	pulmonary alveolar macrophage
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PRCV	porcine respiratory coronavirus
PRDC	porcine respiratory disease complex
PRRSV	porcine reproductive and respiratory syndrome virus
RSV	respiratory syncytial virus
S	spike
SARS CoV	severe acute respiratory syndrome coronavirus
sc	subcutaneous
SD	standard deviation
SEM	standard error of the mean
$TCID_{50}$	50% tissue culture infective dose
TGEV	transmissible gastroenteritis virus
TLR4	Toll-like receptor 4
TNF-α	tumour necrosis factor-α
U	unit
VN	virus-neutralizing

1.

INTRODUCTION

1.1. VIRUSES WITH A ROLE IN THE "PORCINE RESPIRATORY DISEASE COMPLEX"

1.1.1. Porcine reproductive and respiratory syndrome virus

Introduction

In the eighties a new infectious disease of swine, characterized by outbreaks of abortion, stillborn piglets, weak suckling pigs and respiratory disease, suddenly emerged in North America and subsequently in Europe (Keffaber, 1989; Hill, 1990). Two years after the initial report of this "mystery swine disease", the causative virus was isolated in the Netherlands and later named "porcine reproductive and respiratory syndrome virus" or PRRSV (Wensvoort *et al.*, 1991; Collins *et al.*, 1992). PRRSV is a member of the family *Arteriviridae*, which together with the family *Coronaviridae*, belongs to the order *Nidovirales* (Cavanagh, 1997). PRRSV is unrelated to any of the known porcine viruses and its origin remains unclear. European and North American isolates have marked genetic and antigenic differences and represent two distinct genotypes. Both genotypes probably drifted away from a common ancestor. Some authors speculate that this ancestor is a murine arterivirus, namely the lactate dehydrogenase-elevating virus, which may have crossed the species barrier (Plagemann, 2003).

PRRSV is a small enveloped virus containing a capsid and single-stranded RNA comprising 15000 nucleotides (reviewed by Meulenberg, 2000). The virion is depicted in figure 1. The primary structural proteins are the nucleocapsid (N) protein, the matrix (M) protein and the envelope protein glycoprotein 5 (GP5), which forms a dimer with the M protein. GP5 contains neutralizing epitopes and is presumed to be the attachment protein that binds with the cellular receptors. Four other minor envelope proteins have been identified.

Most pigs become infected through the oronasal route after close contact with virus-excreting pigs (Albina, 1997). Aerial transmission can occur, particularly in winter and over distances of less than 3 km. Sows can become infected after insemination with contaminated semen. PRRSV is highly prevalent in swine populations all over the world. Virus infections are continuously maintained on most Belgian farms and different strains can circulate at the same time (Vynckier and Pensaert, 1993; Houben *et al.*, 1995; Mateusen *et al.*, 2002; Larochelle *et al.*, 2003).

Most pigs are protected by maternal antibodies until the age of 4 to 16 weeks and become infected afterwards.

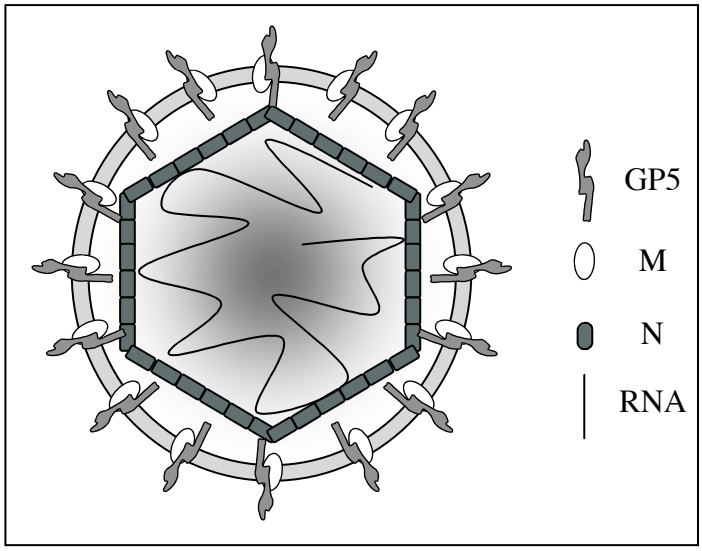

Figure 1. Structure of a PRRSV virion. The icosahedral capsid consists of nucleocapsid (N) proteins, which surround the single-stranded RNA genome. Glycoprotein 5 (GP5) is embedded in the envelope and forms a dimer with the matrix (M) protein. The envelope also contains four different types of minor proteins, which are not shown here.

Pathogenesis of lung infection

The lung is the main target organ of PRRSV. Lung infection sustains cell-free viremia, which results in spread to the lymphoid and reproductive system. Transplacental transmission can occur at the end of gestation and lead to infection of fetuses. Here we will discuss the effects of PRRSV infection on the lungs, with emphasis on features that differ from other respiratory virus infections of swine, such as porcine respiratory coronavirus (PRCV) and influenza virus infections.

Firstly, there is the persistent nature of PRRSV infection in the lungs. PRRSV replicates for at least 5 to 7 weeks in the lungs and peak virus titers are obtained between 7 and 14 days post inoculation (DPI) (Mengeling *et al.*, 1995; Duan *et al.*, 1997b; Beyer *et al.*, 2000; Labarque *et al.*, 2000). In contrast, swine influenza virus is fully cleared from the lungs one week after inoculation (Brown *et al.*, 1993).

Secondly, PRRSV has a specific tropism for sialoadhesin-positive macrophages (Duan *et al.*, 1997a and 1998; Vanderheijden *et al.*, 2003; Delputte and Nauwynck, 2004). Sialoadhesin is an immunoglobulin-like lectin on the membrane of distinct subsets of macrophages, such as resident lung macrophages. Our laboratory identified

this lectin as a specific receptor that mediates the entry of PRRSV in the cell. Blood monocytes do not express this receptor and consequently are refractory to PRRSV infection. Maturation into macrophages coincides with surface expression of sialoadhesin and susceptibility to PRRSV infection. Macrophages are the main target cells in the lungs and no other cell types are infected (Teifke *et al.*, 2001).

A third feature is the massive infiltration of monocytes in the lungs during PRRSV infection. The increase of monocyte-macrophages in bronchoalveolar lavage (BAL) fluids is about 5 times higher during infection with PRRSV, than with PRCV or influenza virus (Van Reeth *et al.*, 1999). Moreover, Labarque *et al.* (2000) found that the infiltration of monocytes is followed a few days later by a five-fold increase of sialoadhesin-positive macrophages in BAL fluids. Presumably, the attracted monocytes differentiate into new PRRSV-susceptible macrophages, which may be a strategy of the virus to maintain virus replication in the lungs.

Finally, PRRSV induces an exceptionally weak innate immune response in the lungs (Murtaugh *et al.*, 2002). The production of proinflammatory cytokines during the early stage of infection is a good parameter to measure the innate immune response. Studies from our laboratory demonstrate that PRRSV is a poor inducer of the proinflammatory cytokines tumour necrosis factor-α (TNF-α) and interferon-α (IFN-α) (Van Reeth *et al.*, 1999). This is in contrast to swine influenza virus, which elicits 10 and 1000-fold higher levels of the respective cytokines in the lungs during the early stage of infection. Other researchers even suggest that PRRSV actively suppresses production of both cytokines in infected cells (Albina *et al.*, 1998a; Lopez-Fuertes *et al.*, 2000; Miller *et al.*, 2004). In addition, PRRSV seems to somewhat evade or postpone an effective specific immune response (Murtaugh *et al.*, 2002). Anti-PRRSV antibodies appear as soon as 7 to 10 DPI in sera and lungs, but are unable to neutralize the virus (Yoon *et al.*, 1995; Albina *et al.*, 1998b; Labarque *et al.*, 2000). Low titers of neutralizing antibodies appear only 3 to 5 weeks later and this correlates with elimination of the virus from the lungs. PRRSV-specific IFN-γ producing T-lymphocytes appear as soon as 3 weeks after inoculation, but their frequency in the blood remains unusually low during the following 7 to 9 weeks, which suggests that PRRSV induces a weak cellular immune response (Meier *et al.*, 2003).

Lung pathology

Gross lung lesions vary from multifocal to diffuse consolidation of lung tissue (Halbur *et al.*, 1995). Typically, lungs fail to collapse and have a red and tan mottled appearance. Microscopically, there is interstitial pneumonia with pronounced thickening of interalveolar septa. The septa are infiltrated with mononuclear cells and the alveolar lining generally appears intact. Alveoli contain a mixture of inflammatory cells and necrotic macrophages. Although PRRSV does not replicate in epithelial cells and most likely causes no direct damage to these cells, hyperplasia of type 2 pneumocytes is a common histopathological feature of PRRSV-infected lungs (Halbur *et al.*, 1995; Teifke *et al.*, 2001).

Disease

PRRSV is considered to be one of the most important primary agents of the "porcine respiratory disease complex" (PRDC) (Thacker, 2001; Choi *et al.*, 2003). The term PRDC refers to multifactorial respiratory disease in pigs, resulting from interactions between primary virus or *mycoplasma* infections, secondary bacterial infections and environmental factors (for review see Brockmeier *et al.*, 2002). Because of its assumed role in multifactorial respiratory disease and the occasional reproductive losses, PRRSV is regarded as the most costly infectious agent in the US swine industry (www.porkscience.org/documents/other/positionprrs.pdf). Since the virus became enzootic, a significant increase of respiratory disease, secondary bacterial infections and poor productivity has also been reported on many European farms (Done and Paton, 1995). Nevertheless, an uncomplicated infection, particularly under experimental conditions and with European isolates, fails to induce overt respiratory disease (Van Reeth *et al.*, 1996; Solano *et al.*, 1997; Labarque *et al.*, 2000 and 2002). Indeed, numerous nursery and grower pigs have been inoculated with different European isolates in our laboratory and infections usually remained mild or subclinical. The most consistent clinical signs were transient fever (40 to 41°C) and decrease of appetite. However, genetic variation between circulating PRRSV strains continues to increase and this might be accompanied with differences in virulence (Forsberg *et al.*, 2002; Grebennikova *et al.*, 2004). Some strains, particularly of the American type, are assumed to be more virulent and can induce severe respiratory distress on their own (Halbur *et al.*, 1995 and 1996). The mechanisms underlying this

apparent difference in pathogenicity are so far unclear. It is nonetheless commonly accepted that PRRSV has to cooperate with secondary agents to induce respiratory disease.

One of the prevailing concepts is that PRRSV infection leads to a decrease of innate defences in the lungs. This presumption follows from the fact that PRRSV infects macrophages, which constitute the first line of defence in the lungs. Indeed, *in vitro* infection of macrophages results in cell death within 24 to 48 hours after inoculation (Paton *et al.*, 1992; Suaréz *et al.*, 1996; Oleksiewicz and Nielsen, 1999). Additionally, studies on lung tissue demonstrated that part of the macrophages surrounding infected cells undergo apoptosis (Sirinarumitr *et al.*, 1998; Sur *et al.*, 1998; Choi and Chae, 2002; Labarque *et al.*, 2003). Thanawongnuwech *et al.* (2000) demonstrated that the capacity of the lungs to clear copper particles from the blood is reduced during PRRSV infection, probably because of destruction of intravascular lung macrophages. Furthermore, some dual inoculation studies revealed that PRRSV infection renders the lungs susceptible to colonization with secondary invaders such as *Bordetella bronchiseptica* and *Streptococcus suis* (Galina *et al.*, 1994; Brockmeier *et al.*, 2000; Halbur *et al.*, 2000; Thanawongnuwech *et al.*, 2000; Schmitt *et al.*, 2001).

However, many other studies, including those from our laboratory, do not confirm the idea of an impaired innate defence. Firstly, PRRSV infection does not lead to increased colonization of the lungs with many other bacteria, such as *Pasteurella multocida, Haemophilus parasuis, Mycoplasma hyopneumoniae* and *Salmonella choleraesuis* upon experimental inoculation (Cooper *et al.*, 1995; Van Alstine *et al.*, 1996; Carvalho *et al.*, 1997; Solano *et al.*, 1997; Segalés *et al.*, 1999; Thacker *et al.*, 1999; Brockmeier *et al.*, 2001). Secondly, the total number of viable alveolar macrophages does not decrease during PRRSV infection (Labarque *et al.*, 2000). On the contrary, infection causes a 5-fold increase of the number of alveolar macrophages between 5 and 52 DPI. This is because at most 3% of total BAL cells become infected with PRRSV and infiltrated monocytes differentiate into a new pool of macrophages during infection (Mengeling *et al.*, 1995; Duan *et al.*, 1997b; Labarque *et al.*, 2000). Finally, previous studies from our laboratory revealed that PRRSV infection does not impair clearance of influenza virus from the lungs (Van Reeth *et al.*, 1996).

Most studies do not support a systemic immunosuppressive effect of PRRSV. One experiment of Li and Yang (2003) suggests that the antibody response against a

commercial classical swine fever vaccine is diminished in PRRSV-infected pigs, compared to uninfected pigs. However, this putative immunosuppressive effect of PRRSV infection is refuted by many other studies (Molitor *et al.*, 1992; Brun *et al.*, 1994; Albina *et al.*, 1998b; De bruin *et al.*, 2000). For example, PRRSV infection did not weaken the development of vaccine-induced protection against pseudorabies virus. On the contrary, some studies even suggest that PRRSV infection enhances the humoral immune response against foreign antigens by stimulating polyclonal proliferation of B-lymphocytes (Vézina *et al.*, 1996; De Bruin *et al.*, 2000; Lamontagne *et al.*, 2001).

In the past, our laboratory has studied the effects of dual infections with PRRSV followed by other respiratory viruses, namely influenza virus or PRCV (Van Reeth *et al.*, 1996 and 2001). Serological data demonstrated that dual infections with these viruses occur often under field circumstances (Van Reeth and Pensaert, 1994a; Houben *et al.*, 1995). Pigs were inoculated with PRRSV and 3 to 14 days later with influenza virus or PRCV. Dual PRRSV-influenza virus and PRRSV-PRCV infections sometimes caused significantly more respiratory disease and growth retardation than each of the virus infections alone. Still, the clinical outcome of these dual infections varied strongly within and between experiments. Within experiments, the proportion of pigs that developed enhanced respiratory disease varied from 20 to 100%. Moreover, the severity of respiratory disease and weight loss differed strongly between experiments. Though our data support that interactions between respiratory viruses potentially aggravate respiratory disease, dual inoculations with PRRSV and other respiratory viruses lack the reproducibility that is required to study the pathogenesis of multifactorial respiratory disease. This is probably due to the fact that even a single experimental infection with a respiratory virus has intrinsic variation in virological, inflammatory and clinical parameters. The variation of a second infection will likely be extra enhanced, as the outcome of this infection depends partly on that of the first infection.

Thus, it remains poorly understood how viruses, like PRRSV, can cooperate with other agents in the induction of severe respiratory disease. This lack of knowledge of the pathogenesis of multifactorial viral respiratory disease was inherited by the author of this thesis and warrants the research described further.

1.1.2. Porcine respiratory coronavirus

Introduction

Porcine respiratory coronavirus (PRCV), a member of the coronaviridae, is a variant of the transmissible gastroenteritis virus (TGEV) with altered tropism from the enteric tract to the respiratory tract. This previously unrecognized coronavirus suddenly emerged in Europe during the early eighties and was first isolated and described by Pensaert *et al*. (1986).

PRCV is an enveloped virus containing a capsid and single-stranded RNA comprising 30000 nucleotides (reviewed by Laude *et al*., 1993). The virion, depicted in figure 2, contains three major structural proteins. The spike (S) protein is a membrane-anchored glycoprotein that protrudes from the envelope as a trimer. This protein induces virus-neutralizing antibodies and is considered to be the attachment protein (Godet *et al*., 1994; Callebaut *et al*., 1996). The glycosylated matrix (M) protein is integrated in the envelope and the phosphorylated nucleocapsid (N) protein is closely associated with the RNA, with which it forms the helical nucleocapsid.

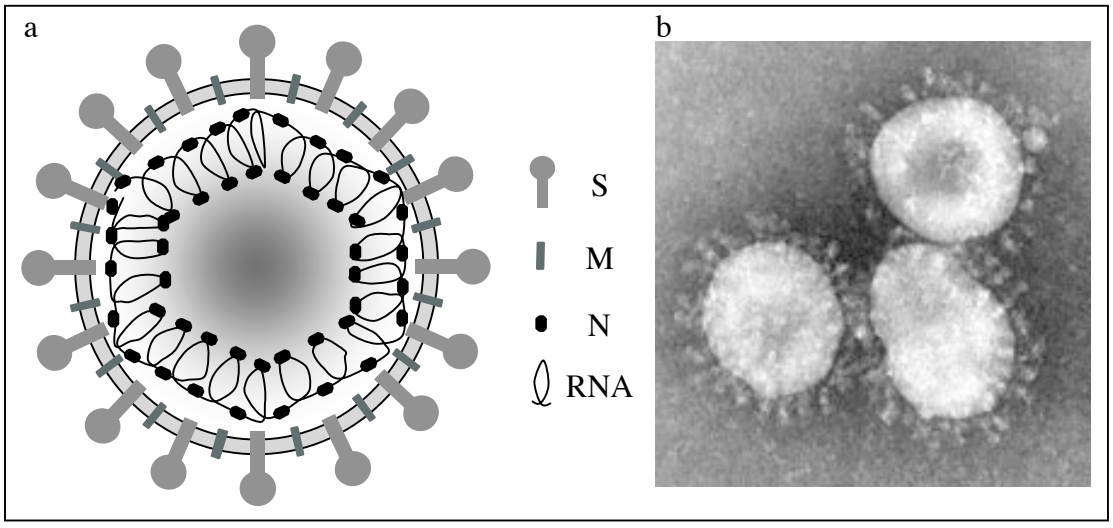

Figure 2. Structure of a PRCV virion (a). Nucleocapsid (N) proteins are complexed with the RNA into a helix. The matrix (M) protein is embedded in the envelope. Spike (S) glycoproteins are inserted in the envelope and form large peplomers, which protrude from the outside of the virion. These peplomers are visible as a "corona" on the outside of the virion, as illustrated in the photograph (b; courtesy of CDC, USA).

The genomes of PRCV and TGEV are highly homologous (96%), except for a large deletion of 672 nucleotides in the S protein gene (Rasschaert *et al.,* 1990). Most

likely, PRCV originated as a deletion mutant of TGEV. The S protein of PRCV lacks 224 amino acids and has a length of 1209 amino acids, whereas that of TGEV has a length of 1431 amino acids. Smaller deletions are also present in the open reading frame 3a. The deletions caused a shift of the tropism from the enteric tract to the respiratory tract and utterly changed the epizootiology, pathogenesis and clinical effects of the virus (Cox *et al.*, 1990b; Ballesteros *et al.*, 1997).

PRCV spreads rapidly through the air and pigs become infected through the oronasal route (Pensaert *et al.*, 1993). Nowadays, PRCV is considered to be enzootically present in swine populations all over the world. Different surveys demonstrate that most Belgian farms harbour the virus and more than 90% of the sows have antibodies (Pensaert *et al.*, 1993; Van Reeth *et al.*, 1994a). Maternal antibodies are protective until the age of 5 to 16 weeks and the majority of pigs become infected afterwards.

Pathogenesis of lung infection

The pathogenesis of PRCV infection differs clearly from that of PRRSV infection. Firstly, PRCV causes a typical acute lung infection (O'Toole *et al.*, 1989; Cox *et al.*, 1990a). Highest virus titers are obtained between 2 and 5 days after inoculation and virus is fully cleared from the lungs less than a week later. The lungs are incontestably the main target organ, as virus titers here are higher than in any other organ. PRCV can also readily be isolated from the trachea, tonsils and nasal mucosa. Low virus titers have been detected in the blood, spleen, mesenteric lymph nodes and the intestines of experimentally inoculated neonates (<1 week old) (O'Toole *et al.*, 1989; Cox *et al.*, 1990a). In older pigs (>5 weeks old) virus replication seems to be restricted to the lungs (Cox *et al.*, 1990b).

Secondly, PRCV is an epitheliotropic virus. The main target cells in the lungs are pneumocytes, bronchiolar epithelial cells and to a lesser extent bronchial epithelial cells (Cox *et al.*, 1990a). PRCV antigens are occasionally detected in lung macrophages, but it is still unclear whether this represents a productive infection or phagocytosis of virus particles and virus-contaminated cell debris. TGEV replicates productively in alveolar macrophages, but this has not been verified for PRCV (Laude *et al.*, 1984). There are indications that PRCV, as other group 1 coronaviruses, uses aminopeptidase-N as a receptor for entry in the cell (Delmas *et al.*, 1993; Tresnan *et*

al., 1996). This enzyme is expressed on the membrane of a wide range of cells, including lung epithelial cells and enterocytes.

Thirdly, the cytokine profiles in the BAL fluids of PRCV-infected lungs differ strikingly from those of PRRSV-infected lungs. PRCV is highly interferonogenic and infection typically induces high levels of IFN-α in the lungs, whereas TNF-α and interleukin-1 (IL-1) levels remain low or undetectable (Van Reeth *et al.*, 1999). The capacity to induce high levels of IFN-α is a common feature of many other coronaviruses, such as TGEV (Baudoux *et al.*, 1998). Particularly the M protein, a glycoprotein abundantly expressed in the envelope of all types of coronaviruses, is a potent inducer of IFN-α in peripheral blood mononuclear cells (Charley and Laude, 1988; de Haan *et al.*, 2003).

Finally, PRCV induces a swift and effective immune response. This is illustrated by the fact that high levels of virus-neutralizing antibodies are mounted in the blood within one week after inoculation and their appearance is followed by complete virus elimination around 7 to 10 days after inoculation (Laude *et al.*, 1993).

Lung pathology

Pathological changes are characterized by lobular red consolidated areas, which are most pronounced in the cranial and cardiac lung lobes (O'Toole *et al.*, 1989; Halbur *et al.,* 1993). Microscopically, there is bronchointerstitial pneumonia with cuffing of mononuclear cells around the small airways and thickening of interalveolar septa. Part of the alveolar and bronchiolar epithelial cells are degenerated and necrotic. Regeneration of lung tissue is visible from 7 days after inoculation and is characterized by hyperplasia of type 2 pneumocytes and bronchiolar epithelial cells.

Disease

It is still a matter of debate whether PRCV causes clinical disease or not. Most studies, including those from our laboratory, indicate that experimental infection causes no or mild respiratory disease (O'Toole *et al.*, 1989; Cox *et al.*, 1990a; Halbur *et al.*, 1993; Van Reeth *et al.*, 1999). Fever and anorexia were sometimes noted, but pigs did not develop obvious respiratory disease. Some researchers reported mild to severe respiratory disease upon experimental inoculation (Duret *et al.*, 1988; van Nieuwstadt and Pol, 1989; Vannier, 1990; Vaughn *et al.*, 1994). The symptoms varied

from sneezing, coughing, tachypnoea, dyspnoea to death. Vaughn *et al.* (1994) suggest that the severity of the clinical signs depends on the strains used, and possibly correlates with slight differences in the genomic deletions, but more research is needed to validate this hypothesis. Pensaert *et al.* (1993) monitored several outbreaks of PRCV in closed swine farms without the appearance of respiratory disease signs. Obviously, many PRCV infections in the field remain subclinical, but in combination with other (unknown) agents disease can occur. This is illustrated by other field studies in which seroconversion of herds to PRCV was associated with the appearance of clinical signs such as fever, anorexia, coughing and difficult breathing (Jestin *et al.*, 1987; Laval *et al.*, 1991; Ulbrich *et al.*, 1991; Bergevoet *et al.*, 1997). Therefore, some authors consider this virus, next to PRRSV and *Mycoplasma hyopneumoniae*, as one of the primary agents of the porcine respiratory disease complex (Brockmeier *et al.*, 2002). Few studies, however, have examined the role of PRCV in multifactorial respiratory disease. One study of Van Reeth and Pensaert (1994b) demonstrated that dual infections of pigs with PRCV followed by influenza virus resulted in more severe clinical signs and lung lesions than infections with only one of these viruses. Little is known about the impact of a PRCV infection on a secondary bacterial infection in the lungs. Although PRCV infections are very common and occur at an age when pigs are challenged with a multitude of other pathogens, the true impact of PRCV on respiratory disease in the field is difficult to assess at this moment.

1.2. LIPOPOLYSACCHARIDE OF GRAM-NEGATIVE BACTERIA AND ITS EFFECTS ON THE RESPIRATORY TRACT

1.2.1. Effects on the lungs of humans and pigs

Introduction

Richard Pfeiffer, a student of Robert Koch, was the first to describe endotoxins in 1892. He found that guinea pigs died after inoculation with dead *Vibrio cholerae* bacteria and that this was due to heat-stable toxins, which were part of the "bacterial body". Several decades later, it became clear that endotoxin was composed of lipid and carbohydrate, and hence the term lipopolysaccharide (LPS) came into usage. The terms endotoxin and lipopolysaccharide are often used as synonyms, but their meaning differs slightly. Endotoxin refers to LPS as it appears in nature, as fragments of the cell wall together with other bacterial compounds (Rylander, 1994). LPS, on the other hand, implies a chemically purified endotoxin, which is obtained by extraction with trichloroacetic acid, phenol or phenol-chloroform-petroleum ether (Galanos *et al.*, 1969; Morrison and Leive, 1975). These LPS preparations are still slightly contaminated with varying amounts of bacterial proteins, lipids and nucleic acids, depending on the method used. LPS is used in most experimental exposure studies and this term will be used further.

Structure

LPS is the main component of the outer membrane of Gram-negative bacteria and is vital to the structural and functional integrity of the cell wall (Rietschel *et al.*, 1994). The general conformation of LPS is presented in figure 3. LPS consists of a phospholipid, called lipid A, that is covalently linked to a hydrophilic heteropolysaccharide (Rietschel *et al.*, 1994). Lipid A is highly hydrophobic and responsible for the endotoxic activity of LPS (Westphal *et al.*, 1985; Rietschel *et al.*, 1993). Lipid A consists of a phosphorylated diglucosamine carrying different fatty acids. The endotoxicity depends on the number, nature and arrangement of the fatty acids and phosphate groups (reviewed by Erridge *et al.*, 2002). The lipid A of *Escherichia* and *Salmonella* species is considered to be highly endotoxic, whereas that of *Pseudomonas* and *Bordetella* species is poorly endotoxic (Erridge *et al.*, 2002). The

polysaccharide of LPS is made up of two parts, an O-polysaccharide with a composition varying between different bacterial species (O-antigens) and a rather invariable core section, which is located between the O-polysaccharide and the lipid A. Due to its amphiphilic nature, LPS forms micelle-like aggregates in solution. These aggregates adopt a lamellar, cubic or hexagonal conformation, depending on the structure of the lipid A, the length of the sugar chain and the environment (Brandenburg *et al.*, 1993; Erridge *et al.*, 2002).

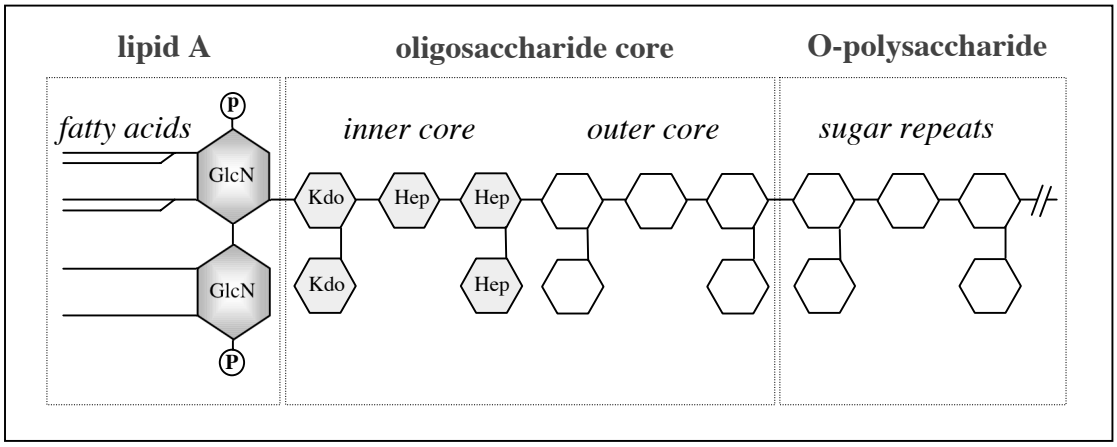

Figure 3. General structure of LPS (adapted from Holst *et al.*, 1996). Lipid A is embedded in the outer membrane of Gram-negative bacteria and consists of a phosphorylated diglucosamine carrying several fatty acid chains. The oligosaccharide core links lipid A to the O-polysaccharide. The inner core contains unusual sugars, such as Kdo and heptose. Both the lipid A and core region are highly conserved, whereas the O-polysaccharide is more variable. The latter contains up to fifty repeats of sugar units, which protrude from the outer membrane. The O-polysaccharide contains antigenic determinants (O-antigens) specific for different bacterial serotypes.
P: phosphate, GlcN: N-acetyl-D-glucosamine, Kdo: 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid, HEP: L-*glycero*-D-*manno*-heptose

Prevalence in the environment

LPS is released from the outer membrane during multiplication, but especially after death and lysis of Gram-negative bacteria (Rietschel *et al.*, 1994). Gram-negative bacteria are ubiquitous in the environment and continuously release LPS. The amount of airborne LPS depends mainly on the amount and source of organic dust in the air (Rylander, 2002). Especially dust derived from faeces, bacteria-contaminated plants such as grain or cotton, and organic waste contains high loads of LPS (Donham, 1991; Wouters *et al.*, 2000; Radon *et al.*, 2002). Table 1 compares the concentrations of airborne LPS in different environments. Airborne LPS can reach exceedingly high

concentrations in animal confinement units. Concentrations increase dramatically when dust is agitated, for example during moving or feeding of pigs (Zhiping *et al.*, 1996; Rylander, 2002). In households, humidifiers with contaminated water appear to be an important source of airborne LPS (Mamolen *et al.*, 1993). LPS that is associated with the respirable fraction of dust (<5 μm) reaches the alveoli upon inhalation (Pearson and Sharples, 1995).

Table 1. Concentrations of airborne LPS in different environments (adapted from Rask-andersen *et al.*, 1989; Zhiping *et al.*, 1996; Hartung and Seedorf, 1999; Hasday *et al.*, 1999; Rylander, 2002).

Location	Source	LPS
agriculture	swine	8-4900 ng/m^3
	dairy cattle	25-50000 ng/m^3
	poultry	33-301 ng/m^3
	animal feed	0.2-1870 ng/m^3
	grain dust	286-721 ng/m^3
home	humidifier	130-390 ng/m^3
	dust	18-50 ng/m^3
other	cotton mill	2-314 ng/m^3
	saw mill	0-4000 ng/m^3
	brewery	60-927 ng/m^3
	waste sewage	1-32170 ng/m^3
	cigarette smoke	120 ng/cigarette
	outdoor air	0.19-0.49 ng/m^3

Effects on the lungs

1) exposure through the blood

LPS is the most potent inflammatory component of Gram-negative bacteria and is presumed to play a pivotal role in many inflammatory disorders, such as the “acute respiratory distress syndrome” (ARDS) in humans (Brigham and Meyrick, 1986; Bhatia and Moochhala, 2004). ARDS is characterized by acute lung injury and hypoxemia, and primarily occurs as a lethal complication of sepsis with Gram-negative bacteria, but can also be triggered by other processes, such as respiratory virus infections (Hammer *et al.*, 1997; Peiris *et al.*, 2003). The acute lung injury is

characterized by damage to endothelial cells, lung oedema and massive sequestration of neutrophils in the vascular, and subsequently in the interstitial and bronchoalveolar compartments of the lungs. Several researchers were able to reproduce the syndrome in pigs by injecting a high dose of LPS (100-250 μg/kg) in the blood (Cohn *et al.*, 1991; Lutz *et al.*, 1998; Carney *et al.*, 2001). LPS acts mainly indirectly by inducing harmful amounts of proinflammatory cytokines and reactive oxygen species, but can also directly cause vascular injury by triggering apoptosis of endothelial cells (reviewed by Bannerman and Goldblum, 2003).

2) exposure through the airways

LPS also affects the lungs after inhalation. There is convincing evidence that inhalation of LPS-containing dust causes airway disease in humans. Certain occupations that imply long-term exposure to organic dust, such as swine farmers, cotton workers and veterinarians are especially at risk (Donham, 1990; Melbostad *et al.*, 1997; Radon *et al.*, 2001). Swine farmers have an increased incidence of chronic bronchitis and suffer more frequently from symptoms such as excessive sputum production, coughing, wheezing and chest tightness. Inhalation of swine dust also causes general symptoms such as fever, malaise and headache (Larsson *et al.*, 1994). This is well illustrated by a case report of Jolie *et al.* (1998b) that describes a transient flu-like disease in veterinary students after visiting a swine farm. This flu-like disease is sometimes referred to as "organic dust toxic syndrome" or "toxic pneumonitis" (Von Essen *et al.*, 1990). A Dutch survey indicates that large animal practitioners experience 2 to 3 times more respiratory symptoms than their colleagues working in small animal practice (Tielen *et al.*, 1996). Also, Andersen *et al.* (2004) found a disturbing correlation between working hours in hog barns and decline of pulmonary function in veterinarians.

Swine dust causes airway inflammation characterized by infiltration of neutrophils and production of proinflammatory cytokines (Larsson *et al.*, 1994). Wang *et al.* (1997) studied the production of proinflammatory cytokines in the lungs of healthy subjects after a stay of 3 hours in a swine confinement building. All subjects developed a significant increase of TNF-α, IL-1 and IL-6 in their BAL fluids. The concentration of LPS in the building was 1.2 $\mu g/m^3$, which compares to concentrations reported on other farms (Jolie, 1998). Inhalation of LPS-containing

dust causes bronchial hyperresponsiveness and impairs lung function, characterized by a decline of the forced expiratory volume in one second (FEV1) (Malmberg and Larsson, 1993; Vogelzang *et al.*, 1998; Larsson *et al.,* 2001).

In recent years, more and more attention has come to the impact of LPS in household dust on allergic disease. Concern was raised by a publication of Michel *et al*. (1996) who found a positive correlation between indoor LPS levels and the severity of asthma in atopic subjects. Asthmatics appear to be particularly sensitive to inhaled endotoxin, and inhalation of relatively low amounts induces both immediate and sustained airflow obstruction (Michel *et al*., 1989). Paradoxically, other researchers postulate that exposure to LPS during childhood decreases the risk of developing atopic asthma later in life (Kuipers *et al*., 2003; Douwes *et al*., 2004). These researchers state that a relatively high level of exposure to LPS in early life may keep allergen sensitisation and asthma from developing by promoting a Th1-type immune response. Indeed, several studies suggest that early childhood exposure to animals, such as indoor pets or farm animals, is associated with a lower prevalence of asthma and hay fever (Johnson and Alford, 2002).

LPS is the most potent inflammatory component of organic dust and is assumed to account for most of the biological effects of dust upon inhalation (Schwartz *et al*., 1995; Zejda *et al*., 1994). Indeed, pretreatment with specific LPS antagonists abolishes most of the inflammatory capacity of grain dust upon inhalation (Jagielo *et al*., 1996 and 1998). Moreover, experimental exposure to an LPS aerosol mimics all the biological effects of inhalation of organic dust in humans. This has been thoroughly reviewed by Thorn (2001). Experimentally, LPS induces the typical neutrophilic airway inflammation, decline of lung function and flu-like symptoms as reported in field cases. The deterioration of lung function is due to LPS-induced bronchoconstriction, bronchial hyperreactivity and to a lesser extent to reduced alveolar-capillary diffusion (Michel, 2000). The threshold dose for inducing these symptoms (30-40 μg) is about 14 times higher than the dose normally encountered in swine confinement units (Thorn, 2001). The reason for this discrepancy is unclear, but could be due to a different bioavailability of nebulized LPS compared to LPS in respirable dust. It is more likely, however, that LPS acts in synergy with other inflammatory components of dust, such as lipoteichoic acid, peptidoglycan or β-1,3-glucan, to induce lung inflammation (Wray *et al*., 2001).

Although it is clear that exposure to LPS-contaminated swine dust contributes to respiratory disease in humans, no such evidence is available for pigs. Few researchers investigated the possible impact of airborne LPS on respiratory health in pigs and a clear link between both has not been established in the field (Donham, 1991; Jolie *et al.*, 1998a). This discrepancy with human literature could be due to a lack of field research, although some authors have suggested that pigs might be less sensitive to airborne LPS than humans (Jolie, 1998). The latter hypothesis was supported by an experiment of Urbain *et al.* (1999) in which pigs inhaled LPS-contaminated dust at a concentration normally found in swine buildings (2.5 $\mu g/m^3$) for 6 days. These pigs did not develop lung inflammation or clinical signs. Inhalation of higher doses of LPS (100 to 1000 μg/kg) caused infiltration of neutrophils, atelectasis and fever (Liggett *et al.*, 1986; Urbain *et al.*, 1996). Apparently, LPS-induced lung inflammation only occurs at inhalation doses that are markedly higher than normally encountered in the environment. Even at these doses, no overt respiratory signs were observed. In spite of the lack of respiratory signs, Urbain *et al.* (1996) demonstrated that bronchial rings, isolated from LPS-exposed pigs, were hyperreactive upon stimulation with histamine. Remarkably, similar doses of LPS (100 to 1000 μg/kg) cause acute respiratory distress and even death when injected in the blood of pigs (Carney, 2001; Urbain *et al.*, 1996). In our hands, an LPS dose of ≥5000 μg/kg is required to consistently induce substantial amounts of proinflammatory cytokines in the lungs of pigs upon intratracheal inoculation (Van Reeth *et al.*, 2000). For comparison, inhalation of 30 μg LPS (≈ 0.43 μg/kg) is already sufficient to induce detectable amounts of proinflammatory cytokines in the lungs of humans (Wesselius *et al.*, 1997).

1.2.2. The lipopolysaccharide receptor complex mediates biological activity

Introduction

Expressed by all Gram-negative bacteria, LPS serves as one of the primary targets of the innate immune system. Recognition of the presence of LPS by cells provides the host with a rapid detection of and reaction towards Gram-negative bacteria. Pattern-recognition receptors bind to conserved structures of microbial pathogens, such as LPS, and discriminate between self and non-self. During the past decade, enormous progress has been obtained in the elucidation of LPS recognition and signaling. According to the current model, recognition of LPS is initialized by the cooperative interplay between the "lipopolysaccharide-binding protein" (LBP), the membrane-bound or soluble forms of "cluster of differentiation 14" (CD14) and the recently identified "Toll-like receptor 4" (TLR4) (for review see Martin, 2000). Together, these proteins form the "LPS receptor complex" which is presented in figure 4. The role of the different components is distinct and can be summarized as follows:

1) LBP: binds free or cell wall-bound LPS and presents it to CD14
2) CD14: receives LPS from LBP and then associates with TLR4
3) TLR4: signals towards the nucleus leading to the release of proinflammatory cytokines and other mediators.

Each component will be discussed in detail below.

LBP, an acute phase protein with a dual role

LBP is a class 1 acute phase protein of 60 kilodalton (kDa), which was first identified in 1986 by Tobias and co-workers. LBP is constitutively present in the plasma. Plasma of healthy humans contains about 2-20 μg/ml and levels increase up to ten times during acute phase responses (Fenton and Golenbock, 1998). LBP in plasma is mainly produced by hepatocytes in the liver. Recently, it was shown that type 2 pneumocytes can also produce LBP after *in vitro* stimulation with the proinflammatory cytokines TNF-α, IL-1 or IL-6 (Dentener *et al.,* 2000).

LBP functions as a "lipid transfer molecule" that extracts single LPS molecules from LPS aggregates or directly from the bacterial outer membrane and transfers it to CD14 or lipoproteins (Wurfel *et al.*, 1994; Ulevitch *et al.*, 1999). LPS released from

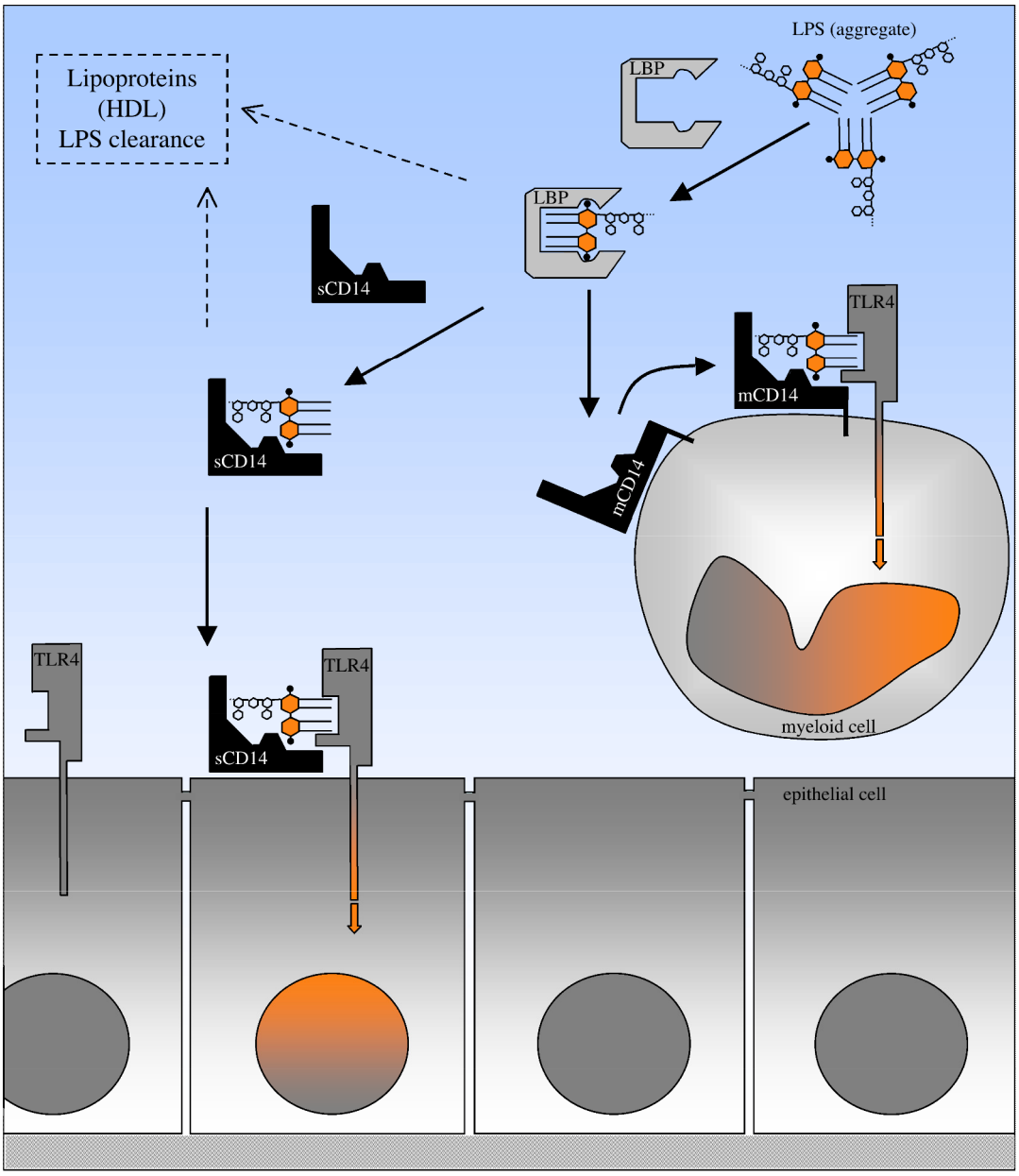

Figure 4. Overview of the different steps of LPS recognition (adapted from Martin, 2000). LPS-binding protein (LBP) extracts a single LPS molecule from an LPS aggregate and transfers it to soluble or membrane-bound CD14 (sCD14, mCD14). The CD14-LPS complex associates with Toll-like receptor 4 (TLR4), which is followed by intracellular signaling and activation of proinflammatory genes. mCD14 is expressed on myeloid cells, but not on epithelial cells. sCD14 can present LPS to TLR4 on epithelial cells and thus render these cells more sensitive to LPS. LBP and sCD14 can also transfer LPS to lipoproteins, such as high-density lipoproteins (HDL), which leads to neutralization and clearance of LPS.

Gram-negative bacteria forms micelle-like aggregates and spontaneous diffusion of single LPS molecules from these aggregates to CD14 occurs at a very low rate. This process is strongly accelerated in the presence of LBP. This way, LBP enhances the proinflammatory effects of LPS 100- to 1000-fold (Hailman *et al.*, 1994). For

example, mice deficient in LBP do not mount an inflammatory response to small amounts of LPS and, as a consequence, are more susceptible to colonization with Gram-negative bacteria (Jack *et al.*, 1997; Le Roy *et al.*, 2001).

A second role of LBP is to transfer LPS to plasma lipoproteins and chylomicrons, leading to neutralization and clearance (Wurfel *et al.*, 1994; Vreugdenhil *et al.*, 2003; Hamann *et al.*, 2005). A dynamic equilibrium is established during endotoxemia, in which LPS is both shuttled to CD14, resulting in cellular activation, and to lipoproteins, resulting in neutralization. The second pathway becomes predominant at high concentrations of LBP, when LBP is more abundant than CD14. This way, high concentrations of LBP can reduce the proinflammatory activity of LPS. Some researchers suggest that low LBP concentrations, which are found during the onset of infection, favour binding to CD14 and subsequent inflammation, whereas high concentrations of LBP, which are mounted in the serum during the acute phase response, favour binding to lipoproteins and reduce the proinflammatory effect of LPS (Gioannini *et al.*, 2003). Indeed, high concentrations of LBP in serum of patients with sepsis can inhibit LPS activation of monocytes (Zweigner *et al.*, 2001). Recently, it becomes more and more evident that LBP also mediates recognition of other bacterial compounds, such as lipoteichoic acid, peptidoglycan and acylated lipopeptides (Schroder *et al.*, 2004).

Less is known about the occurrence and role of LBP in the lungs. It was shown that people suffering from asthma or acute respiratory distress syndrome have elevated levels of LBP in their BAL fluids (Martin *et al.*, 1992; Dubin *et al.*, 1996; Martin *et al.*, 1997; Strohmeier *et al.*, 2001). Most studies in the lungs indicate that LBP enhances the proinflammatory effect of LPS. In rabbits, for example, it was shown that LBP significantly increased TNF-α production and neutrophil infiltration after intratracheal inoculation of a low dose of LPS (Ishii *et al.*, 1993). Intratracheal instillation of a mixture of LPS and LBP markedly induced TNF-α in BAL fluids, whereas LPS alone did not.

Few data are available on LBP in pigs. One study quantified LBP in serum of pigs throughout an experimental infection with *Chlamydia suis* (Sachse *et al.*, 2004). LBP was present in the serum of uninfected pigs in concentrations ranging from 0.5 to 6.5 μg/ml and concentrations increased 3 to 4-fold during infection.

CD14, a receptor for different pathogen-associated molecules

CD14 is a 53 kDa glycoprotein expressed on the surface of myeloid cells (Antal-Szalmas, 2000). CD14 is embedded in the cell membrane via a glycosylphosphatidylinositol (GPI) anchor and has no transmembrane or cytoplasmatic domains. The extracellular domain contains repeated leucine-rich motifs, a characteristic shared with other pattern recognition receptors such as Toll-like receptors. The number of CD14 molecules on the membrane varies according to type, maturation and localization of the cell. Neutrophils generally express 33 times less CD14 on their membranes than monocytes (Antal-Szalmas *et al.*, 1997). In pigs, two subsets of monocytes with respectively high and low CD14 expression have been identified (Chamorro *et al.*, 2000). Presumably, CD14-low monocytes present a more mature "macrophage-like" phenotype. CD14 expression on macrophages depends highly on the localization in the body. Peritoneal and pleural macrophages express high amounts of CD14, whereas alveolar and intestinal macrophages express low to undetectable amounts of CD14 (Hasday *et al.*, 1997; Antal-Szalmas, 2000; Smith *et al.*, 2001).

In 1990, Wright *et al.* were the first to show that the myeloid differentiation marker CD14 functions as an LPS receptor. CD14 binds with high affinity to LPS and this complex shuttles to TLR4 (Jiang *et al.*, 2000). Although unable to activate the cell on its own, CD14 is necessary for recognition of low (clinically relevant) amounts of LPS. Numerous studies in different species have demonstrated that impairment of CD14 function, by neutralization with antibodies or use of knockout animals, suppresses LPS-induced cytokine production, respiratory disease and shock (Ishii *et al.*, 1993; Haziot *et al.*, 1996; Leturcq *et al.*, 1996; Schimke *et al.*, 1998; Tasaka *et al.*, 2003). Treatment with anti-CD14 strongly reduces acute inflammation after intratracheal LPS exposure, but at the same time renders the lungs more susceptible to colonization with Gram-negative bacteria (Frevert *et al.*, 2000).

CD14 also exists in a soluble form, resulting either from the shedding of membrane-bound CD14 or from the production of GPI-free CD14 molecules (Bazil *et al.*, 1989). Like LBP, soluble CD14 is constitutively present in the serum (2-6 μg/ml) and concentrations rise during acute phase responses (Bazil and Strominger, 1991; Bas *et al.*, 2004). Increased levels of soluble CD14 were also found in BAL fluids of

humans suffering from "acute respiratory distress syndrome" and asthma (Dubin *et al.*, 1996; Martin *et al.*, 1997).

Soluble CD14 can accept LPS from LPS/LBP complexes and facilitate LPS-dependent activation of CD14-negative cells, such as epithelial and endothelial cells (Pugin *et al.*, 1993). Indeed, Alexis *et al.* (2000) found a tight correlation between soluble CD14 levels in the BAL fluid and the inflammatory response to inhaled LPS. In contrast, soluble CD14 can also participate with LBP in the transfer of LPS to plasma lipoproteins, thereby inactivating LPS (Wurfel *et al.*, 1995).

CD14 was originally defined as a specific receptor for LPS, but there is increasing evidence that CD14 functions as a receptor for components of various other pathogens. Table 2 presents an overview of ligands shown to bind to CD14.

Table 2. Ligands of different pathogens that bind to CD14.

Origin	Ligand	Reference
Gram-negative bacteria	LPS	Wright *et al.*, 1990
Gram-positive bacteria	lipoteichoic acid	Cleveland *et al.*, 1996
	peptidoglycan	Dziarski *et al.*, 1998
mycobacteria	lipoarabinomannan	Savedra *et al.*, 1996
spirochaetes	lipoproteins	Sellati *et al.*, 1998
fungi	mannan	Tada *et al.*, 2002
viruses	surface antigen of hepatitis B virus	Vanlandschoot *et al.*, 2002
arthropods	chitosans	Otterlei *et al.*, 1994

CD14 recognizes various sugar or glycolipid motifs, acting as a lectin-like receptor. Intriguingly, a recent study found that CD14 also mediates influenza virus-induced cytokine production (Pauligk *et al.*, 2004). A prevailing concept of innate immunity is that CD14 provides a first line, low specificity screening of different microbial ligands, which is followed by a very specific, but less sensitive binding to a second line of pattern recognition receptors (Antal-Szalmas, 2000). The latter were recently identified as Toll-like receptors.

TLR4, the crucial link for LPS signaling

TLR4 is a 100 kDa transmembrane protein characterized by an extracellular leucine-rich domain and an intracellular signaling domain, called the ''Toll domain''

(Rock *et al.*, 1998). TLR4 is related to the Toll protein of the fruit fly (*Drosophila melanogaster*), a protein involved in embryonic development and antifungal defence. Ten human Toll-like receptors (TLR1 to 10) have been identified so far (Takeda *et al.*, 2003). All these are related to the Toll protein of the fruit fly and possess the same intracellular Toll domain. This conserved signaling domain is part of an evolutionary ancient immune response of both insects and vertebrates. The Toll domain is also highly homologous to the signaling domain of the IL-1 receptor. By consequence, LPS signaling operates in a manner that is very close to IL-1 signaling. IL-1 can thus be considered as a cytokine that mimics the effects of LPS.

The group of Bruce Beutler was the first to identify TLR4 as an LPS signaling receptor, a long-time missing link of LPS recognition (Poltorak *et al.*, 1998). TLR4 physically associates with the CD14-LPS complex, which is followed by intracellular signaling and eventually by the production of numerous mediators, such as the proinflammatory cytokines TNF-α and IL-1 (Jiang *et al.*, 2000). More and more researchers argue that the interaction between LPS and TLR4 also requires the presence of "myeloid differentiation protein-2" (MD-2), a secreted protein that is associated with the extracellular part of TLR4 (Gioannini *et al.*, 2004; Miyake, 2004). Although TLR4 can induce a response against high concentrations of LPS (μg to mg/ml), the concerted action of all three receptor components is necessary to engage an inflammatory response against minute amounts of LPS (pg/ml) (Muta and Takeshige, 2001). Recently, it was shown that polymorphisms in the TLR4 gene cause variation in the LPS sensitivity of humans. A small percentage of people is naturally hyporesponsive to LPS and this is associated with specific mutations in the TLR4 gene (Arbour *et al.*, 2000).

TLR4 is expressed on a wide variety of cells, including monocytes, macrophages, epithelial and endothelial cells. One recent study describes the distribution of TLR4 in different organs of pigs using polyclonal antibodies against murine TLR4 (Wassef *et al.*, 2004). In the lungs of pigs, alveolar and intravascular macrophages, monocytes, bronchiolar epithelium and endothelium of large blood vessels stained positive for TLR4.

The remaining members of the TLR family recognize conserved molecules of a wide variety of other pathogens (reviewed by Takeda *et al.*, 2003). TLR2, for example, recognizes peptidoglycan and lipoteichoic acid of Gram-positive bacteria.

Remarkably, some members also recognize RNA and proteins of viral origin. TLR3, for example, recognizes double stranded RNA, which is produced during replication of many viruses (Alexopoulou *et al.*, 2001). Furthermore, it was shown that TLR4 plays a crucial role in the innate immune response against the respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000; Haynes *et al.*, 2001). The fusion protein of RSV binds to TLR4 on monocytes and thus triggers the production of cytokines. Moreover, the clearance of RSV from the lungs is significantly impaired in TLR4-deficient mice.

1.3. INTERACTIONS BETWEEN VIRUSES AND BACTERIAL LIPOPOLYSACCHARIDE IN THE LUNGS

Introduction

Interactions between viruses and bacteria in the induction of severe respiratory disease have been described since the early thirties (Shope, 1931). However, little remains known about the mechanisms whereby respiratory viruses can predispose for disease by secondary agents. This also holds true for PRRSV and PRCV, two viruses which are believed to play a role in the porcine respiratory disease complex (Thacker, 2001; Brockmeier *et al.*, 2002). It is our working hypothesis that virus-induced respiratory signs are caused for an important part by overproduction of inflammatory mediators. Indeed, swine influenza virus induces high levels of the proinflammatory cytokines TNF-α, IL-1 and IL-6 in the lungs and these levels are correlated tightly with the appearance of clinical signs (Van Reeth *et al.*, 1998 and 2002a). PRRSV and PRCV, in contrast, fail to simultaneously induce substantial levels of all three cytokines in the lungs and uncomplicated infections cause no overt respiratory disease (Van Reeth *et al.*, 1999 and 2002b). In an attempt to study interactions between respiratory viruses and secondary agents in a reproducible way, we have performed subsequent inoculations of pigs with either PRRSV or PRCV, followed by a secondary inoculation with LPS. Besides the fact that LPS is the most important inflammatory component of Gram-negative bacteria (Rietschel *et al.*, 1994), there were three additional reasons to choose this agent.

Firstly, lungs of pigs are continuously exposed to LPS under farm conditions, as LPS is present in stable dust in concentrations up to several microgrammes/m^3 air (Rask-Andersen *et al.*, 1989; Zhiping *et al.*, 1996). Also, LPS is released at high concentrations in the lungs during pulmonary infections with Gram-negative bacteria (Pugin *et al.*, 1992). Treatment with antibiotics, especially those belonging to the β-lactam family, can induce a sudden and massive release of LPS from the bacterial cell wall (Periti and Mazzei, 1999).

Secondly, standardized LPS preparations are commercially available and the total amount of exposure in the lungs can easily be controlled, which is not the case for replicating agents, such as viruses and bacteria. Combined inoculations with virus and

LPS, therefore, may avoid the variability resulting from interference of a first virus with replication of a second virus or bacterium.

Thirdly, LPS is a potent inducer of proinflammatory cytokines and exerts many of its biological effects through cytokines (Rietschel *et al.*, 1994). The effects of LPS are dose-related, and relatively high doses are required to induce substantial cytokine production and decreased lung function upon intratracheal inoculation. Low LPS doses, on the other hand, induce only minute amounts of cytokines and no obvious disease.

This chapter contains a summary of the experiments that were performed in our laboratory. An overview hereof is presented in figure 5. In addition, we discuss briefly some studies of other researchers who also examined virus-LPS interactions.

The combination of PRCV and LPS in the lungs of pigs

Studies in our laboratory demonstrated that PRCV sensitizes the lungs to LPS at the very early stage of infection (Van Reeth *et al.*, 2000). Gnotobiotic pigs were inoculated intratracheally with PRCV and 24 hours later with LPS from *E. coli* (20 μg/kg). The effects of separate virus or LPS inoculations were subclinical and failed to induce high or sustained cytokine levels in the lungs. The combination of both agents, on the contrary, resulted in marked laboured breathing, dullness and loss of appetite during the first 12 hours after the LPS inoculation. Prior infection with PRCV truly potentiated the cytokine response to LPS, with 10 to 100 times higher titers of TNF-α, IL-1 and IL-6 in the BAL fluids than after inoculation with each agent alone. Further experiments demonstrated that the PRCV-LPS synergy depends on the interval between PRCV and LPS inoculations. The typical clinical signs, together with high cytokine titers, were seen with an interval of 12 to 24 hours between virus and LPS, but not with shorter intervals. The titers of TNF-α and IL-6, but not of IL-1, were tightly correlated with the clinical signs. An important question that remains is whether PRCV also sensitizes the lungs to LPS at later stages of infection (>24 hours).

Unexpectedly, there was no clear synergistic effect between PRCV and LPS with respect to lung pathological changes. Neutrophil infiltration, macroscopical and microscopical lesions in the lungs of PRCV-LPS inoculated pigs resembled the combined effects of each agent alone without synergy. Also, there was little correlation between these pathological features and disease or cytokines. These

observations were surprising, because a direct relationship between neutrophil sequestration in the lungs and respiratory disorders has been demonstrated in many other studies (Puneet *et al.*, 2005). Activation of neutrophils in the lungs is a key event in the generation of pulmonary injury in ARDS (Aldridge, 2002). Indeed, depletion of neutrophils prevents pulmonary endothelial injury upon intravenous LPS inoculation (Hefling and Brigham, 1981). One attractive hypothesis is that not structural lung damage, but functional lung disturbances, such as bronchoconstriction, are responsible for the difficult breathing after virus-LPS exposure. Interestingly, the combination of TNF-α and IL-1 causes bronchoconstriction through the induction of thromboxanes in rats (Martin *et al.*, 2001).

The combination of PRRSV and LPS in the lungs of pigs

PRRSV is considered to be an important cause of multifactorial disease in pigs (Thacker, 2001; Brockmeier *et al.*, 2002). PRRSV infection in the lungs lasts approximately 5 to 7 times longer than PRCV infection. The remarkably long duration of PRRSV infection increases the chance that the lungs become exposed to other agents, such as LPS, during the ongoing virus replication. The clinical effects of the PRRSV-LPS combination were studied in conventional pigs of 5 to 10 weeks old (Labarque *et al.*, 2002). We used a European strain of PRRSV (Lelystad virus) and LPS from *E. coli* (20 μg/kg). Pigs were inoculated intratracheally with PRRSV, followed by LPS 3, 5, 6 or 9 days later. Pigs inoculated with PRRSV or LPS alone were included as controls.

Exposure of pigs to PRRSV or LPS alone resulted in transient fever (40-40.9°C), but respiratory symptoms were minimal or absent (<45 breaths per minute). In contrast, exposure of PRRSV-infected pigs to LPS resulted in severe respiratory disease, characterized by tachypnoea (45 to 154 breaths per minute), abdominal breathing and dyspnoea in 87% of the pigs. These pigs also showed enhanced general symptoms, such as high fever (≥41.0°C) and depression. Clinical signs started within 1 hour after LPS, reached a climax 2 to 4 hours later and disappeared between 12 and 24 hours after the LPS inoculation.

There is thus a strong synergy between PRRSV and LPS in the induction of respiratory disease. The pathogenesis of this disease has not been studied yet. The clinical signs of the PRRSV-LPS combination are remarkably similar to those of the

PRCV-LPS combination. So, it is tempting to speculate that overproduction of proinflammatory cytokines is also involved in this disease. It is our working hypothesis that both PRRSV and PRCV prime the lungs for enhanced production of

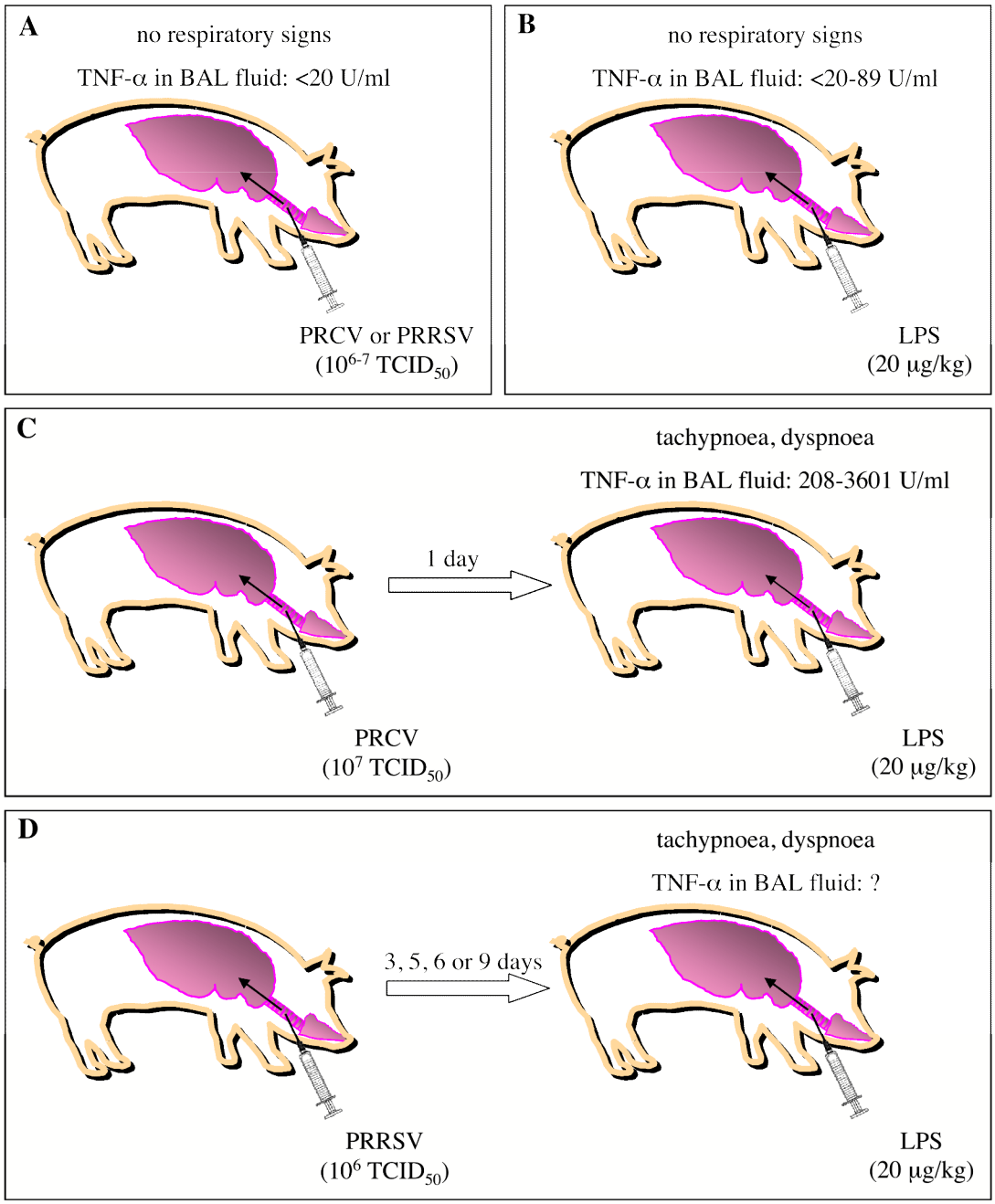

Figure 5. Overview of the virus-LPS experiments performed at the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University (Van Reeth *et al.*, 2000; Labarque *et al.*, 2002). Control pigs were inoculated exclusively with PRCV, PRRSV (A) or bacterial LPS (B). Other pigs were inoculated with virus followed one or several days later by inoculation with LPS (C and D). The virus-LPS combination induced acute respiratory signs, which were not seen after inoculation with virus or LPS alone. Moreover, the PRCV-LPS combination induced massive amounts of the proinflammatory cytokine TNF-α in the lungs. This has not been examined for the PRRSV-LPS combination.

proinflammatory cytokines in response to minute amounts of LPS and thereby initiate clinical disease. Possibly, both viruses sensitize the lungs to LPS in a similar way by increasing different components of the LPS receptor complex, such as LBP and CD14, during infection. These issues will be addressed further in the research presented in this thesis.

Other studies on virus-LPS interactions

To our knowledge, PRRSV and PRCV are the only respiratory viruses that have been shown to synergize with LPS in the induction of respiratory disease. Recently, it has been described that systemic infection of mice with lymphocytic choriomeningitis virus or vesicular stomatitis virus leads to fatal shock upon intraperitoneal inoculation with a sublethal dose of LPS (Nguyen and Biron, 1999; Nansen and Thomsen, 2001). The shock syndrome appeared to result from an overproduction of TNF-α in the blood. Studies with IFN knockout mice indicated that virus-induced interferons, both IFN-α/β and -γ, are responsible for the increased systemic sensitivity to LPS (Doughty *et al.*, 2001; Nansen and Thomsen, 2001).

In vitro studies on virus-LPS interactions have yielded conflicting results. Recently, it was shown that *in vitro* infection of airway epithelial cells with RSV up-regulates TLR4, which in turn leads to an increased LPS response (Monick *et al.*, 2003). It is unknown whether a synergy between RSV and LPS also occurs *in vivo* in the lungs. In 1990, Nain *et al.* demonstrated that influenza virus infection of macrophages leads to transcription and accumulation of TNF-α messenger RNA (mRNA) in the cytoplasm. Subsequent LPS stimulation leads to massive translation of this mRNA and release of TNF-α in the medium. In contrast, many other studies reported a decrease of the LPS response of virus-infected macrophages. This was the case for bovine herpesvirus-1, bovine RSV, African swine fever virus and many other viruses (Tsai *et al.*, 1991; Bienhoff *et al.*, 1992; Whittall and parkhouse, 1997). We (unpublished data) and others (Chiou *et al.*, 2000; Lopez-Fuertes *et al.*, 2000) found that PRRSV-infected macrophages become less responsive to LPS or phorbol myristate acetate, characterized by a decrease of TNF-α production or oxidative burst. This is consistent with the fact that PRRSV infection of macrophages results in cell death within 24 to 48 hours after inoculation (Paton *et al.*, 1992; Suaréz *et al.*, 1996; Oleksiewicz and Nielsen, 1999). Therefore, it is likely that PRRSV-infected

macrophages are not directly responsible for the increased LPS response in the lungs. We hypothesize that this is rather an indirect effect of the PRRSV infection and results from sensitization of uninfected lung cells and/or infiltration of new LPS-responsive cells.

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2.

AIMS OF THE THESIS

Aims of the thesis

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine respiratory coronavirus (PRCV) synergize with bacterial lipopolysaccharide (LPS) in the induction of respiratory disease signs. This has been demonstrated during the very early stage of the PRCV infection (from 12 to 24 hours after inoculation) and during a major part of the PRRSV infection (from 3 to 9 days after inoculation). The main aims of this thesis were to scrutinize the pathogenesis of virus-LPS induced respiratory disease, with special emphasis on the role of proinflammatory cytokines as mediators of virus-LPS disease, and to explore possible mechanism(s) of virus-induced sensitisation to LPS. This was studied for PRRSV and for PRCV, two respiratory viruses of swine with different cellular tropism.

The specific aims of this thesis were:

1) to study the pathogenesis of the PRRSV-LPS induced respiratory disease on a cellular and cytokine level. More specifically, we wanted to examine whether PRRSV, like PRCV, synergizes with LPS in the induction of proinflammatory cytokines in the lungs and whether cytokines are correlated with the appearance of respiratory signs

2) to confirm the role of proinflammatory cytokines, especially tumour necrosis factor-α (TNF-α), in the induction of respiratory signs. Hereto, we compared the effects of different drugs, including a cytokine inhibitor and two prostaglandin inhibitors, on PRRSV-LPS induced clinical signs

3) to examine whether PRRSV infection induces an increase of the LPS recognition proteins lipopolysaccharide-binding protein (LBP) and CD14 in the lungs, as a potential mechanism of increased LPS sensitivity

4) to examine whether PRCV infection induces an increase of LBP and CD14 in the lungs and verify whether this is associated with an increased LPS sensitivity

3.

PATHOGENESIS OF THE RESPIRATORY DISEASE CAUSED BY THE COMBINATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND BACTERIAL LIPOPOLYSACCHARIDE

3.1

INTERACTION BETWEEN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND BACTERIAL LIPOPOLYSACCHARIDE IN THE LUNGS OF PIGS: POTENTIATION OF CYTOKINE PRODUCTION AND RESPIRATORY DISEASE

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is a key agent in multifactorial respiratory disease of swine. Intratracheal administration of bacterial lipopolysaccharide (LPS) to PRRSV-infected pigs results in markedly enhanced respiratory disease, whereas the inoculation of each agent alone results in largely subclinical disease. This study examines whether PRRSV-LPS induced respiratory disease is associated with the excessive production of proinflammatory cytokines in the lungs. Gnotobiotic pigs were inoculated intratracheally with PRRSV followed by LPS at 3, 5, 7, 10 or 14 days of infection and euthanized 6 hours after the LPS inoculation. Controls were inoculated with PRRSV or LPS only or with phosphate-buffered saline. Virus titers, (histo)pathological changes in the lungs, numbers of inflammatory cells, and bioactive tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 levels in bronchoalveolar lavage fluids were examined. All pigs inoculated with PRRSV-LPS developed severe respiratory disease, whereas the controls that were inoculated with PRRSV or LPS alone did not. PRRSV infection significantly enhanced cytokine production in response to LPS. Peak TNF-α, IL-1 and IL-6 titers were 10 to 100 times higher in the PRRSV-LPS inoculated pigs than in the pigs inoculated with PRRSV or LPS alone and the titers correlated with the respiratory signs. The levels of neutrophil infiltration and the pathological changes detected in the lungs of PRRSV-LPS inoculated pigs resembled the combined effects of the single PRRSV and LPS inoculations with no synergistic interaction between both agents. These data demonstrate a synergy between PRRSV and LPS in the induction of proinflammatory cytokines and an association between induction of these cytokines and disease.

Introduction

European strains of porcine reproductive and respiratory syndrome virus (PRRSV) fail to cause respiratory disease as such. Nevertheless, PRRSV is considered one of the most important etiological agents in multifactorial respiratory disease of swine, both in Europe and in the United States (Thacker, 2001). Few studies, however, have been able to reproduce clinical respiratory disease by experimental inoculation with PRRSV followed by a secondary virus or bacterium (Galina *et al.*, 1994; Van Reeth *et al.*, 1996; Thacker *et al.*, 1999; Brockmeier *et al.*, 2000). Variation in the severity of clinical signs and lack of reproducibility are the main problems with this type of studies. Even a single experimental infection with respiratory viruses results in intrinsic variation in virological, inflammatory and clinical parameters. Therefore, a second infection may enhance this variation, as the outcome of the second infection is in part dependent on that of the first infection.

We have previously developed an alternative dual inoculation model consisting of a primary inoculation with PRRSV followed by inoculation with a non-replicating agent, namely lipopolysaccharide (LPS) from *Escherichia coli* (Labarque *et al.*, 2002). LPS is a major component of the outer membrane and the main endotoxin of Gram-negative bacteria. Intratracheal administration of LPS (20 μg/kg body weight) to PRRSV-infected pigs resulted in severe respiratory disease, characterized by tachypnoea, abdominal breathing, dyspnoea and high fever. In contrast, the single PRRSV or LPS inoculations resulted in subclinical or mild disease. This model proved to be reproducible, in contrast to the classic dual infection models consisting of inoculation with PRRSV followed by inoculation with a second replicating agent. In addition, we believe that the PRRSV-LPS combination has practical relevance. Most pigs become infected with PRRSV between 4 and 16 weeks of age and the virus persists in the lungs for up to 40 days after inoculation (Labarque *et al.*, 2000; Mateusen *et al.*, 2002). Also, most pigs are exposed to LPS under farm conditions, as LPS is present in stable dust at concentrations ranging up to 4.9 $\mu g/m^3$. Furthermore, LPS is released at high concentrations in the lungs during pulmonary infections with Gram-negative bacteria (Pugin *et al.*, 1992; Zhiping *et al.*, 1996).

The proinflammatory cytokines interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are important mediators of several respiratory

diseases. IL-1 and TNF-α are among the first cytokines that are produced in the lungs during an infection. They cause infiltration and activation of leukocytes in the lungs, increased microvascular permeability and pulmonary dysfunctions (Ulich *et al.*, 1991; Bielefeldt-Ohmann, 1995; Wang *et al.*, 1997). IL-1 and TNF-α also induce a cascade of secondary cytokines, such as IL-6. IL-6 is a potent inducer of acute-phase proteins in the liver (Murtaugh *et al.*, 1996). Although IL-6 is generally considered a proinflammatory cytokine, it also has some anti-inflammatory properties (Tilg *et al.*, 1994). IL-6 can down-regulate the production of IL-1 and TNF-α and suppress their activity by inducing IL-1 receptor antagonists and soluble TNF-α receptors. Furthermore, the production of each of the three cytokines in the lungs has been associated with general signs of disease such as fever, depression and anorexia.

The present study was undertaken to test the hypothesis that PRRSV-LPS induced respiratory disease is associated with the excessive production of proinflammatory cytokines in the lungs. Therefore, we compared the production of IL-1, TNF-α and IL-6 in the lungs of pigs after dual inoculation with PRRSV and LPS with that after the inoculation with each agent alone. Correlations between cytokine levels and respiratory signs, macroscopic and microscopic lung pathology, and the infiltration of inflammatory cells in the bronchoalveolar spaces were examined.

Materials and methods

Virus and LPS preparations

PRRSV (Lelystad strain) (Wensvoort *et al.*, 1991) was used in the present study. The virus used for inoculation was at the fifth passage in alveolar macrophages, which had been obtained from 4- to 6-week-old gnotobiotic pigs. The inoculation dose was 10^6 tissue culture infective doses ($TCID_{50}$)/pig.

LPS of a non-enteropathogenic strain of *Escherichia coli* (serotype 0111:B4, trichloroacetic acid extraction, 90% purity) was obtained from Difco Laboratories (Detroit, USA) and was used at a dose of 20 μg/kg body weight. This dose was based on data from earlier experiments and was selected because it caused no clinical disease and minimal IL-1 and TNF-α production in the lungs (Van Reeth *et al.*, 2000). Virus and LPS were diluted in sterile pyrogen-free phosphate-buffered saline (PBS; Gibco, Merelbeke, Belgium) to obtain a 3 ml inoculum.

Pigs, experimental design and sampling

Thirty-eight caesarean-derived colostrum-deprived pigs at the age of 4 weeks were used. They were housed in individual Horsefall-type isolation units with positive pressure ventilation and fed with commercial ultrahigh-temperature-treated cow's milk. All inoculations were performed intratracheally with a 20-gauge needle that was inserted through the skin cranial to the sternum.

The pigs were allocated to 4 groups (table 1). Fourteen pigs were inoculated with PRRSV and 3 (n = 2), 5 (n = 3), 7 (n = 6), 10 (n = 2) or 14 (n = 1) days later with LPS (PRRSV-LPS group). These pigs were euthanized at 6 hours after the LPS inoculation. This time point was chosen because previous virus-LPS experiments showed that cytokine production peaks at 3 to 8 hours after the LPS inoculation and declines afterwards (Van Reeth *et al.*, 2000). Fourteen pigs were inoculated exclusively with PRRSV and euthanized at 3 (n = 3), 5 (n = 3), 7 (n = 3), 10 (n = 4) or 14 (n = 1) days after inoculation (PRRSV control group). Five pigs were inoculated exclusively with LPS and euthanized 6 hours later (LPS control group). Five pigs were mock inoculated with PBS and euthanized 6 hours later (PBS control group). All pigs were clinically monitored until euthanasia.

Samples from the left lung were collected for virological, histopathological and standard bacteriological examinations. The right lung was used for lung lavage by an earlier described method (Van Reeth *et al.*, 1998). Recovered BAL fluids were separated into cells and cell-free fluids by centrifugation (400 × *g*, 10 min, 4°C). For four of the six pigs that were inoculated with PRRSV and 7 days later with LPS, both the left and right lungs were used for lung lavage.

Clinical and pathological examinations

Pigs were monitored for clinical signs daily throughout the experiment and every hour after the LPS inoculation. At the moment of euthanasia a respiratory disease score was attributed to each pig. Scores ranged from 0 to 4: 0 = normal; 1 = tachypnoea when stressed; 2 = tachypnoea at rest; 3 = tachypnoea and dyspnoea at rest; 4 = severe tachypnoea and dyspnoea with laboured, jerky breathing.

Macroscopic lung lesions were evaluated by visual inspection. For histopathological examination, samples of the cardiac and diaphragmatic lung lobes

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

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

Cytokine bioassays

Cell-free BAL fluids were concentrated 20 times by dialysis against a 20% w/v solution of polyethylene glycol (MW 20000) and cleared of residual virus by centrifugation at 100000 × *g* before analysis in cytokine bioassays. IL-1, IL-6 and TNF-α bioassays have been described in detail elsewhere (Helle *et al.*, 1988; Van Reeth *et al.*, 1999).

IL-1 was assayed by its capacity to stimulate proliferation of D10(N4)M cells in the presence of concanavalin A (Grade IV, Sigma, Bornem, Belgium) and recombinant human interleukin-2 (Genzyme, Cambridge, MA, USA). The percentage of proliferation was determined by the thiazolyl blue (MTT) conversion procedure and optical densities were measured. The number of biological units/ml of BAL fluid was determined as the dilution that produced 50% maximal proliferation. To confirm specificity of the bioassay, D10 cells were incubated with monoclonal rat anti-mouse IL-1 receptor type 1 antibodies (Genzyme).

TNF-α activity was measured in a cytotoxicity assay with PK(15) subclone 15 cells (a gift from G. Bertoni, Bern, Switzerland) in the presence of actinomycin D. The plates were stained with crystal violet and read spectrophotometrically. The number of biological units/ml of BAL fluid was defined as the dilution that produced 50% cytotoxicity. Specificity was demonstrated by neutralization of samples with rabbit anti-human TNF-α antibodies (Innogenetics, Zwijnaarde, Belgium).

IL-6 was assayed by its capacity to stimulate proliferation of B9 cells (a gift from L. A. Aarden, Amsterdam, the Netherlands). The percentage proliferation was determined by the thiazolyl blue (MTT) conversion procedure and optical densities were measured. The number of biological units/ml of BAL fluid was determined as the dilution that produced 50% maximal proliferation. To confirm specificity of the bioassay, samples were neutralized with goat anti-porcine IL-6 antibodies (R&D systems, Abingdon, UK).

Bioassays were done with two-fold dilutions of samples in 96-well microtitration plates. Laboratory standards were run in each bioassay. Samples were tested in two or three individual bioassays and geometric means were calculated.

Virological and bacteriological examinations

Tissue samples from the diaphragmatic lobe of the left lung were used for virological and bacteriological examinations. PRRSV titrations were performed on alveolar macrophages using standard methods (Wensvoort *et al.*, 1991). For bacteriology, samples of lung tissue were plated on bovine blood agar and cultured aerobically. A nurse colony of coagulase-positive *Staphylococcus* species was streaked diagonally on each plate. Plates were inspected for bacterial growth after 48 and 72h. Colonies were then identified by standard techniques.

Statistical analysis

Standard two-sample Mann-Whitney tests were used to compare respiratory disease scores and cytokine titers. Correlation coefficients were calculated using the Spearman rank correlation test. P-values <0.05 were considered significant. Statistical analyses were performed using SPSS 6.1.

Results

The lungs of all pigs were free of bacteria by culture. PRRSV titers are presented in table 1. PRRSV was isolated from the lungs of all virus-inoculated pigs, but not from pigs inoculated with LPS or PBS only. There were no differences in virus titers between the pigs inoculated with PRRSV and LPS combined and the pigs inoculated with PRRSV alone, or at the different time points after inoculation with PRRSV.

Clinical signs

Mean respiratory scores are presented in table 1. Pigs that received PBS or LPS only remained asymptomatic. Pigs inoculated with PRRSV only showed no respiratory signs at any day after inoculation. They showed mild anorexia and dullness between 3 and 5 days after inoculation.

In contrast, all PRRSV-LPS inoculated pigs developed marked respiratory signs. All pigs were clinically normal before the LPS inoculation, but developed tachypnoea, dyspnoea with laboured, abdominal breathing and depression within 1 to 2 hours after the LPS inoculation. These signs were still present at the time of euthanasia. There were no differences in disease severity among the pigs inoculated with LPS at 3, 5, 7, 10 or 14 days after inoculation with PRRSV. Respiratory disease scores were significantly ($P < 0.05$) higher for the PRRSV-LPS group than for any other group.

Table 1. Respiratory scores, virus titers and numbers of inflammatory cells in BAL fluids.

Inoculation with	n	Euthanasia after		Mean resp. score[1] ± SD	Mean virus titer ± SD (log_{10} $TCID_{50}$[2]/g)	Mean no. of BAL cells ± SD ($\times 10^6$)	
		PRRSV (days)	LPS (hours)			neutrophils	mononuclear cells
PBS	5	-[3]	-	0 ± 0	negative	2 ± 2	115 ± 52
PRRSV	3	3d	-	0 ± 0	4.4 ± 1.1	5 ± 8	154 ± 115
	3	5d	-	0 ± 0	5.4 ± 1.3	4 ± 3	117 ± 42
	3	7d	-	0 ± 0	6.0 ± 0.6	7 ± 2	216 ± 71
	4	10d	-	0 ± 0	5.7 ± 0.9	38 ± 38	409 ± 219
	1	14d	-	0	6.0	3	337
LPS	5	-	6h	0 ± 0	negative	303 ± 105	233 ± 60
PRRSV-LPS	2	3d	6h	2 ± 1.4	6.0 ± 0	18 ± 23	121 ± 22
	3	5d	6h	3 ± 0	5.1 ± 2.2	296 ± 163	208 ± 14
	6	7d	6h	3.2 ± 0.8	5.5 ± 0.3	320 ± 263	275 ± 144
	2	10d	6h	2.5 ± 0.7	5.9 ± 1.3	380 ± 474	486 ± 142
	1	14d	6h	3	5.7	576	483

(1) respiratory scores were determined immediately before euthanasia and ranged from 0 to 4 (see text for the calculation of scores)
(2) 50% tissue culture infective dose
(3) not applicable

Macroscopic and microscopic lung pathology

PBS control pigs did not have macroscopic or microscopic lung pathologies (figure 1). Lungs of PRRSV-inoculated pigs had a mottled appearance with multifocal red and tan areas. Multifocal interstitial pneumonia was found microscopically. Interalveolar septal thickening with infiltration of mononuclear cells was the major feature and increased from 3 to 14 days after PRRSV inoculation. Inoculation with LPS only resulted in milder pneumonic lesions. Macroscopic lesions were characterised by focal areas of atelectasis and interlobular oedema. The characteristic histopathological features were thickening of the interalveolar septa, although it was less pronounced than that after inoculation with PRRSV, and bronchiolar infiltration with neutrophils and mononuclear cells. Intra-alveolar oedema and focal transudation of erythrocytes were occasionally seen.

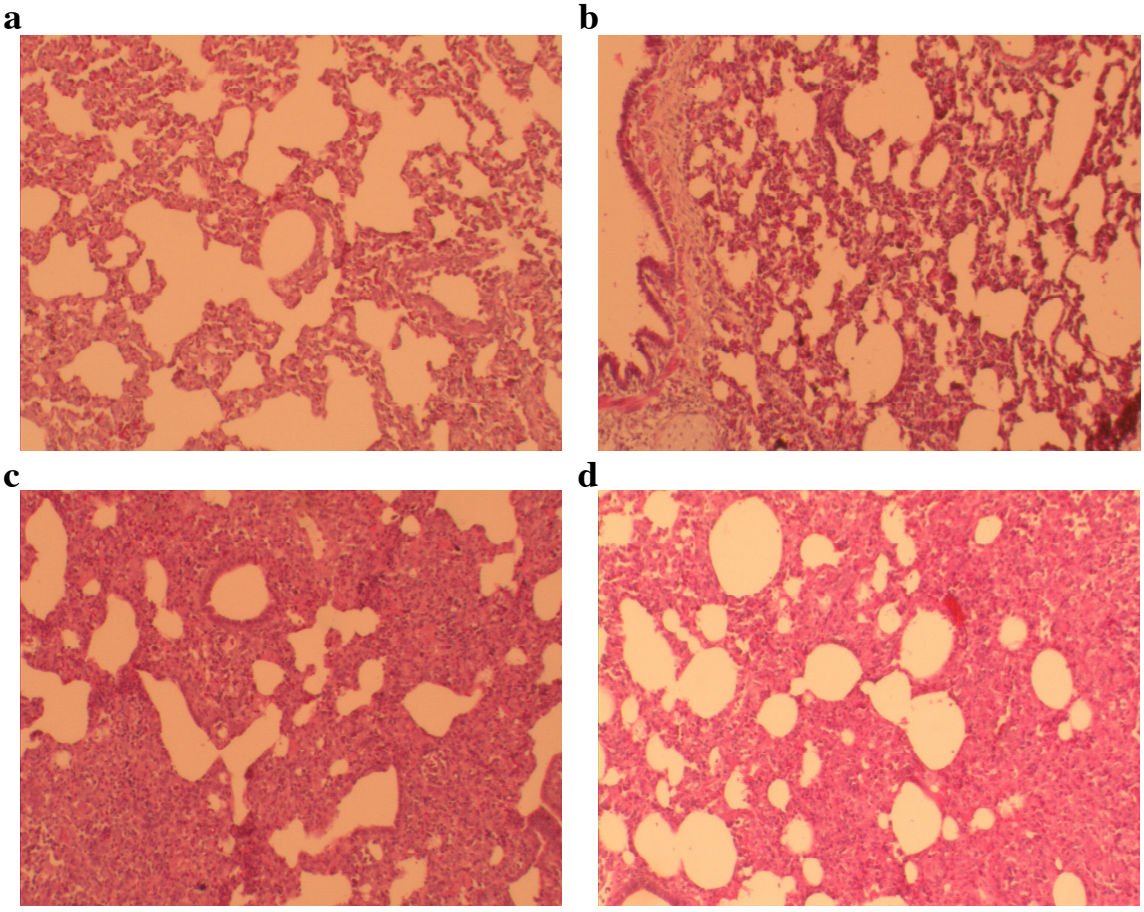

Figure 1. Haematoxylin-eosin staining (× 100) of the lungs of pigs inoculated with PBS only (a), LPS only (b), PRRSV only (10 days after inoculation) (c), and the combination of PRRSV and LPS over a 10-day interval (d). Interalveolar septal thickening was comparable in pigs inoculated with PRRSV-LPS and pigs inoculated with PRRSV alone.

The macroscopic and microscopic lung lesions after PRRSV-LPS inoculation resembled the combination of the lesions seen after inoculation with PRRSV and LPS alone. Lungs were mottled with small red and tan areas and interlobular oedema. Microscopically, there was thickening of the interalveolar septa due to the infiltration of mononuclear cells and neutrophils. The degree of septal thickening was comparable to that seen after inoculation with PRRSV only.

Infiltration of inflammatory cells

BAL cells of the PBS control pigs consisted mainly of mononuclear cells (mean of 115 × 10^6 cells) and few neutrophils (mean of 2 × 10^6 cells) (table 1). PRRSV-inoculated pigs showed an influx of mononuclear cells in the bronchoalveolar spaces and this influx increased from 3 to 14 days after inoculation. Starting at 7 days after inoculation with PRRSV, the mean number of mononuclear cells was at least two times higher in PRRSV-inoculated pigs than in PBS control pigs. The number of neutrophils was comparable to that in PBS control pigs, except for one pig with 91 × 10^6 neutrophils. The LPS inoculation induced infiltration of both neutrophils (mean of 303 × 10^6 cells) and mononuclear cells (mean of 233 × 10^6 cells).

PRRSV-LPS inoculated pigs showed an influx of both mononuclear cells and neutrophils. The amount and kinetics of the mononuclear cell infiltration were comparable to those in the PRRSV control pigs. Neutrophil numbers, on the other hand, were generally comparable to those in the LPS control pigs. Only 3 of the 14 PRRSV-LPS inoculated pigs had a higher number of neutrophils (567-786 × 10^6) than the LPS control pigs. One pig showed no neutrophil infiltration at all (1 × 10^6) and three pigs showed only minor neutrophil infiltration (19-44 × 10^6) compared to the LPS control pigs. Two of these pigs were inoculated with LPS 3 days after PRRSV inoculation, which explains the low mean number of neutrophils in this group.

Biologically active IL-1, TNF-α and IL-6 in BAL fluids

Figure 2 shows the IL-1, TNF-α and IL-6 titers in BAL fluids of individual pigs after inoculation with PRRSV-LPS, PRRSV only and LPS only. PBS control pigs had no detectable IL-1, TNF-α or IL-6. Ten out of fourteen PRRSV-inoculated pigs had elevated titers of IL-1 with the highest titers (183-339 U/ml) at 10 days after inoculation. Only 3 of these 14 pigs (which were euthanized 7, 10 and 14 days after

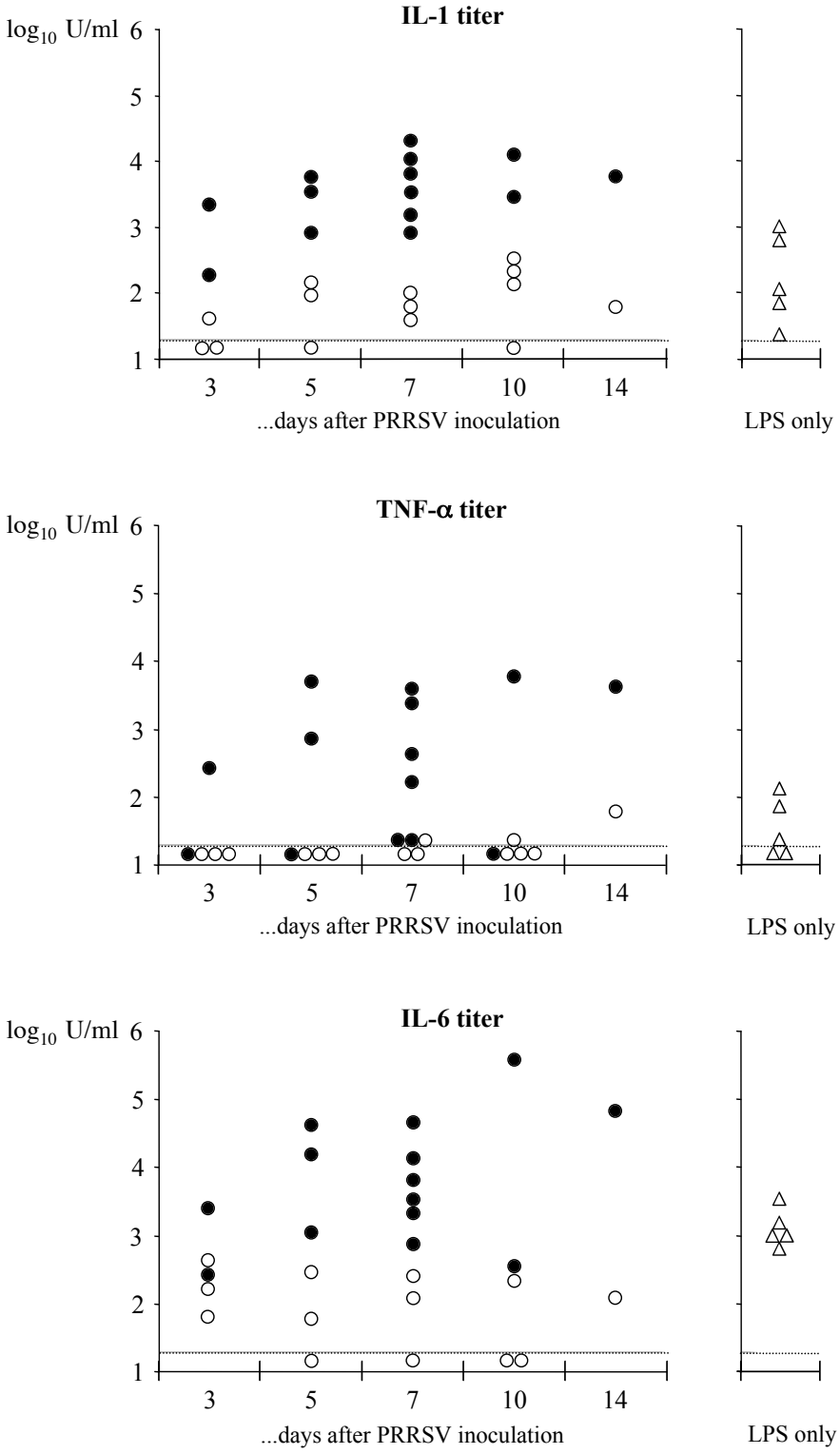

Figure 2. Titers of proinflammatory cytokines in BAL fluids of PRRSV-LPS inoculated pigs and pigs inoculated with PRRSV only or LPS only. Each dot corresponds to one pig: (●) pigs inoculated with LPS at the indicated day after PRRSV inoculation, (○) pigs inoculated with PRRSV only, (△) pigs inoculated with LPS only. Pigs inoculated with LPS were euthanized 6 hours later. The dotted line represents the detection limit.

inoculation, respectively) had detectable TNF-α titers (28-61 U/ml). Ten pigs had detectable IL-6 titers (61-343 U/ml). LPS inoculation induced the production of all three cytokines in the lungs. IL-1 (28-1022 U/ml) and IL-6 (1276-2659 U/ml) were detected in all five pigs and TNF-α (28-133 U/ml) was detected in three pigs.

Compared to the pigs inoculated with PRRSV or LPS alone, 10 of 14 PRRSV-LPS inoculated pigs showed significantly ($P < 0.05$) increased titers of at least one of the three cytokines. In nine pigs, the titers of IL-1 (2172-20480 U/ml), TNF-α (164-6047 U/ml) and IL-6 (2511-378724 U/ml) were strongly increased; and in one pig only the titer of IL-1 (2840 U/ml) was increased. The highest cytokine titers were detected in pigs inoculated with LPS 5 to 14 days after the PRRSV inoculation, and they were 10 to 100 times higher than the cytokine titers of the control pigs inoculated with PRRSV or LPS only. Four PRRSV-LPS inoculated pigs, on the other hand, did not show enhanced cytokine production. These pigs had negligible levels of TNF-α (<20-31 U/ml), and the levels of IL-1 (191-1571 U/ml) and IL-6 (266-2425 U/ml) were comparable to those of the LPS control pigs.

The left and right lungs of pigs that were inoculated with PRRSV-LPS and whose both lung halves were lavaged showed no difference in cytokine titers or cell counts ($P > 0.05$, data not shown).

Table 2 presents the correlation between respiratory scores, cytokine levels and numbers of inflammatory cells in BAL fluids.

Table 2. Correlation coefficients between respiratory scores, cytokine titers and numbers of inflammatory cells in BAL fluids.

	Correlation with					
	Resp. score	IL-1 titer	TNF-α titer	IL-6 titer	Neutrophils no.	Mononuclear cells no.
Resp. score	1	0.81	0.70	0.71	0.59	ns(1)
IL-1 titer	-(2)	1	0.75	0.85	0.80	0.43
TNF-α titer	-	-	1	0.84	0.74	0.40
IL-6 titer	-	-	-	1	0.84	ns
Neutrophils no.	-	-	-	-	1	0.61
Mononuclear cells no.	-	-	-	-	-	1

(1) no significant correlation ($P > 0.05$)
(2) not applicable

The levels of all three cytokines were tightly correlated with each other and with the respiratory scores and the neutrophil numbers. There was, however, little correlation between neutrophil numbers and respiratory scores. The number of infiltrated mononuclear cells did not correlate with cytokine levels or respiratory scores. The four PRRSV-LPS inoculated pigs that did not have increases in cytokine levels also had lower neutrophil numbers (1-129 × 10^6). The cytokine titers and BAL cell numbers did not correlate with the virus titers (data not shown).

Discussion

This study demonstrates that a PRRSV infection sensitizes the lungs for the production of proinflammatory cytokines upon exposure to LPS. Moreover, the cytokine titers were tightly correlated with the appearance of respiratory signs. We have previously documented a similar phenomenon for another respiratory virus of swine that causes subclinical disease, porcine respiratory coronavirus (PRCV) (Van Reeth *et al.*, 2000). Like PRRSV, PRCV infection enhanced the production of TNF-α and IL-1 in response to LPS, and the levels of both cytokines correlated with the severity of disease. The pathogenesis of PRRSV-LPS induced disease appears to be similar to the pathogenesis of PRCV-LPS induced disease. As IL-1 and TNF-α have overlapping effects and potentiate the effects of each other, we consider them both as central mediators in virus-LPS induced disease. IL-6 levels in the lungs of pigs inoculated with both virus and LPS were assessed for the first time in the present study, and they were also found to be markedly enhanced. IL-6 is probably induced as a secondary cytokine in response to IL-1 and TNF-α, which may explain the tight correlation between IL-6 levels and IL-1 and TNF-α levels. Because IL-6 has both pro- and anti-inflammatory activities, it may either contribute to disease or counteract the activities of IL-1 and TNF-α.

We have indications that the tachypnoea and dyspnoea resulting from PRRSV-LPS or PRCV-LPS inoculations are due to a functional process, such as bronchoconstriction, rather than to structural lung damage. Firstly, the onset of respiratory signs is hyperacute. In another PRRSV-LPS inoculation study, it was shown that respiratory signs started within 1 hour after LPS, reached a climax 2 to 4 hours later and were clearly diminished 12 hours later (Labarque *et al.*, 2000).

Secondly, microscopic lung lesions of PRRSV-LPS inoculated and PRRSV-inoculated pigs did not differ much. Pigs of both groups had interstitial pneumonia typical of PRRSV infection, and LPS inoculation had little extra effect. The inoculation with LPS as such caused a marked increase in the number of neutrophils in BAL fluids, but there were no differences in neutrophil numbers between pigs inoculated with PRRSV-LPS and those inoculated with LPS alone. Thirdly, it is well known that IL-1 and TNF-α can cause bronchial hyperreactivity (Anticevich *et al.*, 1995; Okada *et al.*, 1995) and bronchoconstriction (Martin *et al.*, 2001), leading to asthma-like symptoms. Moreover, TNF-α and IL-1 were shown to synergize in the induction of bronchoconstriction in the rat lung (Martin *et al.*, 2001). Therefore, simultaneous overproduction of these cytokines after PRRSV-LPS inoculation may cause increased and sustained contraction of bronchi, which may explain the acute respiratory signs.

We cannot explain why four PRRSV-LPS inoculated pigs, which showed clear respiratory signs, had only low cytokine titers and negligible neutrophil infiltration. There were no consistent differences in PRRSV titers or the numbers of mononuclear cells in BAL fluids between these and the other pigs. Because LPS exerts its effect locally, our initial hypothesis was that the LPS inoculum probably did not reach the right lung in those pigs and that cytokine production and neutrophil infiltration might have been restricted to the left lung. To test this hypothesis we lavaged both the left and right lungs of four PRRSV-LPS inoculated pigs. There were no differences in cytokine levels or neutrophil infiltration between the two lung halves. Therefore, it can be assumed that the LPS inoculum is distributed equally between both lung halves in most pigs. The true reason for the variability in cytokine production and neutrophil infiltration among PRRSV-LPS inoculated pigs is unclear.

There have been few studies on the interactions between viruses and LPS *in vivo*. To our knowledge, PRRSV and PRCV are the first respiratory viruses shown to act synergistically with LPS in the induction of respiratory disease and cytokines. Recently, it has been described that systemic infection of mice with lymphocytic choriomeningitis virus or vesicular stomatitis virus leads to fatal shock upon intraperitoneal inoculation with a sublethal dose of LPS (Nguyen and Biron, 1999; Nansen and Thomsen, 2001). It appeared that the shock syndrome was caused by the overproduction of TNF-α. Mice inoculated with virus-LPS had 3- to 50-fold higher

serum TNF-α levels compared to those in the sera of mice inoculated with LPS only. By use of knockout mice, it was demonstrated that virus-induced interferon was responsible for the increased sensitivity to LPS (Doughty et al., 2001; Nansen and Thomsen, 2001). Both interferon-α/β and -γ were able to sensitize mice to systemic LPS exposure. It is unlikely, however, that interferon-α is involved in the sensitization of PRRSV-infected pigs to LPS, because interferon-α production is minimal during infection with PRRSV (Albina *et al*., 1998; Van Reeth *et al*., 1999).

It remains to be seen whether the PRRSV-induced infiltration of the lungs with mononuclear cells contributes to the increased LPS responsiveness. PRRSV induces pronounced infiltration of monocytes in the lungs, reaching a peak at 25 days after inoculation (Labarque *et al*., 2000). In mice, it was shown that monocytes infiltrating the lungs in response to monocyte chemo-attractant protein-1 (MCP-1) have increased levels of expression of CD14, the LPS receptor, and become primed for enhanced TNF-α production in response to LPS (Maus *et al*., 2001). It is possible that PRRSV-attracted monocytes are an important source of cytokines upon LPS exposure and that they are responsible for the enhanced cytokine response compared to the response of uninfected lungs. In this study, the number of mononuclear cells in the bronchoalveolar spaces did not correlate with the respiratory signs. There are two important considerations in this regard. Firstly, the BAL cell profiles in PRRSV-LPS inoculated pigs were partly the result of the LPS inoculation and as such did not reflect the situation before the LPS inoculation. Secondly, we have counted mononuclear cells in the BAL fluids and not in the interstitium, while interstitial monocytes may be important targets for LPS.

In conclusion, respiratory viruses like PRRSV, which do not cause respiratory signs on their own, can sensitize the lungs for the production of proinflammatory cytokines and respiratory signs upon exposure to bacterial endotoxin. This interaction may be important in the development of multifactorial respiratory disease, as is often seen in the field.

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3.2

EFFECT OF A CYTOKINE INHIBITOR AND PROSTAGLANDIN INHIBITORS ON VIRUS-LIPOPOLYSACCHARIDE INDUCED RESPIRATORY DISEASE

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Van Gucht, S., Labarque, G. and Van Reeth, K.

Abstract

The porcine reproductive and respiratory syndrome virus (PRRSV) synergizes with bacterial lipopolysaccharide (LPS) in the induction of proinflammatory cytokines and respiratory disease. We sought to confirm that the excessive production of these cytokines is responsible for the acute respiratory signs after PRRSV-LPS exposure. Hereto, we studied the effect of pentoxifylline, a phosphodiesterase inhibitor, on PRRSV-LPS induced cytokine production and disease. Pentoxifylline is known to suppress the production of TNF-α and other proinflammatory cytokines. The clinical effects of two prostaglandin inhibitors, namely meloxicam and flunixin meglumine, were also examined. Pentoxifylline, but not the prostaglandin inhibitors, significantly reduced fever and respiratory signs of PRRSV-LPS inoculated pigs from 2 to 6 hours after the LPS inoculation. The levels of tumour necrosis factor-α and interleukin-1 in the lungs of pentoxifylline-treated PRRSV-LPS inoculated pigs were moderately reduced compared to untreated PRRSV-LPS inoculated pigs, but were still markedly higher than in control pigs inoculated with PRRSV or LPS only. The beneficial effect of pentoxifylline on the respiratory disease could not be attributed solely to the limited reduction of proinflammatory cytokines in the lungs. We conclude that pentoxifylline is not a good tool to study the role of proinflammatory cytokines in virus-LPS induced respiratory disease.

Introduction

The porcine reproductive and respiratory syndrome virus (PRRSV) synergizes with bacterial lipopolysaccharide (LPS) in the induction of proinflammatory cytokines and respiratory disease (chapter 3.1). We wanted to confirm that the excessive production of these cytokines in the lungs is responsible for the acute respiratory signs after PRRSV-LPS exposure. Hereto, we tested the effect of a known cytokine inhibitor on PRRSV-LPS induced disease. Pentoxifylline (Torental®, Hoechst) is a non-selective phosphodiesterase-inhibitor that suppresses the production of tumour necrosis factor-α (TNF-α) (Noel *et al.*, 1990; Lin *et al.*, 2004) and according to some reports also interleukin-1 (IL-1), IL-6 and IL-8 (Neuner *et al.*, 1994). Moreover, pentoxifylline has been successfully used to suppress systemic TNF-α levels in pigs (Gibson *et al.*, 1991). Additionally, we also examined the effects of two non-steroidal anti-inflammatory drugs (NSAIDs), namely meloxicam (Metacam®, Boehringer Ingelheim) and flunixin meglumine (Finadyne®, Schering-Plough). Both NSAIDs are registered to treat inflammation in swine. They inhibit the synthesis of prostaglandins and thromboxanes, which are eicosanoid mediators of inflammation (Odensvik *et al.*, 1989; Schmidt and Banting, 2000; Hirsch *et al.*, 2003).

Materials and Methods

Five-week-old conventional pigs were inoculated intratracheally with 10^6 50% tissue culture infective doses ($TCID_{50}$) of the Lelystad strain of PRRSV and 5 days later with LPS (20 μg/kg, derived from *E. coli* serotype 0111:B4, Sigma-Aldrich). Ten hours and one hour before the LPS inoculation, pigs were treated with pentoxifylline (120 mg/kg orally, n = 15), meloxicam (1.5 mg/kg im, n = 8) or flunixin meglumine (5.5 mg/kg im, n = 3) or they were left untreated (n = 17). Untreated PRRSV-inoculated (n = 7), LPS-inoculated (n = 8) and non-inoculated pigs (n = 8) were also included. Clinical signs were monitored at -10, -1, 0, 2, 4, 6, 8, 10 and 12 hours after the LPS inoculation and evaluated using a scoring system. Pigs were scored for fever (0: ≤39.9°C, 1: ≥40°C - ≤40.9°C, 2: ≥41°C), tachypnoea (0: ≤45, 1: ≥46 - ≤59, 2: ≥60), abdominal thumping (0: absent, 1: present) and dyspnoea (0: absent, 1: present). The total score per pig was obtained at each time point by

adding the scores for the different parameters and ranged from 0 to 6. At 4 hours after the LPS inoculation, 14 pigs of the PRRSV-LPS group (7 pentoxifylline-treated and 7 untreated), 3 of the PRRSV group and 4 of the LPS group were euthanized. Bioactive levels of TNF-α, IL-1 and IL-6, and numbers of inflammatory cells in bronchoalveolar lavage (BAL) fluids were determined as described previously (Van Gucht *et al.*, 2003).

Results

The evolution of clinical scores in all groups is presented in figure 1. Treatment with pentoxifylline significantly reduced fever and respiratory signs, but side effects like nervousness and tremor were seen in 40% of the pigs. Meloxicam and flunixin meglumine had no significant effect on fever or respiratory signs.

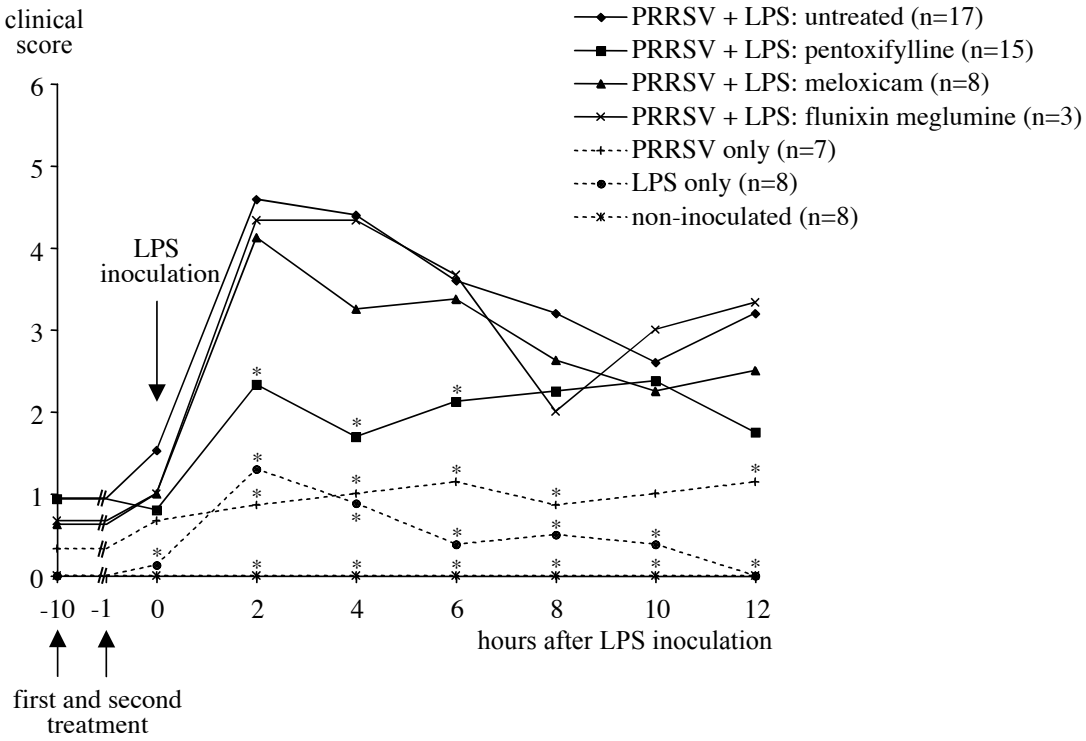

Figure 1. Effect of pentoxifylline, meloxicam and flunixin meglumine treatment on clinical signs of PRRSV-LPS inoculated pigs. Clinical scores are calculated as described in the text. Values with an asterisk are significantly different from the untreated PRRSV-LPS group (Mann-Whitney test, $P < 0.05$)

The results of cytokine titrations and BAL cell enumerations are presented in table 1 and 2. Mean TNF-α, IL-1 and IL-6 levels were 11 to 126 times higher in PRRSV-

LPS inoculated pigs than in pigs inoculated with PRRSV or LPS only. Pentoxifylline treatment of PRRSV-LPS inoculated pigs reduced the mean TNF-α and IL-1 levels 5- and 3-fold respectively, but these levels were still 26- and 3.5-fold higher than those of the singly inoculated pigs. Pentoxifylline treatment had no effect on IL-6 levels or infiltration of inflammatory cells in the lungs.

Table 1. Effect of pentoxifylline treatment on titers of proinflammatory cytokines in BAL fluids of PRRSV-LPS inoculated pigs at 4 hours after the LPS inoculation.

Inoculation with	Treatment	n	Mean clin. score(1) ± SEM	Mean BAL cytokine titers ± SEM (U/ml)		
				TNF-α	IL-1	IL-6
PRRSV-LPS	PTX(2)	7	$1.7^{a} \pm 0.5$	$1369^{a} \pm 912$	$4037^{a} \pm 1356$	$31196^{a} \pm 23168$
PRRSV-LPS	untreated	7	$4.7^{b} \pm 0.4$	$6561^{b} \pm 1789$	$12359^{b} \pm 194$	$32802^{a} \pm 23683$
PRRSV	untreated	3	$0.7^{a} \pm 0.3$	$<40^{c} \pm 0$	$490^{c} \pm 668$	$382^{b} \pm 625$
LPS	untreated	4	$0.8^{a} \pm 0.3$	$52^{c} \pm 39$	$1149^{c} \pm 614$	$1443^{b} \pm 456$

(1) clinical scores were determined immediately before euthanasia and ranged from 0 to 6 (see text for the calculation of scores), (2) pentoxifylline

[a, b, c] values with different superscripts are significantly different (Mann-Whitney test, $P < 0.05$)

Table 2. Effect of pentoxifylline treatment on numbers of inflammatory cells in BAL fluids of PRRSV-LPS inoculated pigs at 4 hours after the LPS inoculation.

Inoculation with	Treatment	n	Mean BAL cells ± SEM ($\times 10^6$)	
			monomorphonuclear cells	neutrophils
PRRSV-LPS	PTX(1)	7	$545^{a} \pm 101$	$1316^{a, b} \pm 381$
PRRSV-LPS	untreated	7	$669^{a} \pm 118$	$1377^{a} \pm 209$
PRRSV	untreated	3	$772^{a} \pm 157$	$315^{b} \pm 146$
LPS	untreated	4	$659^{a} \pm 100$	$1039^{a, b} \pm 255$

(1) pentoxifylline

[a, b, c] values with different superscripts are significantly different (Mann-Whitney test, $P < 0.05$)

Discussion

This study demonstrated that pentoxifylline, a phosphodiesterase inhibitor, was more effective for the treatment of virus-LPS induced disease than classic prostaglandin synthesis inhibitors. Levels of TNF-α and IL-1 in PRRSV-LPS inoculated pigs were reduced by pentoxifylline treatment, but they were still considerably higher than those of the singly inoculated control pigs. The beneficial effect of pentoxifylline on the respiratory disease could thus not be attributed solely to

the limited reduction of proinflammatory cytokines in the lungs. Possibly, other mechanisms contributed to the clinical improvement of pentoxifylline-treated pigs. Pentoxifylline can also inhibit neutrophil activation, improve blood perfusion and cause bronchodilatation (Tighe *et al.*, 1990; Cortijo *et al.*, 1993). Moreover, pentoxifylline was recently found to inhibit translocation of nuclear factor-κB to the nucleus of alveolar epithelial cells upon LPS stimulation (Haddad *et al.*, 2002).

Myers *et al.* (2002) studied the effect of pentoxifylline on acute lung inflammation caused by *Actinobacillus pleuropneumoniae* infection in swine. They found that a dose of 20 mg/kg sc had no effect on the expression of proinflammatory cytokines in the lungs. Higher doses (200 mg/kg, sc) induced side effects such as vomiting, diarrhoea and tremor. *In vitro*, pentoxifylline could fully abrogate transcription of TNF-α mRNA in porcine alveolar macrophages, but such inhibitory concentrations were not achievable in swine due to side effects. These researchers concluded that pentoxifylline was a poor inhibitor of proinflammatory cytokine production in swine, which agrees to some extent with our study.

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

We conclude that pentoxifylline is not a good tool to study the role of proinflammatory cytokines in virus-LPS induced respiratory disease. More specific cytokine inhibitors are needed to confirm the role of proinflammatory cytokines in the acute respiratory disease.

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4.

EFFECT OF VIRUS INFECTION ON THE EXPRESSION OF DIFFERENT COMPONENTS OF THE LIPOPOLYSACCHARIDE RECEPTOR COMPLEX IN THE LUNGS

4.1

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS INFECTION INCREASES CD14 EXPRESSION AND LIPOPOLYSACCHARIDE-BINDING PROTEIN IN THE LUNGS OF PIGS

Viral Immunology (2005) 18, 117-127

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is a respiratory virus of swine that plays an important role in multifactorial respiratory disease. European strains of PRRSV cause mild or no respiratory signs on their own, but can sensitize the lungs for the production of proinflammatory cytokines and respiratory signs upon exposure to bacterial lipopolysaccharide (LPS). The inflammatory effect of LPS depends on the binding to the LPS receptor complex. Therefore, we quantified the amounts of CD14 and LPS-binding protein (LBP) in the lungs of pigs throughout a PRRSV infection. Twenty-four gnotobiotic pigs were inoculated intranasally with PRRSV (10^6 50% tissue culture infective doses per pig, Lelystad strain) or phosphate-buffered saline (PBS) and euthanized 1 to 52 days later. Lungs were examined for CD14 expression (immunofluorescence and image analysis), LBP (ELISA) and virus replication. PRRSV infection caused a clear increase of CD14 expression from 3 to 40 days post inoculation (DPI) and LBP from 7 to 14 DPI. Both parameters peaked at 9-10 DPI (40 and 14 times higher than PBS control pigs, respectively) and were correlated tightly with virus replication in the lungs. Double immunofluorescence labelings demonstrated that resident macrophages expressed little CD14 and that the increase of CD14 expression in the PRRSV-infected lungs was probably due to infiltration of highly CD14-positive monocytes in the interstitium. As both CD14 and LBP potentiate the inflammatory effects of LPS, their increase in the lungs could explain why PRRSV sensitizes the lungs for the production of proinflammatory cytokines and respiratory signs upon exposure to LPS.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a respiratory *arterivirus* of swine that has a strict tropism for differentiated macrophages (Duan *et al.*, 1997). In spite of the fact that European strains of PRRSV fail to cause respiratory disease on their own (Van Reeth *et al.*, 1999), the virus is considered an important cause of multifactorial respiratory disease (Thacker, 2001). However, little is known about the mechanisms of interaction between PRRSV and secondary agents in the lungs.

We have previously demonstrated that PRRSV sensitizes the lungs for the production of proinflammatory cytokines and respiratory signs upon exposure to lipopolysaccharides (LPS) (Labarque *et al.*, 2002; Van Gucht *et al.*, 2003). LPS are endotoxins of Gram-negative bacteria. They are present in high concentrations in organic dust of swine confinement units and they are released locally in the lungs during infections with Gram-negative bacteria (Pugin *et al.*, 1992; Zhiping *et al.*, 1996). Treatment with some antibiotics can even enhance the release of LPS from the bacterial cell wall (Periti and Mazzei, 1999). Intratracheal administration of LPS (20 μg/kg body weight) to PRRSV-infected pigs results in severe respiratory signs, characterized by tachypnoea, abdominal breathing, dyspnoea, high fever and depression (Labarque *et al.*, 2002). Pigs exposed to PRRSV or LPS only, in contrast, develop no or mild respiratory signs. Also, PRRSV-LPS induced respiratory disease is associated with an excessive production of proinflammatory cytokines in the lungs (Van Gucht *et al.*, 2003). Following exposure to LPS, the production of interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the lungs is 10 to 100 times higher in PRRSV-infected pigs than in uninfected pigs. In previous experiments, pigs were exposed to LPS from 3 to 14 days after PRRSV inoculation (Labarque *et al.*, 2002; Van Gucht *et al.*, 2003). The synergy between PRRSV and LPS occurred at all time intervals, but was most pronounced between 5 and 14 days after PRRSV inoculation.

LPS exert their inflammatory effects after binding to “cluster of differentiation 14” (CD14), a specific LPS receptor which is expressed on monocytes and macrophages and to a lesser extent on neutrophils (Antal-Szalmas *et al.*, 1997). CD14 is a so-called “pattern recognition receptor”. This is a receptor that recognizes conserved molecules

of several pathogens, such as LPS from Gram-negative bacteria, lipoteichoic acid from Gram-positive bacteria and chitosans from fungi and insects thereby initiating the innate immune response against these organisms (Antal-Szalmas, 2000). Numerous studies in different species have demonstrated that impairment of CD14 function, by neutralization with antibodies or use of knockout animals, suppresses LPS-induced cytokine production, respiratory disease and shock (Ishii *et al.*, 1993; Haziot *et al.*, 1996; Leturcq *et al.*, 1996; Schimke *et al.*, 1998; Frevert *et al.*, 2000; Tasaka *et al.*, 2003). In humans and mice, the CD14-LPS complex binds to Toll-like receptor 4 (TLR4) (Heumann and Roger, 2002). TLR4 has an intracellular tail that activates messenger molecules, eventually leading to the activation of several proinflammatory genes.

Binding of LPS to CD14 is enhanced by LPS-binding protein (LBP), a soluble acute phase protein produced by liver and lung epithelial cells (Fenton and Golenbock, 1998; Dentener *et al.*, 2000). Plasma of healthy humans contains about 2-20 μg/ml LBP and levels increase ten times during acute phase responses. LBP facilitates the transfer of LPS from bacterial membranes to the cell surface receptor CD14 and catalyzes the binding of LPS to CD14 (Hailman *et al.*, 1994). This way, LBP increases the biological effects of LPS 100- to 1000-fold. LBP plays a role in the pathogenesis of the "adult respiratory distress syndrome" and asthma (Martin *et al.*, 1997; Strohmeier *et al.*, 2001). CD14 and LBP are both important components of the so-called "LPS receptor complex".

A PRRSV infection causes a marked infiltration of the lungs with monocytes (Van Reeth *et al.*, 1999; Labarque *et al.*, 2000). Monocytes express CD14 on their membranes and produce proinflammatory cytokines in response to LPS. Also, LBP is induced during the acute phase response of different infections. Therefore, we hypothesize that, as a consequence of the PRRSV infection, CD14 expression and LBP levels increase in the lungs, which may lead to LPS sensitization.

In this study, we quantified the levels of CD14 expression and LBP in the lungs of pigs throughout a PRRSV infection. Further, the cells expressing CD14 were characterized using monocyte-macrophage markers.

Materials and methods

Pigs, experimental design and sampling

Twenty-four colostrum-deprived pigs (age: 4 weeks) delivered by caesarean section were used in the study. They were housed in individual Horsefall-type isolation units with positive-pressure ventilation and fed with commercial ultrahigh-temperature-treated cow's milk.

Nineteen pigs were inoculated intranasally with 10^6 50% tissue culture infective doses ($TCID_{50}$) of the Lelystad strain in 3 ml phosphate-buffered saline (PBS; Gibco, Merelbeke, Belgium) (1.5 ml in each nostril). A fifth passage on porcine alveolar macrophages of the Lelystad strain of PRRSV (Wensvoort *et al.*, 1991) was used. The remaining five pigs were mock-inoculated with PBS. PRRSV-inoculated pigs were euthanized at 1 (n = 1), 3 (n = 2), 5 (n = 2), 7 (n = 2), 9 (n = 2), 10 (n = 1), 14 (n = 3), 20 (n = 1), 25 (n = 1), 30 (n = 1), 35 (n = 1), 40 (n = 1) or 52 (n = 1) days post inoculation (DPI). PBS control pigs were euthanized at 1 (n = 1), 7 (n = 1), 14 (n = 1), 30 (n = 1) or 52 (n = 1) DPI.

Tissue samples from the apical, cardiac and diaphragmatic lung lobes of the left lung were collected for virological and bacteriological examinations and immunofluorescence staining. For immunofluorescence staining, samples were embedded in methylcellulose medium, frozen at -70°C and cryostat sections of 5 to 8 µm were made. The right lung was used for lung lavage by an earlier described method (Van Reeth *et al.*, 1998). Recovered bronchoalveolar lavage (BAL) fluids were cleared from cells and debris by centrifugation (400 × *g*, 10 min, 4°C). Cell-free BAL fluids were then concentrated 20 times by dialysis against a 20% w/v solution of polyethylene glycol (MW 20000) and again centrifuged at 100000 × *g*.

Virological and bacteriological examinations

PRRSV titrations were performed on porcine alveolar macrophages using standard methods (Wensvoort *et al.*, 1991). PRRSV antigen-positive cells in lung tissue sections were quantified using monoclonal antibodies (mAbs) against the nucleocapsid (WBE1 and WBE4-6) and a streptavidin-biotin immunofluorescence technique (Labarque *et al.*, 2000). A distinction was made between viral antigen-positive single cells and foci. Foci were defined as clusters of viral antigen-positive

cells and cellular debris in the tissue. Because the number of cells was difficult to determine, each cluster was counted as one viral antigen-positive focus.

For bacteriology, samples of lung tissue were plated on bovine blood agar and cultured aerobically. A nurse colony of coagulase-positive *Staphylococcus* species was streaked diagonally on each plate. Plates were inspected for bacterial growth after 48 and 72 hours. Colonies were then identified by standard techniques.

BAL cell quantification

The total amount of cells recovered from the BAL fluids was counted in a Türk chamber. The percentage of neutrophils was determined using Diff-Quick® (Baxter, Düdingen, Switzerland) staining of cytocentrifuge preparations. The percentage of SWC3a- and sialoadhesin-positive cells was determined using flow cytometric analysis (Becton Dickinson FACSCalibur™, BD Cellquest software). SWC3a (mAb 74-22-15) is expressed on the cell membrane of monocytes, macrophages and neutrophils (Thacker *et al.*, 2001) and sialoadhesin (mAb 41D3) is expressed exclusively on the cell membrane of differentiated macrophages (Vanderheijden *et al.*, 2003). Resident macrophages of uninfected lungs are sialoadhesin-positive, whereas newly infiltrated monocyte-macrophages are sialoadhesin-negative (Labarque *et al.*, 2000). The number of sialoadhesin-negative monocyte-macrophages was determined by subtracting the number of neutrophils, determined by Diff-Quick® staining, and the number of sialoadhesin-positive cells from the number of SWC3a-positive cells.

BAL cells (5 x 10^6) were incubated with optimal dilutions (in 10% goat serum) of 74-22-15 or 41D3 antibodies respectively for 1 hour at 4°C. Subsequently, BAL cells were incubated with fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse polyclonal antibodies (4 μg/ml, 10% goat serum) (Molecular Probes, Eugene, Oregon, USA) for 1 hour at 4°C. Three washings were done with cold PBS after each incubation. BAL cells which were exclusively incubated with FITC-labeled goat anti-mouse polyclonal antibodies were included as controls. Ten thousand cells were analysed for each sample.

CD14 quantification

Immunofluorescence staining for CD14 was performed on sections of the apical (n = 1), cardiac (n = 2) and diaphragmatic (n = 2) lobes of each lung using mouse mAb MIL2 (Thacker *et al.*, 2001). Sections were fixed in 4% paraformaldehyde for 10 min at room temperature, incubated with an optimal dilution (in 10% goat serum) of MIL2 antibodies and thereafter with FITC-labeled goat anti-mouse polyclonal antibodies (4 µg/ml, 10% goat serum) (Molecular Probes, Eugene, Oregon, USA). Sections were mounted in a glycerin-PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazobicyclo-2.2.2-octane (DABCO) (Janssen Chimica, Beerse, Belgium). Antibodies were diluted in PBS with 10% goat serum. All incubations were performed at 37°C for 1 hour. After fixation and incubation with the respective antibodies, sections were rinsed in PBS (4 × 5 min). Specificity of the CD14 staining was determined by deletion of MIL2 antibodies and use of irrelevant mouse mAbs.

Fifteen pictures (1 picture ≈ 0.1 mm^2) of the interstitium of each section were taken randomly using a fluorescence microscope (× 400) (Leica DM RBE, Leica Microsystems GmbH, Wetzlar, Germany), a Sony® 3CCD colour video camera (Sony Corporation, Tokyo, Japan) and Adobe® Photoshop® 5.0 LE (Adobe Systems, San Jose, California, USA). Pictures were converted to black and white using the image analysis program Scion Image 1.62C (Scion Corporation, Frederick, Maryland, USA). Positive cells (green fluorescence) were converted to black pixels whereas negative cells and background were converted to white pixels. The number of black pixels, which depends on the number of positive cells and the amount of CD14 they express, was counted. The average number of black pixels was calculated for each lung (5 sections, 15 pictures/section) and expressed as a ratio compared to the number of black pixels in a reference sample. A section of the apical lung lobe of the PBS control pig euthanized at 1 DPI was used as the reference sample.

Characterization of CD14-positive cells

Double immunofluorescence staining for CD14 (mAb MIL2, IgG2b isotype) and sialoadhesin (mAb 41D3, IgG1 isotype) (Vanderheijden *et al.*, 2003) or SWC3a (mAb 74-22-15, IgG1 isotype) (Pescovitz *et al.*, 1984) was performed on sections of the cardiac and diaphragmatic lung lobes. Sections were fixed in 100% methanol for 15 min and dried for 20 min at -20°C. Sections were incubated consecutively with

optimal dilutions (in 10% goat serum) of 41D3 or 74-22-15 antibodies, FITC-labeled goat anti-mouse IgG1 polyclonal antibodies (4 μg/ml, 10% goat serum) (Santa Cruz Biotechnology, Santa Cruz, California, USA), biotinylated MIL2 antibodies (10 μg/ml), streptavidin-Texas Red (10 μg/ml) (Molecular probes, Eugene, Oregon, USA) and Hoechst 33342 (10 μg/ml) (Molecular probes, Eugene, Oregon, USA). Sections were mounted in a glycerin-PBS solution (0.9:0.1, v/v) with 2.5% DABCO (Janssen Chimica, Beerse, Belgium). All incubations were performed at 37°C for 1 hour. After fixation and incubation with the respective antibodies, sections were rinsed in PBS (4 × 5 min). Specificity of the double labelings was determined by deletion of primary antibodies and use of irrelevant mouse mAbs.

Digital images were taken using a Leica TCS SP2 laser scanning spectral confocal system linked to a Leica DM IRB inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Immunohistochemical staining for CD14

Lung tissue sections were fixed in 100% methanol for 15 min and dried for 20 min at -20°C. Sections were incubated for 30 min with a 0.5% (v/v) hydrogen peroxide-sodium azide solution to quench endogenous peroxidase activity. Sections were incubated consecutively with an optimal dilution (in 10% sheep serum) of MIL2 antibodies (1 h, 37°C), biotinylated sheep anti-mouse polyclonal antibodies (1:200, 1 h, 37°C) (Amersham Biosciences, Little Chalfont, UK), streptavidin-biotinylated horseradish peroxidase complex (1:200, 30 min, 37°C) (Amersham Biosciences, Little Chalfont, UK) and 3,3'-diaminobenzidine (DAB)/hydrogen peroxide chromogen substrate (5 min, room temperature) (Sigma-Aldrich, Steinheim, Germany). Sections were counter-stained with haematoxylin. After fixation and incubation with the respective reagents, sections were rinsed in TRIS-buffered saline (3 × 5 min). Sections were mounted with DPX (Fluka, Buchs, Switzerland). Specificity of the CD14 staining was confirmed by replacement of MIL2 antibodies by irrelevant mouse mAbs.

LBP quantification

LBP was quantified in BAL fluids using an ELISA kit for LBP of different species, including swine LBP (Hycult biotechnology, Uden, the Netherlands).

Statistical analysis

Differences between mean BAL cell numbers, CD14 ratios and LBP levels of PRRSV-inoculated pigs and PBS control pigs were analysed using the Student's *t* test. Correlation coefficients (ρ) between virus replication, CD14 expression and LBP levels were calculated using the Spearman rank correlation test. P values <0.05 were considered significant. Statistical analyses were performed using SPSS (version 6.1) software.

Results

The lungs of all pigs were free of bacteria by culture. Clinical signs were not observed, except for mild anorexia and dullness between 3 and 5 DPI.

Virus replication

All PBS control pigs were negative for PRRSV. Mean virus titers in the lungs at different days after the PRRSV inoculation are shown in table 1. Infectious virus was detected in the lungs of PRRSV-inoculated pigs euthanized between 1 and 40 DPI, except in one pig euthanized at 30 DPI. Virus titers were highest between 7 and 14 DPI ($10^{5.8}$ to $10^{6.6}$ $TCID_{50}$/g) and decreased slowly thereafter ($10^{5.1}$ to $10^{1.0}$ $TCID_{50}$/g). Virus titers of the apical, cardiac and diaphragmatic lung lobes were similar.

Figure 1 shows the evolution of the mean number of viral antigen-positive cells and foci in the lungs throughout the PRRSV infection. Viral antigen-positive cells and foci were observed from 3 to 25 DPI and from 3 to 14 DPI, respectively. Mean numbers of both singly infected cells (39/mm^2 lung tissue) and infected foci (29/mm^2 lung tissue) peaked at 9 DPI. No infected cells were detected in the lungs of PBS control pigs.

BAL cell quantification

The evolution of the number of different types of BAL cells throughout the PRRSV infection is shown in table 1. PBS control pigs had 114 to 256 x 10^6 BAL cells. Ninety-four percent of these cells were sialoadhesin-positive macrophages, 2.7% were sialoadhesin-negative monocyte-macrophages and 1% were neutrophils.

The remaining cells (2%) were negative for SWC3a. Most of these cells had low granularity and small size and were presumably lymphocytes.

During PRRSV infection, all types of BAL cells increased significantly. Total numbers of BAL cells increased from 9 to 52 DPI and were 2- to 5-fold higher compared to the PBS control pigs. Most pronounced were increases in the numbers of sialoadhesin-negative monocyte-macrophages. The highest numbers of these cells were detected between 10 and 20 DPI and were 32- to 55-fold higher compared to the PBS control pigs. During the late stage of infection from 25 to 52 DPI, the numbers of sialoadhesin-positive macrophages increased 3- to 4-fold compared to the PBS control pigs. The numbers of neutrophils were increased between 7 and 52 DPI. In most PRRSV-infected pigs, except the pig euthanized at 10 DPI, neutrophils represented only a minor fraction (1 to 15%) of total BAL cells. The highest numbers of SWC3a-negative cells were detected between 7 and 52 DPI and were 10- to 40-fold higher compared to the PBS control pigs.

CD14 quantification

The evolution of CD14 expression in the lungs throughout the PRRSV infection is presented in figure 1. CD14 expression in the lungs of PBS control pigs varied little (ratio of 0.4 to 1.5). Throughout the PRRSV infection, CD14 ratio's increased from 3 to 9 DPI, peaked at 9 DPI (ratio of 40.1) and returned to the level of the PBS control pigs at 40 DPI.

Characterization of CD14-positive cells

Results of the double stainings and immunohistochemical staining are presented in figures 2 and 3. In the lungs of PBS control pigs, cells with high CD14 expression were scarce (15 ± 11 cells/mm^2) and distributed as round, single cells in the interstitium. More than 90% of resident macrophages (sialoadhesin-positive) expressed almost no visible CD14.

During infection, the number of highly CD14-positive cells increased and these cells formed clusters in the interstitium. Between 9 and 14 DPI, the frequency of highly CD14-positive cells and the size of the clusters were greatest. Extensive areas of the interstitium were filled with highly CD14-positive cells, whereas bronchial walls and lumina contained almost no CD14-positive cells. More than 95% of the

Table1. Mean virus titers and numbers of BAL cells in the lungs during PRRSV infection.

Inoculation with	Number of pigs	Euthanasia at…DPI with PRRSV	Virus titers ± SD (log_{10} $TCID_{50}$[(1)]/g lung tissue)	BAL cells ± SD (× 10^6)				
				total	$sial^+$ macro[(2)]	$sial^-$ mono-macro[(3)]	neutro[(4)]	$SWC3a^-$ cells[(5)]
PBS	5	n.a.[(6)]	negative	187 ± 51	176 ± 49	5.0 ± 1.7	1.9 ± 0.5	3.7 ± 1.0
PRRSV	1	1	5.8	152	137	4.2	1.5	9.1*
	2	3	5.3 ± 0.1	132 ± 33	115 ± 35	6.9 ± 0.8	2.4 ± 1.2	7.0* ± 1.4
	2	5	5.4 ± 0.8	140 ± 31	116 ± 24	5.6 ± 0.9	2.8 ± 0.6	15.5* ± 4.9
	2	7	5.9 ± 0.2	261 ± 39	154 ± 27	40.1* ± 4.5	6.5* ± 0.7	60.0* ± 8.5
	2	9	6.6 ± 0.7	351* ± 23	137 ± 85	91.1* ± 47.8	42.5* ± 17.7	83.0* ± 38.2
	1	10	5.8	750*	248	195.5*	247.5*	60.0*
	3	14	5.9 ± 0.3	583* ± 269	260 ± 193	161.3* ± 20.4	27.7* ± 24.5	134.7* ± 36.0
	1	20	5.1	590*	270	272.8*	11.8*	35.4*
	1	25	5.0	687*	488*	123.2*	27.5*	48.1*
	1	30	negative	782*	649*	7.9	7.8*	117.3*
	1	35	4.0	990*	673*	158.8*	9.9*	148.5*
	1	40	1.0	642*	469*	19.3*	64.2*	89.9*
	1	52	negative	717*	617*	7.4	7.2*	86.0*

(1) 50% tissue culture infective dose, (2) sialoadhesin-positive macrophages, (3) sialoadhesin-negative monocyte-macrophages,
(4) neutrophils, (5) SWC3a-negative cells with low granularity and small size, presumably lymphocytes, (6) not applicable,
(∗) values marked with an asterisk differ significantly ($P < 0.05$) from those of the PBS control pigs

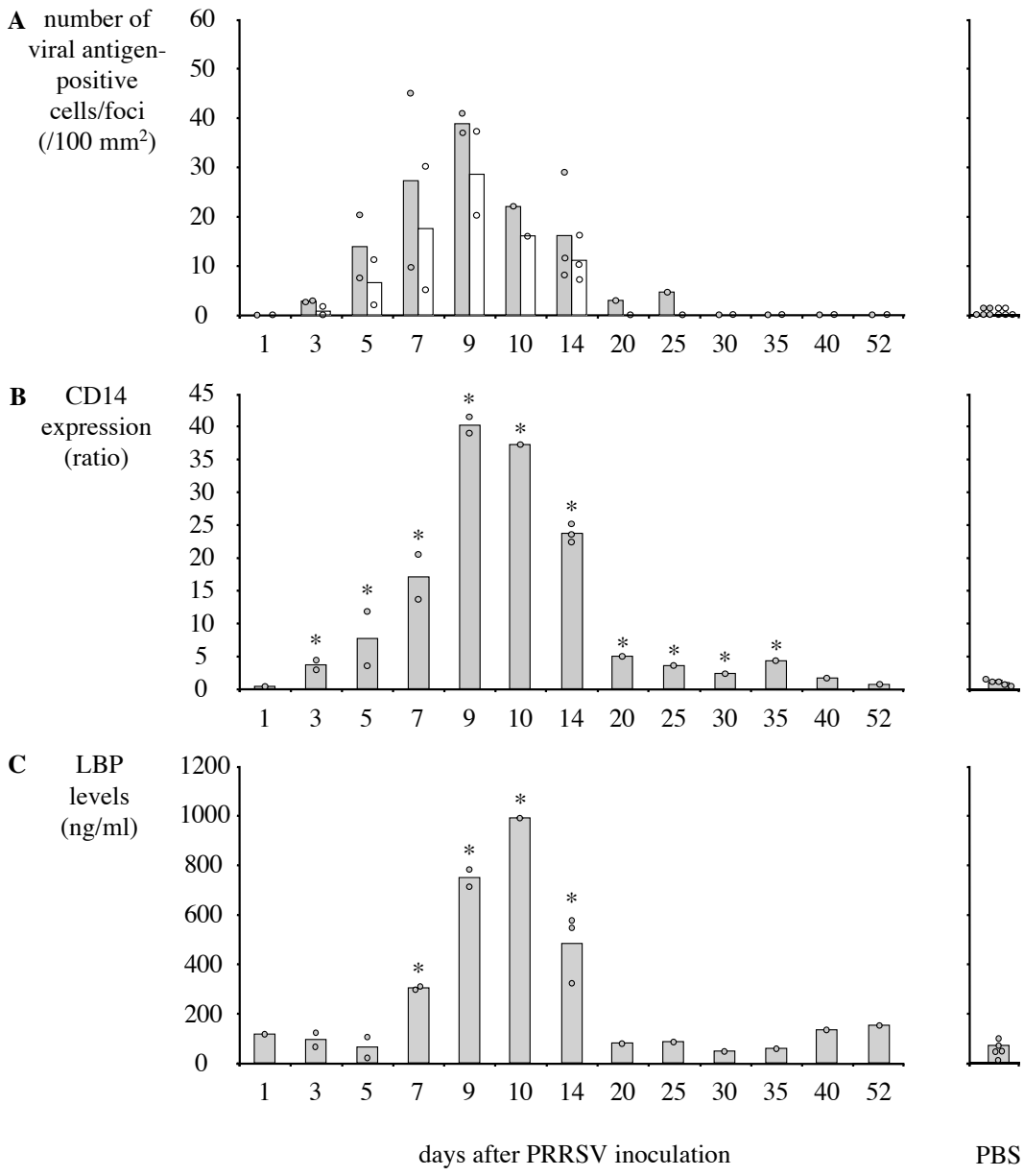

Figure 1. Evolution of virus replication (a), CD14 expression (b) and LBP levels (c) in the lungs throughout a PRRSV infection. Each dot corresponds to one pig and the bars represent the mean at each time point. In the first graph, grey bars/dots represent the number of viral antigen-positive cells and white bars/dots represent the number of viral antigen-positive foci. Mean CD14 ratios and LBP levels marked with an asterisk (∗) differ significantly ($P < 0.05$) from those of the PBS control pigs.

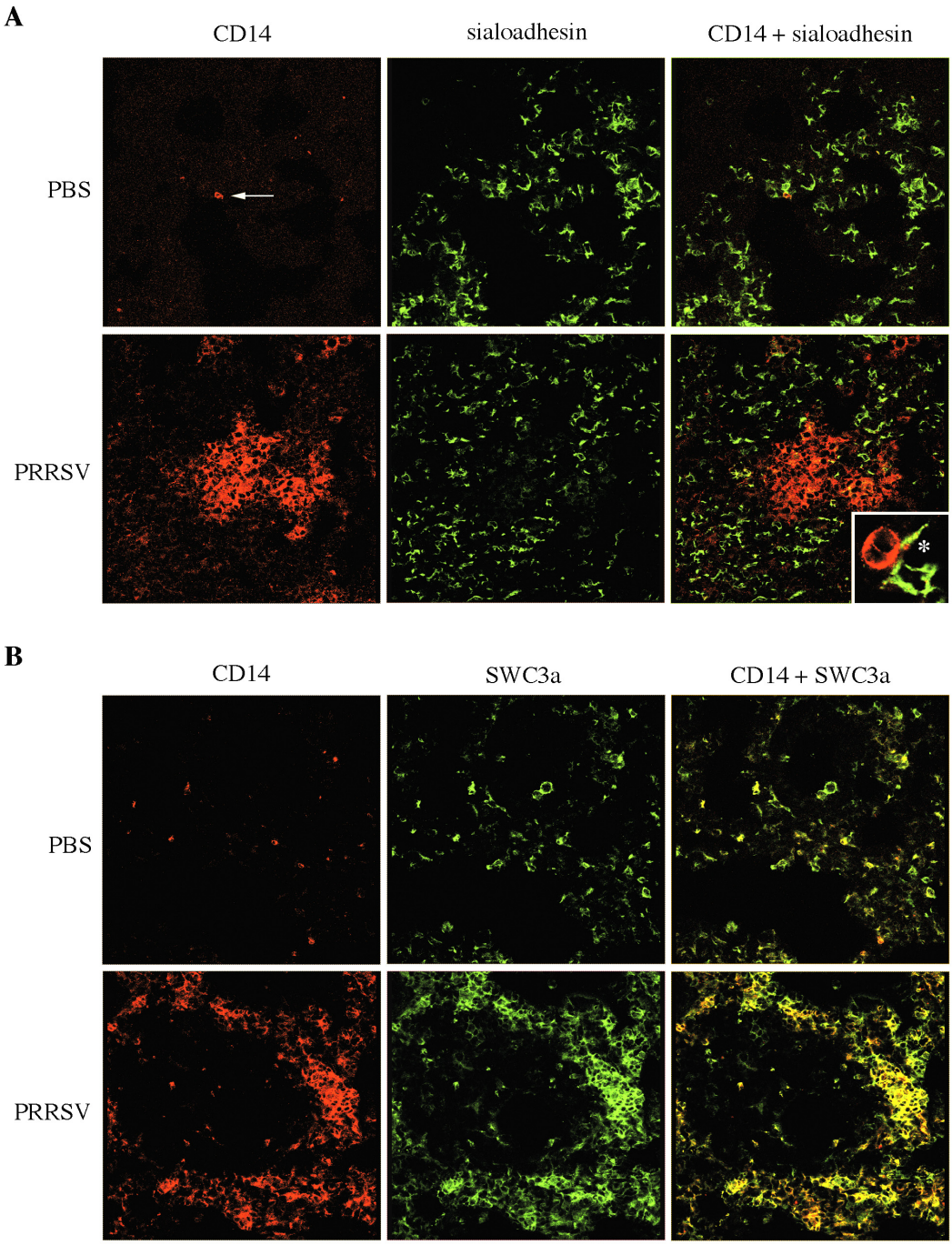

Figure 2. Double immunofluorescence staining (× 400) for CD14-sialoadhesin (a) and CD14-SWC3a (b) of the lung tissue of a PBS- and a PRRSV-inoculated pig at 14 DPI. Sialoadhesin is a marker for differentiated macrophages and SWC3a for both monocytes and macrophages. The PBS-inoculated pig has few highly CD14-positive cells as indicated by the arrow. The majority of resident sialoadhesin-positive macrophages express little CD14. Their signal is too weak to be visible on the photograph. The PRRSV-inoculated pig shows a clear increase of highly CD14-positive cells. These cells are sialoadhesin-negative, clustered in the interstitium and have typical round morphology as shown by the picture at higher magnification (∗; × 3200). Most highly CD14-positive cells are positive for SWC3a as indicated by the yellow colour in the merge picture.

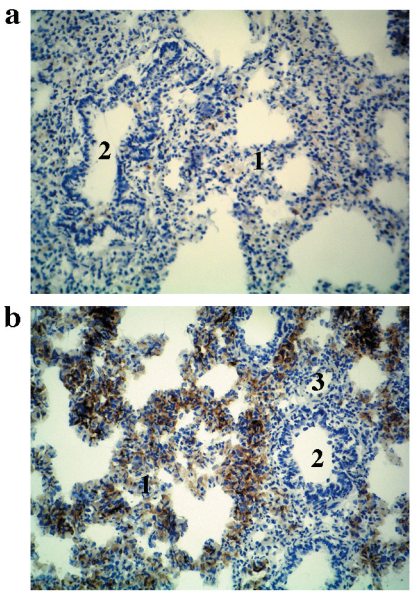

Figure 3. Immunohistochemical staining (× 200) for CD14 of the lung tissue of a PBS- (a) and a PRRSV-inoculated (b) pig at 14 DPI. The alveolar interstitium, bronchioli and large bloodvessels are indicated respectively by the numbers 1, 2 and 3. Highly CD14-positive cells (brown colour) are clustered in the alveolar interstitium of the PRRSV-inoculated pig.

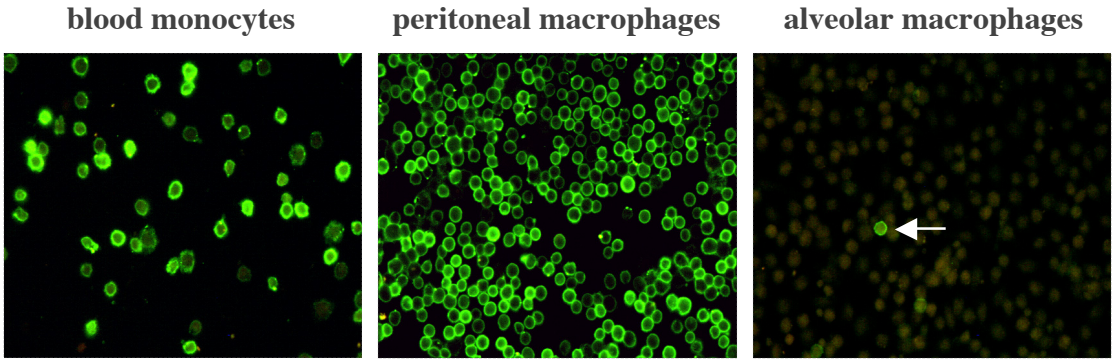

Figure 4. Immunofluorescence staining (× 400) for CD14 of blood monocytes (a), peritoneal macrophages (b) and alveolar macrophages (c) freshly isolated from a PBS-inoculated pig. The majority of monocytes and peritoneal macrophages express high amounts of CD14 on their membranes (green colour). Most of the alveolar macrophages (>90%) show weak CD14 expression, only a minority of cells express high amounts of CD14 (arrow).

highly CD14-positive cells were SWC3a-positive and siaoladhesin-negative. These cells were round with a round to kidney-shaped nucleus, corresponding to a monocyte-like phenotype. These cells differed clearly from the resident macrophages, which were large, irregular, SWC3a- and sialoadhesin-positive. A minority (<5%) of the highly CD14-positive cells also expressed sialoadhesin.

The relatively weak expression of CD14 on alveolar macrophages in uninfected lungs is illustrated by figure 4. This figure shows a CD14 staining of blood monocytes, peritoneal and alveolar macrophages isolated from a PBS-inoculated pig. Most blood monocytes and peritoneal macrophages expressed high amounts of CD14 on their membranes, which contrasted clearly with the weak expression on alveolar macrophages.

LBP quantification

The evolution of LBP levels in the lungs throughout the PRRSV infection is presented in figure 1. All pigs had detectable levels of LBP in their BAL fluids. PBS control pigs had 71 ± 63 ng LBP/ml. PRRSV-infected pigs euthanized between 7 and 14 DPI had 4 to 14 times higher levels (303-989 ng/ml) of LBP than PBS control pigs. At the other stages of infection, LBP levels were comparable to those of PBS control pigs.

Correlations

CD14 ratios were tightly correlated with the number of viral antigen-positive cells ($\rho = 0.88$, $P < 0.01$), the number of viral antigen-positive foci ($\rho = 0.85$, $P < 0.01$), and virus titers ($\rho = 0.79$, $P < 0.01$). LBP levels were also correlated with these parameters, but correlation coefficients were lower ($\rho = 0.67$, $\rho = 0.72$ and $\rho = 0.72$, respectively). CD14 ratios and LBP levels were weakly correlated with each other ($\rho = 0.61$, $P < 0.01$).

Discussion

This study demonstrates that PRRSV causes a clear increase of CD14 and LBP in the lungs of pigs. Both parameters peaked at 9-10 DPI and were correlated tightly with virus replication in the lungs. CD14 and LBP are important components of the LPS receptor complex and several studies found a correlation between the amount of CD14 and LBP in the lungs and the sensitivity of the lungs to LPS (Martin *et al.*, 1992; Ishii *et al.*, 1993; Maus *et al.*, 2001; Jiang *et al.*, 2003; Moriyama *et al.*, 2004). Although not proven, we believe that the increase of both receptor components in the lungs could be an important cause of the enhanced LPS responsiveness during PRRSV infection (Van Gucht *et al.*, 2003). To our knowledge, this is the first study

that describes the evolution of CD14 expression and LBP levels in the lungs throughout a respiratory virus infection.

The biological effect of LPS depends on two antagonistic processes. On the one hand, LPS can bind to scavenger molecules leading to internalization and degradation without cytokine production (Stamme and Wright, 1999; Augusto *et al.*, 2003; Jiang *et al.*, 2003; Alcorn and Wright, 2004). On the other hand, LPS can bind to CD14 leading to intracellular signaling, stimulation of inflammatory genes and cytokine production. So, the inflammatory effect of LPS depends on the balance between scavenger molecules and signaling receptors (Jiang *et al.*, 2003). Control pigs show little CD14 expression in the lungs, which may explain their low LPS sensitivity. It is likely, therefore, that an important part of the inhaled LPS is bound by scavenger molecules and degraded without causing inflammation in the lungs of such pigs. During PRRSV infection, the abundant CD14 expression will probably increase the chance that LPS binds to CD14 leading to massive cytokine production and clinical signs.

In uninfected lungs, only few cells expressed high levels of CD14. The majority of macrophages (>90%) expressed low levels of CD14 (see figures 2 to 4). The CD14 expression of these cells was often difficult to distinguish from the background of the surrounding tissue. Still, flow cytometric studies show that the majority of alveolar macrophages bear CD14 on their membrane (own observations; Thacker *et al.*, 2001; Murtaugh and Foss, 2002), but compared to blood monocytes or peritoneal macrophages the CD14 signal is weak (Ziegler-Heitbrock *et al.*, 1994; Hasday *et al.*, 1997; McCullough *et al.*, 1999; Maus *et al.*, 2001; Jiang *et al.*, 2003). In humans for example, freshly isolated alveolar macrophages express only 9% of the amount of CD14 expressed by blood monocytes (Hasday *et al.*, 1997). Moreover, it was shown that viral infection of human alveolar macrophages reduces CD14 expression on their membranes (Hopkins *et al.*, 1996). Expression of CD14 depends highly on the localization and microenvironment of the cell. For example, intestinal macrophages, in contrast to peritoneal macrophages, lack CD14 expression and, as a consequence, are unresponsive to LPS (Smith *et al.*, 2001). This is beneficial because, otherwise, intestinal macrophages would constantly be activated by the high LPS content in the gut lumen. As lungs are continuously exposed to environmental LPS, it is possible

that a similar suppression of CD14 expression on resident lung macrophages helps to prevent chronic lung inflammation.

During the PRRSV infection, there was a gradual increase of highly CD14-positive cells in the interstitium with a peak at 10 DPI. Because most cells were round, clustered in the interstitium and expressed markers for monocytes (CD14, SWC3a), but not for macrophages (sialoadhesin), we believe that these cells were infiltrated monocytes attracted by PRRSV. Most of these cells did not contain PRRSV antigens and therefore were probably not infected (data not shown). Macrophages, which were irregularly shaped, scattered in the interstitium and sialoadhesin-positive, usually had low CD14 expression. It is likely that the process of differentiation into macrophages coincides with a decrease of CD14 expression, a process which has been previously described in pigs (Basta *et al.*, 1999; Sanchez *et al.*, 1999; Chamorro *et al.*, 2000). In the present study, a few sialoadhesin-positive macrophages also expressed high levels of CD14. These cells may have been at an intermediate stage of differentiation.

We have reproduced the PRRSV-LPS synergy in both gnotobiotic and conventional pigs (Labarque *et al.*, 2002; Van Gucht *et al.*, 2003). Here, we studied CD14 expression in the lungs of gnotobiotic pigs which were kept under germ-free conditions and low environmental LPS. To study whether this high sanitary status had an effect on CD14 expression, we compared the lungs of conventional pigs (age: 6 and 12 weeks, n = 5) with those of the control pigs used in this study. The pattern and intensity of CD14 staining of lung tissue sections differed little between both types of pigs. Moreover, infection of conventional pigs with PRRSV increased the amount of CD14 in the lungs 8 to 32 times at 6 DPI. Therefore, we believe that our observations on gnotobiotic pigs also apply to pigs kept under conventional circumstances.

We observed a marked increase of sialoadhesin-negative monocyte-macrophages in the bronchoalveolar spaces throughout the PRRSV infection. Their numbers were increased from 7 to 40 DPI and highest numbers were detected between 10 and 20 DPI. CD14 expression in the lung interstitium increased from 3 to 9 DPI and was back to normal at 40 DPI. So, it seems that the increase of monocyte-macrophages in the bronchoalveolar spaces is secondary to the increase of CD14 expression in the lung interstitium with a delay of some days. It is likely that CD14-positive monocytes, which have infiltrated the interstitium during the first two weeks of infection (high virus replication), differentiate into macrophages with low CD14 expression and

migrate into the bronchoalveolar spaces. This is in agreement with the high number of sialoadhesin-positive macrophages in the bronchoalveolar spaces at the late stage of PRRSV infection (25-52 DPI), when virus replication is low and CD14 expression in the lung tissue has decreased strongly.

In this study, immunofluorescence staining of tissue sections was used to quantify CD14 expression in the lungs. This technique allows to visualize CD14 expression in the different compartments (bronchoalveolar, interstitial and intravascular) of the lungs. In preliminary studies, we have performed flow cytometric analysis of CD14 on BAL cells of pigs euthanized at 9, 10 and 14 DPI with PRRSV (n = 6) and of PBS-inoculated pigs (n = 5). In the PRRSV-infected pigs, we found a 2- to 3- fold increase of the number of CD14-positive cells. The mean fluorescence intensity of these cells was slightly higher (× 1.7) than that of the alveolar macrophages of the control pigs. The flow cytometric analysis of BAL cells, therefore, confirmed the data obtained by immunofluorescence staining on lung tissue sections, though the increase of CD14 was higher with the latter technique (23- to 40-fold compared to control pigs). A possible explanation for this difference is that the majority of highly CD14-positive cells were clustered in the interalveolar and peribronchial interstitium and not in the bronchoalveolar compartment (see figures 2 and 3).

LBP, another component of the LPS receptor complex, was increased from 7 to 14 DPI. At the other stages of infection, the LBP concentration in the lungs was comparable with that of the PBS control pigs. A PRRSV infection sensitizes the lungs for the effects of LPS as early as 3 DPI. So, LBP could have contributed to the increased sensitivity for LPS between 7 and 14 DPI, but not between 3 and 7 DPI. CD14, on the other hand, was increased from 3 DPI onwards and could also account for the sensitisation at the earlier stages of infection. LPS induced the highest cytokine titers at 5 to 14 days after PRRSV inoculation (Van Gucht *et al.*, 2003) and at these time points CD14 was also most abundant in the lungs. So, the amount of CD14 appeared to correlate better with the LPS response than the LBP levels.

According to the literature, LBP can be produced by hepatocytes in the liver or by type 2 pneumocytes in the lungs in response to proinflammatory cytokines such as IL-1 and IL-6 (Fenton and Golenbock, 1998; Dentener *et al.*, 2000). Asai *et al.* (1999) reported that serum levels of haptoglobin, another acute phase protein, together with serum levels of IL-6 were increased significantly between 7 and 21 days after PRRSV

inoculation. We did not study LBP levels in serum, but it is possible that LBP levels, like haptoglobin levels, are increased in serum during a PRRSV infection. Increased LBP levels in serum could account for the increased levels in the lungs. However, local production of LBP can not be excluded, as both IL-1 and IL-6 are produced in the lungs during a PRRSV infection and can stimulate the production of LBP in pneumocytes (Van Gucht *et al.*, 2003).

Earlier research in our laboratory demonstrated a similar synergy between porcine respiratory coronavirus (PRCV) and LPS in the induction of respiratory signs and cytokines in the lungs (Van Reeth *et al.*, 2000). PRCV, like PRRSV, causes a subclinical infection of the lungs of swine and preliminary data suggest that PRCV-infected lungs also show an increase of CD14 expression. So, the increase of CD14 expression and synergy with LPS are probably not unique for PRRSV and could be a common feature of different respiratory viruses. However, PRRSV replicates for 5 to 7 weeks in the lungs, while PRCV replication lasts only 7 to 10 days. Therefore, interactions with endotoxins are more likely for PRRSV than for PRCV.

There have been few studies on the interactions between other respiratory viruses and LPS *in vivo*. Recently, it was shown that *in vitro* infection of airway epithelial cells with respiratory syncytial virus (RSV) results in an up-regulation of TLR4, which in turn leads to an increased LPS response (Monick *et al.*, 2003). TLR4 is a component of the LPS receptor complex that is essential for transmembrane signaling of the LPS signal (Heumann and Roger, 2002). It is unknown whether a synergy between RSV and LPS also occurs at the lung level. It is possible that PRRSV as well increases TLR4 expression in the lungs, but specific antibodies to demonstrate porcine TLR4 are not available at this moment. In contrast to CD14 and LBP, TLR4 is crucial in the LPS signaling cascade. The interaction between TLR4 and LPS is, however, potently enhanced by CD14 and LBP and these receptor components are necessary to respond to low doses (≤10 ng/ml) of LPS, which are more likely to occur *in vivo* (Muta and Takeshige, 2001; Tsan *et al.*, 2001).

In conclusion, we propose the following mechanism for the clinical synergy between PRRSV and LPS. During infection, PRRSV attracts massive amounts of monocytes into the lungs which express high levels of CD14. This increase of CD14 and possibly also the increase of LBP, both important components of the LPS receptor complex, could explain why PRRSV sensitizes the lungs for the production

of proinflammatory cytokines and respiratory signs upon exposure to bacterial LPS. However, the true significance of CD14 and LBP in the sensitisation of the lungs for LPS remains to be proven.

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4.2

PORCINE RESPIRATORY CORONAVIRUS INFECTION INCREASES CD14 AND LIPOPOLYSACCHARIDE-BINDING PROTEIN IN THE LUNGS: ASSOCIATION WITH ENHANCED LIPOPOLYSACCHARIDE SENSITIVITY

Manuscript in preparation

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Abstract

Porcine respiratory coronavirus (PRCV) is a respiratory virus of swine, related to the severe acute respiratory syndrome coronavirus of humans. CD14 and lipopolysaccharide-binding protein (LBP) recognize bacterial lipopolysaccharide (LPS) and potently enhance its endotoxic activity. This study aimed to quantify CD14 and LBP in the lungs of pigs throughout a PRCV infection and verify whether this is associated with an increased response to LPS. Gnotobiotic pigs were inoculated intratracheally with PRCV (n = 34) or phosphate-buffered saline (PBS; n = 5) and euthanized 1 to 15 days post inoculation (DPI). Cell-associated CD14 was quantified in lung tissue sections by immunofluorescence microscopy and image analysis. Soluble CD14 (flow cytometric assay) and LBP (ELISA) were quantified in bronchoalveolar lavage (BAL) fluids. In an additional experiment, 7 pigs were inoculated intratracheally with PRCV and 3 (n = 3) or 7 (n = 4) days later with LPS (20 μg/kg). Control pigs were inoculated exclusively with LPS (n = 3). Pigs were euthanized 4 hours after the LPS inoculation and levels of tumour necrosis factor-α and interleukin-6 were determined in the BAL fluids. Infectious PRCV in the lungs was detected from 1 to 9 DPI and the amounts of cell-associated CD14, soluble CD14 and LBP were markedly increased from 1 to 12 DPI. Highest amounts of cell-associated CD14 in lung tissue sections were found at 1-2 DPI (mean 10-fold increase compared to PBS control pigs) and between 5 and 12 DPI (mean 15-fold increase), while soluble CD14 and LBP peaked between 4 and 9 DPI (mean 4-fold and 35-fold increases, respectively). The cell types expressing CD14 varied throughout the infection. High levels of CD14 expression were subsequently found on monocyte-like cells (1-2 DPI), macrophage-like cells (3-12 DPI) and pneumocytes (7-9 DPI). Four out of seven pigs that were inoculated with PRCV and 3 or 7 days later with LPS developed acute respiratory distress and high cytokine titers (× 6) in the BAL fluids, in contrast to pigs inoculated with LPS alone. This adds to an earlier study in which we demonstrated an enhanced LPS response at 1 day after PRCV inoculation. We propose that the massive increase of LBP and CD14 in the lungs during both the early and late stage of PRCV infection enhances the response to subsequent LPS exposure.

Introduction

Coronaviruses cause infections of the respiratory tract of many species, including humans and swine. The porcine respiratory coronavirus (PRCV) is highly prevalent in swine populations all over the world (for review see Laude *et al.*, 1993). Typically, pigs become infected at the age of 5 to 16 weeks and the virus replicates in the lungs for up to 10 days. PRCV shares several characteristics with the "severe acute respiratory syndrome" coronavirus (SARS CoV), a genetically related virus that recently emerged in humans (Holmes, 2003; Nicholls J.M. *et al.*, 2003). Both viruses have a tropism for lung epithelial cells and cause bronchointerstitial pneumonia and necrotizing alveolitis. Uncomplicated infections often remain mild or subclinical, but may evolve into severe respiratory disease.

We have previously shown that PRCV synergizes with bacterial lipopolysaccharide (LPS) in the induction of severe respiratory disease at the very early stage of infection (Van Reeth *et al.*, 2000). 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 at a 12 to 24 hours interval. This led to excessive production of proinflammatory cytokines in the lungs and the simultaneous appearance of acute respiratory distress, which did not occur after inoculation with PRCV or LPS only. It is still unknown, however, whether such a synergy between PRCV and LPS would also occur at later stages of the PRCV infection. This is of particular interest as the clinical course of coronavirus infections sometimes aggravates at an advanced stage of infection for unknown reasons. For example, humans infected with SARS coronavirus can develop the "acute respiratory distress syndrome" (ARDS) after the first week of infection (Peiris *et al.*, 2003). While it is unknown which factors are responsible for the "clinical worsening" at this late stage of infection, excessive production of cytokines seems to be involved (Beijing Group of National Research Project for SARS, 2003; Ng *et al.*, 2004; Wong *et al.*, 2004; Salto-Tellez *et al.*, 2005).

LPS induces proinflammatory cytokines after binding to its specific receptor complex (reviewed by Heumann and Roger, 2002). LPS-binding protein (LBP) and CD14 are major components of this complex. LBP is a soluble acute phase protein

produced by the liver and lung epithelial cells (Fenton *et al.*, 1998; Dentener *et al.*, 2000). LBP extracts single LPS molecules from bacterial membranes or LPS aggregates and transfers them to CD14. CD14 is a "pattern recognition receptor" which is expressed on the membranes of monocytes and macrophages and to a lesser extent on neutrophils (Antal-Szalmas, 2000). CD14 also exists in soluble from, which is enzymatically cleaved from the membrane or directly secreted from the cytoplasm (Bufler *et al.*, 1995). Membrane-bound or soluble CD14 binds LPS with high affinity and presents it to Toll-like receptor 4 (TLR4), which leads to activation of different proinflammatory genes. Studies in different species have demonstrated that impairment of CD14 or LBP function, by neutralization with antibodies or use of knockout animals, suppresses LPS-induced cytokine production, respiratory disease and shock (Ishii *et al.*, 1993; Frevert *et al.*, 2000; Tasaka *et al.*, 2003). Moreover, an increase of CD14 and LBP in the lungs can enhance LPS sensitivity and thus contribute to the development of inflammatory lung diseases, such as ARDS and asthma (Dubin *et al.*, 1996; Martin *et al.*, 1997; Strohmeier *et al.*, 2001). Alexis *et al.* (2001) found a tight correlation between levels of CD14 (both soluble and membrane-bound) in the lungs of humans and the inflammatory response to inhaled LPS.

We hypothesize that infection with PRCV will increase the amount of CD14 and LBP in the lungs, which may lead to LPS hypersensitivity.

The objectives of this study were to quantify the amount of CD14 and LBP in the lungs during the course of a PRCV infection. Both cell-associated and soluble CD14 were quantified and CD14-positive cells were characterized using different markers. In addition, we examined the effects of LPS exposure in the lungs at 3 and 7 days after PRCV inoculation.

Materials and Methods

Virus and LPS preparations

The Belgian 91V44 isolate of PRCV was used at the second passage in swine testis (ST) cells (Van Reeth and Pensaert, 1994). The virus stock was purified by sucrose density gradient centrifugation and contained <1.5 endotoxin units/ml by the gel-clot *Limulus* amoebocyte lysate assay (Pyrogent plus, BioWhittaker, Walkersville, USA). The inoculation dose was 10^7 50% tissue culture infective doses ($TCID_{50}$) per pig.

LPS of *Escherichia coli* (serotype 0111:B4, trichloroacetic acid extraction, 90% purity) was obtained from Sigma-Aldrich (St. Louis, USA) and used at a dose of 20 µg/kg body weight. 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 (Van Reeth *et al.*, 2000; Labarque *et al.*, 2002; Van Gucht *et al.*, 2003). Virus and LPS were diluted in sterile pyrogen-free phosphate-buffered saline (PBS; Gibco, Merelbeke, Belgium) to obtain a 3 ml inoculum.

Pigs, experimental design and sampling

Fourty-nine caesarean-derived colostrum-deprived pigs at the age of 3.5 weeks were used. The pigs originated from 6 sows and were housed in individual Horsefall-type isolation units with positive-pressure ventilation and fed with commercial ultrahigh-temperature-treated cow's milk.

The pigs were allocated to 4 groups. 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) days after inoculation (PRCV group). Seven pigs were inoculated with PRCV and 3 (n = 3) or 7 (n = 4) days later with LPS (PRCV-LPS group). These pigs were euthanized at 4 hours after the LPS inoculation. Previous experiments showed that this time point is optimal for detection of *de novo* synthesis of cytokines upon LPS inoculation (Van Reeth *et al.*, 2000). Three pigs were inoculated exclusively with LPS and euthanized 4 hours later (LPS group). Five pigs were mock-inoculated with PBS and euthanized 4 hours later (PBS group). All inoculations were performed intratracheally with a syringe and 20-gauge needle that was inserted through the skin cranial to the sternum.

The right lung was used for lung lavage as described previously (Van Reeth *et al.*, 1998). Recovered BAL fluids were separated into cells and cell-free fluids by centrifugation (400 × *g*, 10 min, 4°C). Cell-free BAL fluids were concentrated 20 times by dialysis against a 20% w/v solution of polyethylene glycol (MW 20000) and again centrifuged at 100000 × *g*. Tissue samples from the apical, cardiac and diaphragmatic lung lobes of the left lung were collected for virological and bacteriological examinations and immunofluorescence staining. For immunofluorescence staining, samples were embedded in methylcellulose medium,

frozen at -70°C and cryostat sections of 5 to 8 μm were made. Serum from all pigs was collected at euthanasia.

Clinical signs

All pigs were observed daily for clinical signs throughout the experiment. In addition, LPS-inoculated pigs were observed immediately before and every hour after the LPS inoculation for clinical signs. A score was attributed to tachypnoea (0: <60, 1: 60-90, 2: >90), abdominal thumping (0: absent, 1: present), severe dyspnoea (0: absent, 1: present), anorexia (0: absent, 1: present) and depression (0: absent, 1: present). The total score per pig was obtained by adding the scores for each parameter and ranged from 0 to 6.

Virological and bacteriological examinations

The amount of infectious PRCV in lung tissue homogenates of pooled samples of apical, cardiac and diaphragmatic lung lobes was determined by virus titration in swine testis (ST) cells according to standard procedures (Van Reeth and Pensaert, 1994). Bacteriological examinations of lung tissue samples were performed as described earlier (Van Gucht *et al*., 2003).

Antibody titration

PRCV-neutralizing antibodies in sera were titrated using a virus-neutralization assay as described by Voets *et al*. (1980). Two-fold dilutions of sera were mixed with the Purdue strain of transmissible gastroenteritis virus (TGEV) and inoculated on the swine kidney cell line SK6. Absence of cytopathic effect indicated the presence of neutralizing antibodies. PRCV and TGEV are closely related and neutralizing antibodies are fully cross-reactive.

BAL cell analysis

Total cell numbers in BAL fluids were counted in a Türk chamber. The percentage of neutrophils was determined using Diff-Quick® (Baxter, Düdingen, 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) as described earlier (Van Gucht *et*

al., 2005). Sialoadhesin (mAb 41D3) is expressed exclusively on the membrane of differentiated macrophages and SWC3a (mAb 74-22-15) is expressed on monocytes, macrophages and neutrophils (Thacker *et al.*, 2001; Vanderheijden *et al.*, 2003). Resident macrophages of uninfected lungs are sialoadhesin-positive, whereas newly infiltrated monocyte-macrophages are sialoadhesin-negative (Labarque *et al.*, 2000). 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 (Van Zaane and Hulst, 1987; Kirkham *et al.*, 1996).

Cytokine analysis

The bioassays for tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) have been described in detail elsewhere (Helle *et al.*, 1988; Van Reeth *et al.*, 1999). TNF-α activity was measured in a cytotoxicity assay with PK(15) subclone 15 cells and IL-6 was assayed by its capacity to stimulate proliferation of B9 cells. 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.

Quantification of cell-associated CD14 in lung tissue sections

The amount of cell-associated CD14 in lung tissue sections was quantified using immunofluorescence microscopy and image analysis as described previously (Van Gucht *et al.*, 2005). Briefly, 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 (Thacker *et al.*, 2001). Specificity of the CD14 staining was determined by deletion of MIL2 antibodies and use of irrelevant mouse mAbs.

Fifteen pictures (1 picture ≈ 0.1 mm^2) of the interstitium of each section were taken randomly using a fluorescence microscope (× 400) (Leica DM RBE, Leica Microsystems GmbH, Wetzlar, Germany), a Sony® 3CCD colour video camera (Sony Corporation, Tokyo, Japan) and Adobe® Photoshop® 5.0 LE (Adobe Systems, San

Jose, California, USA). Pictures were converted to black and white using the image analysis program Scion Image 1.62C (Scion Corporation, Frederick, Maryland, USA). Positive cells (green fluorescence) were converted to black pixels whereas negative cells and background were converted to white pixels. The number of black pixels, which depends on the number of positive cells and the amount of CD14 they express, was counted. The average number of black pixels was calculated for each lung (6 sections, 15 pictures/section) and expressed as a ratio compared to the number of black pixels in a reference sample. A section of the apical lung lobe of one of the PBS control pigs was used as the reference sample.

Quantification of soluble CD14 in BAL fluids

A flow cytometric assay, adapted from Antal-Szalmas *et al.* (2001), was used to quantify soluble CD14 in BAL fluids. This assay is based on the competition between membrane-bound CD14 on macrophages and soluble CD14 in BAL fluids for binding to anti-CD14 antibodies. The sensitivity is 30 to 120 ng soluble CD14/ml. Briefly, BAL fluids were mixed with MIL2 antibodies, resulting in an antibody concentration of 1 μg/ml. PBS was used as a negative control (no soluble CD14). The BAL fluid-MIL2 mixtures were incubated for 30 minutes at 37°C to allow binding of soluble CD14 with the antibodies. Porcine alveolar macrophages (5×10^6, obtained from conventional pigs at the age of 4 weeks) were then incubated with the BAL fluid-MIL2 mixtures for 1 hour at 4°C. Subsequently, the cells were incubated with FITC-labeled goat-anti-mouse polyclonal antibodies (4 μg/ml, 10% goat serum) (Molecular Probes, Eugene, Oregon, USA) for 1 hour at 4°C. The cells were washed 3 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 analysed for each sample. BAL fluids that contain soluble CD14 reduce the availability of MIL2 antibodies for binding to CD14 on the macrophages, resulting in a decrease of the MFI. The amount of soluble CD14 in BAL fluids was expressed as a ratio compared to the control sample without soluble CD14 (PBS). This ratio was calculated by dividing the MFI of the negative control sample (PBS) with the MFI of the BAL fluid samples.

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; mAb CY-90, Sigma-Aldrich, St. Louis, USA). Cytokeratin 18 is expressed in bronchiolar epithelial cells and type 2 pneumocytes (Schlichenmaier *et al.*, 2002).

Double immunofluorescence stainings for CD14 (mAb MIL2, IgG2b isotype) and sialoadhesin (mAb 41D3, IgG1 isotype), CD14 and SWC3a (mAb 74-22-15, IgG1 isotype) or CD14 and cytokeratin 18 (mAb CY-90, IgG1 isotype) were performed on sections of the cardiac and diaphragmatic lung lobes according to a previously described method (Van Gucht *et al.*, 2005). Briefly, sections were fixed in methanol and incubated consecutively with optimal dilutions of 41D3, 74-22-15 or CY-20 antibodies, FITC-labeled goat anti-mouse IgG1 polyclonal antibodies (4 μg/ml, 10% goat serum) (Santa Cruz Biotechnology, Santa Cruz, California, USA), biotinylated MIL2 antibodies (10 μg/ml), streptavidin-Texas Red (10 μg/ml) (Molecular probes, Eugene, Oregon, USA) and Hoechst 33342 (10 μg/ml) (Molecular probes, Eugene, Oregon, USA). Specificity of the double stainings was determined by deletion of primary antibodies and use of irrelevant mouse mAbs.

Digital images were taken using a Leica TCS SP2 laser scanning spectral confocal system linked to a Leica DM IRB inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

LBP quantification

LBP was quantified in BAL fluids and sera using an ELISA for LBP of different species, including swine (Hycult biotechnology, Uden, the Netherlands).

Statistical analysis

Standard two-sample Mann-Whitney tests were used to compare values between groups. P-values <0.05 were considered significant. Statistical analyses were performed using SPSS 11.0.

Results

Clinical signs during PRCV infection

Clinical signs were not observed in PBS control pigs. Seventeen out of thirty-four PRCV-inoculated pigs did not develop any general or respiratory signs after PRCV inoculation. Mild and transient abdominal thumping and/or increased breathing rates were occasionally seen in 12 pigs between 1 and 12 DPI (score 1 to 2). Only 5 pigs showed obvious respiratory disease (score >2) between 3 and 8 DPI. They developed tachypnoea, dyspnoea, depression and/or anorexia. Coughing was observed in 4 of the 17 pigs with clinical signs between 4 and 8 DPI.

Virus titers in the lungs

The evolution of virus titers in the lungs is shown in figure 1. PBS control pigs were negative for PRCV. 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 6 out of 10 pigs. Mean titers were highest (5.4 to 7.3 $TCID_{50}$/g lung tissue) from 1 to 5 DPI and strongly decreased at 7 DPI (3.3 $TCID_{50}$/g lung tissue) and 9 DPI (2.4 $TCID_{50}$/g lung tissue). The lungs of all pigs were free of bacteria by culture.

Anti-PRCV antibodies in serum

Mean antibody titers in sera are shown in table 1. Neutralizing antibodies were absent in PBS control pigs and in PRCV-inoculated pigs from 1 to 4 DPI. Two of three pigs had low antibody titers (16) at 5 DPI. All pigs had seroconverted by 7 DPI with titers ranging from 48 to 192. At later stages of infection (9-15 DPI), all pigs had high antibody titers (248 to 2560).

Evolution of BAL cells during PRCV infection

Mean numbers of the different types of BAL cells are presented in table 1. PBS control pigs had 64 to 142 × 10^6 BAL cells. Ninety-three percent of these cells were sialoadhesin-positive macrophages, 3.6% were sialoadhesin-negative monocyte-macrophages, 1% were T-lymphocytes and less than 1% were neutrophils or B-lymphocytes.

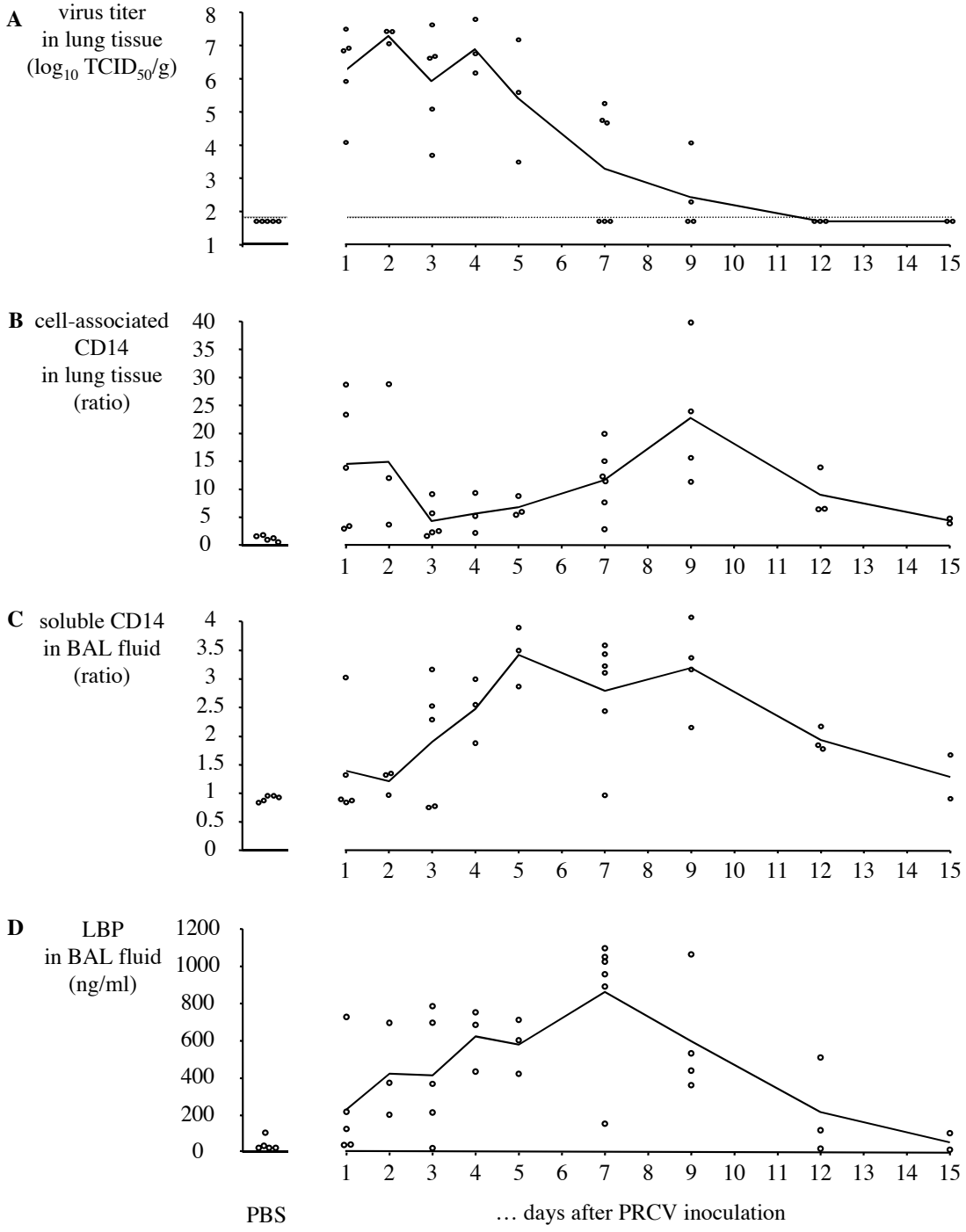

Figure 1. Evolution of virus titers (a), cell-associated CD14 in lung tissue (b), soluble CD14 in BAL fluids (c) and LBP in BAL fluids (d) throughout a PRCV infection. Each dot corresponds to one pig and the solid line represents the mean at each time point. The dotted line represents the detection limit for virus titrations.

Table 1. Mean numbers of different types of BAL cells and serum antibody titers during PRCV infection.

Inoculation with	No. of pigs	Euthanasia at…DPI with PRCV	BAL cells ± SD (× 10^6)						VN titer[6] ± SD
			total	sial$^+$ macro[1]	sial$^-$ mono-macro[2]	neutro[3]	T-lympho[4]	B-lympho[5]	
PBS	5	n.a.[7]	96 ± 32	79 ± 0.8	3.5 ± 1.8	0.4 ± 0.1	1.4 ± 1.3	0.7 ± 0.5	<2 ± 0
PRCV	5	1	164* ± 29	123 ± 18	20 ± 8	17* ± 15	2.3 ± 0.3	2.2 ± 1.1	<2 ± 0
	3	2	142* ± 19	74 ± 9	19 ± 2	13* ± 9	1.4 ± 0.1	1.6 ± 0.8	<2 ± 0
	5	3	148* ± 50	101 ± 40	15 ± 17	10* ± 13	1.9 ± 1.5	1.0 ± 0.7	<2 ± 0
	3	4	179* ± 45	66 ± 30	53 ± 35	9* ± 0.7	3.7 ± 0.8	2.0 ± 1.0	<2 ± 0
	3	5	225* ± 80	103 ± 35	101* ± 51	7* ± 5	20 ± 6	3.4 ± 0.9	11 ± 7
	6	7	304* ± 83	102 ± 50	44* ± 30	15* ± 8	51* ± 32	8.1* ± 5.4	114* ± 51
	4	9	407* ± 216	92 ± 32	131* ± 71	79* ± 108	52* ± 39	9.2* ± 2.9	422* ± 151
	3	12	179 ± 86	52 ± 27	53 ± 32	25* ± 19	12 ± 6	2.4* ± 1.3	1493* ± 798
	2	15	237 ± 89	148 ± 106	58 ± 6	6 ± 2	15 ± 3	2.9 ± 0.9	1600* ± 960

(1) sialoadhesin-positive macrophages, (2) sialoadhesin-negative monocyte-macrophages, (3) neutrophils, (4) CD3-positive T-lymphocytes, (5) IgM-positive B-lymphocytes, (6) virus-neutralizing antibody titer in serum, (7) not applicable,

(*) values marked with an asterisk differ significantly ($P < 0.05$) from those of the PBS control pigs

During PRCV infection, total cells increased significantly ($P < 0.05$) 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, which was probably due to rapid infiltration of blood monocytes towards the infected sites. Mean numbers peaked at 9 DPI and were 37 times higher than in the PBS control pigs. Neutrophils increased significantly ($P < 0.05$) 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 (240×10^6). T-lymphocytes and B-lymphocytes increased significantly ($P < 0.05$) from 5 to 9 and 7 to 12 DPI respectively.

Evolution of cell-associated CD14 in lung tissue sections during PRCV infection

The evolution of the amount of cell-associated CD14 in lung tissue sections is presented in figure 1. The amount of cell-associated CD14 in the lung tissue of PBS control pigs varied little (ratio of 0.4 to 1.6). Mean ratios were significantly ($P < 0.05$) increased at 1 and 2 DPI (10 times higher than in the PBS control pigs). CD14 expression in tissue sections varied however strongly between pigs during the first two days of infection, with increases in 3 out of 5 pigs at 1 DPI and in 2 out of 4 pigs at 2 DPI. Mean CD14 ratios were rather low at 3 and 4 DPI, but rose again significantly ($P < 0.05$) between 5 and 12 DPI with a peak at 9 DPI (15 times higher than in the PBS control pigs).

Identification of CD14-positive cells in tissue sections of PRCV-infected lungs

Figure 2 shows the distribution of different types of CD14-positive cells in lung tissue sections of PRCV-inoculated and PBS control pigs. Cells with high CD14 expression were scarce (13 ± 12 cells/mm^2) in the lungs of PBS control pigs. Typically, they had a monocyte-like phenotype, characterized by round cell morphology, kidney-shaped nucleus and expression of SWC3a, but not of sialoadhesin. Epithelial cells (cytokeratin 18-positive) were CD14-negative and most (>90%) resident macrophages (sialoadhesin-positive) expressed little CD14 on their membranes.

During infection there was an increase of 3 types of highly CD14-positive cells. (i) The first two days of infection, there was an accumulation of highly CD14-positive monocyte-like cells. These cells were clustered around and inside bronchioli and to a lesser extent around small bronchi. They were round, had round to kidney-shaped nuclei and were SWC3a-positive and sialoadhesin-negative. (ii) At later stages, there was a progressive increase of highly CD14-positive macrophage-like cells, which were clustered or scattered in the alveolar tissue. These cells were large, had round nuclei and were SWC3a-positive and sialoadhesin-negative. These cells were seen from 3 to 12 DPI, but were most abundant at 7 and 9 DPI. (iii) At 7 and 9 DPI, CD14-positive "type 2 pneumocytes" appeared in the alveolar tissue. These cells were positive for cytokeratin 18, but 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 expressed CD14.

Evolution of soluble CD14 in BAL fluids during PRCV infection

The evolution of soluble CD14 levels in BAL fluids during PRCV infection is presented in figure 1. 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 pigs. At later stages of infection, soluble CD14 was detected in BAL fluids of almost all pigs. Mean soluble CD14 ratios were significantly ($P < 0.05$) increased from 2 to 12 DPI and highest levels were detected between 5 and 9 DPI. At this stage of infection, ratios were 3 to 4 times higher compared to the PBS control pigs.

Evolution of LBP levels in BAL fluids and sera during PRCV infection

The evolution of LBP levels in BAL fluids is presented in figure 1. PBS control pigs had mean LBP levels of 25 ± 46 ng/ml. Mean LBP levels were significantly increased from 1 to 9 DPI with a peak of 863 ± 354 ng/ml at 7 DPI. Values returned to normal at 15 DPI.

The sera of PBS control pigs had mean LBP levels of 1722 ± 271 ng/ml and serum levels did not change upon infection.

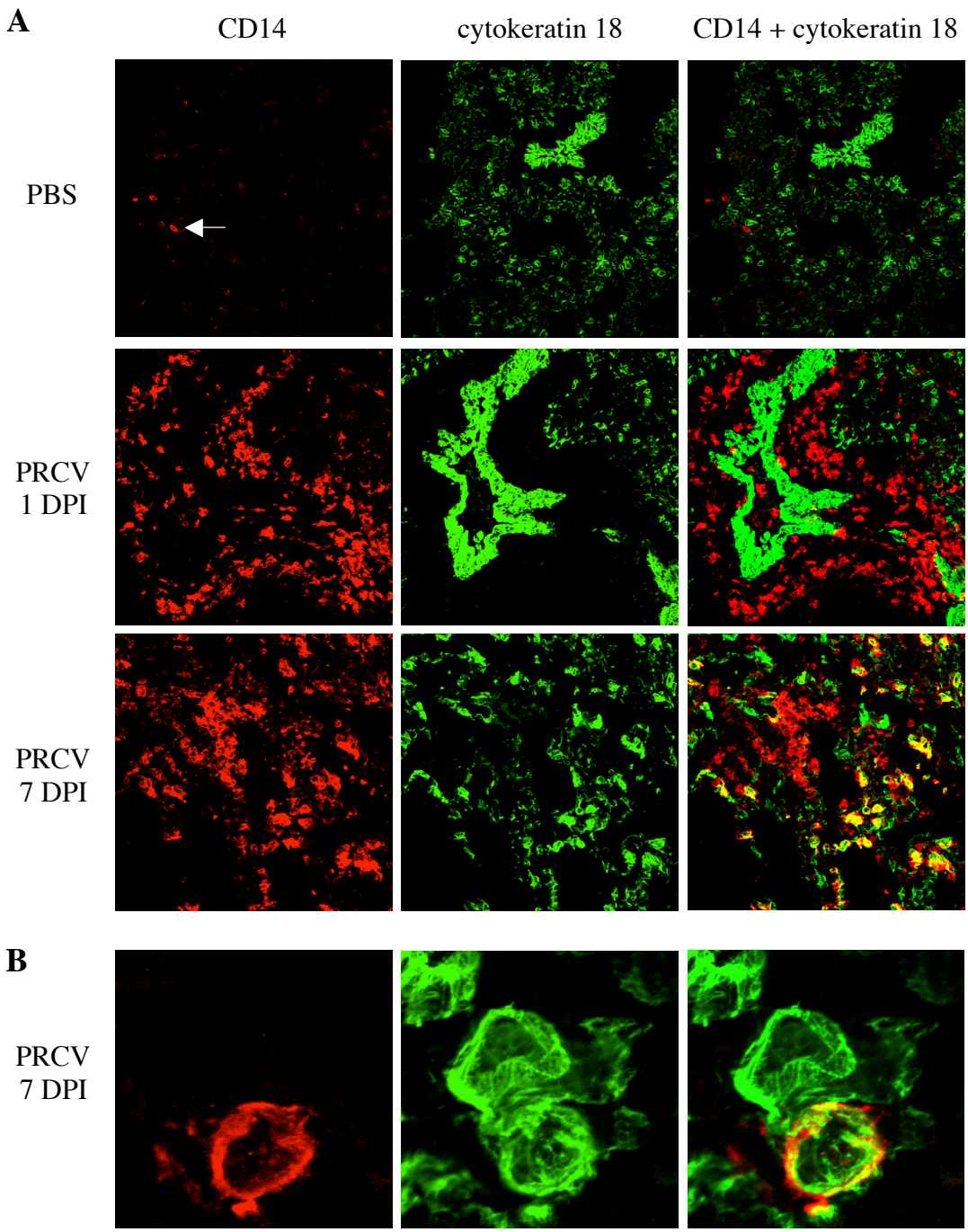

Figure 2. Double immunofluorescence staining for CD14 and cytokeratin 18 in the lung tissue of PBS- and PRCV-inoculated pigs. Figure 2a (× 400) demonstrates that PBS-inoculated pigs contain few highly CD14-positive cells in the lung tissue (arrow). At 1 DPI, there is a massive accumulation of highly CD14-positive monocyte-like cells near the bronchioli. At 7 DPI, the alveolar tissue contains numerous clusters of highly CD14-positive macrophage-like cells. At this time point of infection, part of the type 2 pneumocytes also express CD14, as indicated by the yellow colour in the merge picture. Figure 2b (× 1600) illustrates in more detail the expression of CD14 in a cytokeratin 18-positive pneumocyte.

Clinical signs and cytokine production after inoculation with PRCV and LPS

Table 2 compares virus titers, clinical scores, neutrophil numbers and cytokine titers in the lungs of PRCV-LPS inoculated pigs with those of pigs inoculated with LPS, PRCV or PBS alone. Pigs inoculated with PRCV or PBS alone are the same as described higher.

PBS control pigs had no detectable TNF-α and low levels of IL-6 (<20 to 100 U/ml) in their BAL fluids. PRCV-inoculated pigs had low to moderate levels of TNF-α (<20 to 435 U/ml) and substantial IL-6 levels (<20-20479 U/ml) at 3 DPI. At 7 DPI, TNF-α was undetectable and IL-6 levels had decreased strongly (43-997 U/ml). Pigs inoculated with LPS only showed no respiratory or general signs before or after the LPS inoculation. LPS induced a massive increase of neutrophils, low levels of TNF-α (20-42 U/ml) and moderate levels of IL-6 (220-2456 U/ml) in the BAL fluids.

Two of three pigs that were inoculated with PRCV and 3 days later with LPS developed severe respiratory disease, characterized by abdominal thumping, dyspnoea and tachypnoea (60-90 breaths/min) after the LPS inoculation. The remaining pig developed no signs of respiratory disease upon the LPS inoculation. All pigs were depressed after the LPS inoculation. Clinical signs were not observed before the LPS inoculation. TNF-α and IL-6 titers of the pigs with respiratory signs were at least 6 times higher than the mean titers of the corresponding PRCV and LPS control pigs. Cytokine titers of the pig without respiratory signs were comparable to those of the singly inoculated control pigs. Remarkably, no virus was isolated from the lungs of this pig. Neutrophil numbers of PRCV-LPS inoculated pigs were comparable to those of pigs inoculated with LPS only.

Two of the four pigs which were inoculated with PRCV and 7 days later with LPS developed severe respiratory disease, characterized by abdominal thumping, dyspnoea and severe tachypnoea (>90 breaths/min) after the LPS inoculation. The remaining two pigs developed no respiratory signs after the LPS inoculation. All pigs were depressed after the LPS inoculation. One pig that developed severe respiratory disease upon LPS inoculation, also showed mild tachypnoea (score = 1) before the LPS inoculation. Clinical signs were not observed in the other pigs before the LPS inoculation. 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 and LPS control pigs. Cytokine titers of the pigs without respiratory signs were comparable to those of the

pigs inoculated with LPS only and the neutrophil numbers in their BAL fluids were even markedly lower. Virus titers varied strongly at 7 days after PRCV inoculation, but this was not correlated with the LPS response.

Table 2. Virus titers, clinical scores, neutrophil infiltration and cytokine production in the lungs of PRCV-LPS inoculated pigs and control pigs.

Inoculum	Time of euthanasia after inoculation with		Virus titer (log_{10} $TCID_{50}$[(1)]/g)	Clin. score[(2)]	Neutrophils in BAL fluid ($\times 10^6$)	Cytokines in BAL fluid	
	PRCV (days)	LPS (h)				TNF-α (U/ml)	IL-6 (U/ml)
PBS	-[(3)]	-	<1.7	0	0.4	<20	71
	-	-	<1.7	0	0.4	<20	96
	-	-	<1.7	0	0.3	<20	58
	-	-	<1.7	0	0.5	<20	100
	-	-	<1.7	0	0.1	<20	<20
PRCV	3	-	6.7	0	5	435	4662
	3	-	6.6	1	7	160	8032
	3	-	7.6	0	35	295	20479
	3	-	3.7	0	13	47	3144
	3	-	5.1	0	0.8	<20	116
	7	-	4.8	2	30	<20	744
	7	-	4.7	2	14	<20	109
	7	-	<1.7	0	12	<20	997
	7	-	5.3	4	16	<20	249
	7	-	1.5	2	11	<20	43
	7	-	<1.7	0	6	<20	58
LPS	-	4	<1.7	0	148	20	220
	-	4	<1.7	0	932	42	2456
	-	4	<1.7	0	420	33	715
PRCV-LPS	3	4	7.7	**4**[*]	136	**1798**	**95276**
	3	4	7.0	**4**[*]	80	**2032**	**47277**
	3	4	<1.7	1	495	333	549
	7	4	2.6	**3**[*]	1274	**756**	**16890**
	7	4	<1.7	**4**[*]	403	**251**	**6312**
	7	4	4.2	1	12	<20	726
	7	4	<1.7	1	38	39	1264

(1) 50% tissue culture infective dose, (2) clinical scores were determined immediately before euthanasia and ranged from 0 to 6 (see text for the calculation of scores), (3) not applicable
values in bold represent a synergistic interaction between PRCV and LPS
(∗) these pigs had no or mild clinical signs (score 0 to 1) immediately before the LPS inoculation, but developed severe respiratory disease within 1 hour after the LPS inoculation (score 3 to 4)

Discussion

PRCV infection caused a significant increase of CD14 and LBP in the lungs. This increase was detected as early as 1 day after virus inoculation and remained significant until 12 days after inoculation. The cell types expressing CD14 varied throughout the PRCV infection. High levels of CD14 expression were subsequently found on monocyte-like cells (1-2 DPI), macrophage-like cells (3-12 DPI) and pneumocytes (7-9 DPI). In the beginning of infection, there was an accumulation of highly CD14-positive monocyte-like cells near the bronchioli (see figure 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, the majority of infected cells were pneumocytes, whereas infected bronchioli became rare. This agrees with the fact that we seldom observed peribronchiolar clusters of highly CD14-positive monocytes at later stages than 2 DPI. Surprisingly, highly CD14-positive pneumocytes appeared in the lung tissue at the late stage of infection. At this stage there was pronounced hyperplasia of type 2 pneumocytes and part of these cells expressed high levels of CD14. This was unexpected, as CD14 is considered to be a specific marker for myeloid cells (Martin *et al.*, 1994). Few researchers reported CD14 expression in other cell types. Two reports describe that lung epithelial cells of mice can be induced to express CD14 upon stimulation with IL-1 or TNF-α (Fearns and Loskutoff, 1997; Fearns and Ulevitch, 1998). In our study, however, the latter cytokines were not found in the BAL fluids of lungs with CD14-positive pneumocytes.

Two soluble components of the LPS receptor complex, namely soluble CD14 and LBP, increased significantly in the BAL fluids during PRCV infection. Soluble CD14 can present LPS to TLR4 on epithelial cells and thus render these cells sensitive to low amounts of LPS (Heumann and Roger, 2002). Bronchial and bronchiolar epithelial cells are among the first cells in the lungs that come into contact with LPS, 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.

Soluble CD14 and LBP are constitutively produced by the liver and circulate in the blood (Fenton *et al.*, 1998; Bas *et al.*, 2004). Still, we have indications that the increase of soluble CD14 and LBP in the BAL fluids is due to local production in the

lungs and not to plasma leakage from the blood. The increase of soluble CD14 and LBP levels in BAL fluids, for example, is not correlated with an increase of other plasma proteins, such as haptoglobin (unpublished data). Moreover, serum LBP levels did not rise during PRCV infection. Dentener *et al*. (2000) demonstrated that type 2 pneumocytes produce LBP in response to cytokines such as TNF-α and IL-6. Both cytokines are produced locally in the lungs during PRCV infection and could thus have triggered local LBP production.

The increase of soluble CD14 in the BAL fluids during infection could 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 (Bazil and Strominger, 1991; Hasday *et al*., 1997). Interestingly, the accumulation of highly CD14-positive monocytes near the bronchioli during the first 2 days of infection is followed by a two-fold increase of soluble CD14 in the BAL fluids on the third day of infection. Moreover, this increase of soluble CD14 coincides with a decrease of cell-associated CD14 in the lung tissue.

CD14 and LBP recognize bacterial LPS and potently enhance its endotoxic activity in the lungs. Several studies found a correlation between the amount of CD14 and LBP in the lungs and the sensitivity to LPS (Dubin *et al*., 1996; Martin *et al*., 1997; Alexis *et al*., 2001; Strohmeier *et al*., 2001). Indeed, part of the PRCV-inoculated pigs developed acute respiratory signs and high levels of TNF-α and IL-6 in the lungs after LPS exposure at 3 and 7 days of infection. These results add to an earlier study in which we demonstrated a similar synergy at 1 day of infection (Van Reeth *et al*., 2000). Thus, it seems that PRCV can sensitize the lung to LPS during the entire infection (1 to 7 DPI). The synergy in the induction of cytokines appeared to be stronger at 1 and 3 days of infection, but more experiments are necessary to confirm this.

Three of seven PRCV-inoculated pigs responded poorly to the LPS exposure. One of these pigs was exposed to LPS at 3 days after virus inoculation. No virus was found in the lungs of this pig, whereas high virus titers are expected at this time point after virus inoculation. The reason for this is unclear, but it indicates that productive virus infection is mandatory to sensitize the lungs to LPS at 3 DPI. Indeed, in contrast to the other pigs at 3 DPI, there was no increase of soluble CD14 or LBP in the BAL fluid of this pig. The 2 remaining “low responders” were exposed to LPS at 7 days

after PRCV inoculation. Their low LPS response was not correlated with lower virus titers or lower levels of soluble CD14 and LBP in the BAL fluids. So far, we can not explain the pronounced differences in LPS sensitivity at the end of infection. Moreover, the strikingly low number of neutrophils in the BAL fluids of the "low responder" pigs, suggests that most of the LPS was neutralized before it could activate cells. The biological effects of LPS depend on the balance between LPS receptor proteins and scavenger molecules (Hampton *et al.*, 1991; Stamme and Wright, 1999; Martin, 2000; Iovine *et al.*, 2002; Jiang *et al.*, 2003). Possibly, scavenger mechanisms were stimulated at the end of infection and this could have countered the effects of LBP and CD14 in these pigs.

Variation in clinical development at an advanced stage of infection is also a feature of SARS CoV infection of humans. Peiris *et al.* (2003) describe that 38% of SARS CoV-infected patients developed severe pulmonary disease around 9 days of infection, whereas viral pneumonia resolved in other patients. Several authors suggested that the clinical worsening of SARS patients during the second week of infection is due to an "immunopathological response" and not to direct viral damage (Holmes, 2003; Hsueh *et al.*, 2003; Peiris *et al.*, 2003). SARS patients typically develop ARDS at a time point when virus load is decreasing and specific antibodies are mounted. In our study, we found a strong increase of CD14 expression on pneumocytes and macrophages at a similar time point of infection. We hypothesize that the SARS CoV-associated hyperinflammation and acute respiratory distress might have a similar aetiology as the LPS hyperreactivity in PRCV-infected pigs. The expression of pattern recognition receptors has not been studied in SARS CoV-infected lungs, but a massive load of "activated" macrophages is a typical histological feature of these lungs. Although not proven, several authors suggest that SARS may be caused by overproduction of proinflammatory cytokines by infiltrated macrophages (Nicholls *et al.*, 2003; Van Bever *et al.*, 2004).

In earlier experiments, we found that infection with porcine reproductive and respiratory syndrome virus (PRRSV), another respiratory virus of swine, also increases CD14 and LBP in the lungs, which is likewise associated with enhanced LPS sensitivity. The evolution of CD14 expression and LBP levels differs somehow between both virus infections. Firstly, the increase of CD14 in PRRSV-infected lungs is mainly due to infiltration of monocyte-like cells, whereas most macrophages

express little CD14 and pneumocytes remain CD14-negative. Secondly, the increase of CD14 and LBP in the lungs is tightly correlated with PRRSV replication. The highest amounts are found at the peak of virus replication and levels decrease strongly as PRRSV titers decline at the end of infection. This is clearly different for PRCV-infected lungs, where high levels of CD14 and LBP are found at the end of infection. The reasons for these differences are unclear. Nevertheless, it is tempting to speculate that the increase of these LPS recognition molecules is involved in the enhanced LPS sensitivity during both virus infections.

In conclusion, we found a strong increase of cell-associated CD14, soluble CD14 and LBP in the lungs of PRCV-infected pigs. We also demonstrated a synergy between PRCV and LPS in the induction of respiratory signs and proinflammatory cytokines at both early and late stages of infection. CD14 and LBP are primary components of the LPS receptor complex and their increase in the lungs could account for the increased LPS sensitivity during virus infection. The true role of CD14 and LBP in the virus-LPS synergy will be explored in further studies.

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5.

GENERAL DISCUSSION

General discussion

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine respiratory coronavirus (PRCV) are respiratory viruses of swine, that are important primary agents of multifactorial respiratory disease. The research in this thesis was inspired by the apparent paradox that both viruses are involved in respiratory disease in the field, but experimental infections cause no or mild disease. In order to cause overt respiratory disease, PRRSV and PRCV need to interact with secondary agents, which are usually absent under experimental conditions. This is not surprising as animal experiments are performed in sterile rooms, with minimal dust concentrations and optimal climate. We believe that an important secondary factor could be bacterial lipopolysaccharide (LPS). LPS is the most important inflammatory component of Gram-negative bacteria and is present in organic dust of swine stables (Rietschel *et al*., 1994; Rylander, 2002). Also, there is convincing evidence that natural exposure to LPS-contaminated swine dust causes airway inflammation and lung function decline in humans (reviewed by Thorn, 2001).

In an attempt to study multifactorial respiratory viral disease, we have performed subsequent inoculations of pigs with either PRRSV or PRCV, followed by a secondary inoculation with LPS (Van Reeth *et al*., 2000; Labarque *et al*., 2002; Van Gucht *et al*., 2003, 2005a and b). In our experiments, the interval between virus and LPS inoculations ranged from 3 to 14 days for PRRSV and from 1 to 7 days for PRCV. Virus-infected pigs that are exposed to LPS develop severe respiratory and general disease, whereas uninfected pigs do not. The first virus-LPS induced disease signs are usually vomiting and shivering at 40 minutes to 1 hour after LPS. Respiratory signs start within 1 hour after LPS, reach a climax 2 to 4 hours later and are clearly decreasing 12 hours later. Typical signs include severe tachypnoea (54-154 breaths/minute), dyspnoea, high fever (≥41°C) and depression. The clinical synergy between PRRSV and LPS has proven to be very reproducible. Since the initial experiment, 108 pigs in 12 different experiments have been inoculated with both PRRSV and LPS, together with the appropriate control pigs. The clinical synergy was observed in more than 85% of pigs. The synergy between PRCV and LPS has been reproduced in 16 out of 19 pigs spread over 3 experiments.

A main aim of this thesis was to unravel the pathogenesis of virus-LPS induced respiratory disease, with special emphasis on the possible role of proinflammatory cytokines. For this purpose, gnotobiotic pigs were inoculated intratracheally with PRRSV and 3 to 14 days later with LPS (chapter 3.1). As expected, PRRSV-infected pigs developed acute respiratory signs upon intratracheal LPS inoculation, in contrast to pigs inoculated with PRRSV or LPS only. Moreover, peak tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 titers were 10 to 100 times higher in PRRSV-LPS inoculated pigs than in the singly inoculated pigs and this excessive cytokine production was associated with disease. Though a poor cytokine inducer as such, PRRSV sensitized the lungs for the production of proinflammatory cytokines upon LPS stimulation. In contrast, neutrophil infiltration, macroscopic and microscopic lesions in the lungs of PRRSV-LPS inoculated pigs resembled the combined effects of the single PRRSV and LPS inoculations without synergy. The histological lung lesions of PRRSV-infected pigs were little aggravated by subsequent LPS exposure. This suggests that the difficult breathing of PRRSV-LPS inoculated pigs resulted from functional disturbances such as bronchoconstriction, rather than from structural lung damage.

The pathogenesis of PRRSV-LPS induced disease appears to be similar to the pathogenesis of PRCV-LPS induced disease. The combination of PRCV and LPS did not markedly enhance neutrophil infiltration or lung lesions, but did cause an exaggerated production of proinflammatory cytokines in the lungs, similar to the PRRSV-LPS combination (Van Reeth *et al*., 2000; chapter 4.2). The profile and quantity of cytokines in the lungs was similar for both combinations. These clear resemblances suggest that both viruses sensitize the lungs to LPS in a similar way.

The strong association between the levels of proinflammatory cytokines, particularly TNF-α, and the respiratory signs after virus-LPS exposure, led us to assume that these cytokines where somehow responsible for the observed respiratory disease. This hypothesis was fed by previous studies with swine influenza virus. Van Reeth *et al*. (1998 and 2002) found that the appearance of clinical signs during influenza virus infection is tightly correlated with the levels of interferon-α (IFN-α), TNF-α, and IL-6 in the BAL fluids. Indeed, IL-1 and TNF-α can cause bronchial hyperreactivity leading to asthma-like symptoms (Kips *et al*., 1992; Thomas *et al*., 1995). Moreover, the combination of TNF-α and IL-1 causes bronchoconstriction

through the induction of thromboxanes in rats (Martin *et al.*, 2001). Possibly, overproduction of these cytokines after virus-LPS inoculation causes increased and sustained contraction of bronchi, which may explain the acute respiratory signs. However, in a preliminary experiment, we were unable to bear out such a process. In that experiment, treatment of PRRSV-LPS exposed pigs with the bronchodilatory drugs atropine and/or clenbuterol (β_2-agonist) did not improve respiratory signs. Besides airflow obstruction, LPS exposure can also hamper alveolar-capillary diffusion (Herbert *et al.*, 2002). More refined techniques such as lung function tests and blood gas analysis are necessary to identify the cause(s) of difficult breathing after virus-LPS exposure.

In an attempt to confirm the role of proinflammatory cytokines in virus-LPS induced respiratory disease, PRRSV-infected pigs were treated with pentoxifylline prior to the moment of LPS exposure (chapter 3.2). Pentoxifylline is a xanthine that inhibits the intracellular phosphodiesterase enzyme, resulting in decreased production of proinflammatory cytokines (Neuner *et al.*, 1994). Unexpectedly, pentoxifylline had only a minor effect on cytokine production in the lungs, but at the same time caused a significant reduction of respiratory signs and fever. Possibly, pentoxifylline inhibited other disease mediators or directly caused bronchodilatation, leading to improved airflow (Cortijo *et al.*, 1993). We should bear in mind that virus-LPS exposure probably induces many other types of mediators, which might potentiate the biological effects of proinflammatory cytokines. LPS stimulation of human monocytes, for example, leads to increased transcription of an impressive 118 genes of which many cytokines, chemokines and enzymes (Suzuki *et al.*, 2000). Fourteen percent of LPS-induced transcripts coded for unknown proteins. Moreover, Germonpre *et al.* (1999) revealed that monocyte-macrophages release substance P, a neuropeptide with potent bronchoconstrictory activity, upon LPS exposure.

The significance of the interaction between viruses and LPS in respiratory disease in the field is difficult to assess and may depend on several factors, such as the level of virus replication in the lungs and the level of LPS exposure. Exposure to LPS is variable and depends on several factors, such as concentration of stable dust, load of Gram-negative bacteria in the lungs and use of antibiotics. In our experiments, LPS was administered to pigs at a dose of 20 µg/kg body weight. Most pigs weighed 5-10 kg and thus received 100-200 µg LPS. Assuming an environment with an LPS

concentration of 4.9 μg/m^3 air (Zhiping *et al.*, 1996) and a respiratory volume of 0.3 m^3/hour, pigs of the same weight as in our experiments would be exposed to a total dose of airborne LPS of approximately 35 μg per day. Thus, one could argue that pigs in the field are exposed to lower doses of airborne LPS than those used experimentally. Urbain *et al.* (1999) demonstrated that pigs, in contrast to humans, do not develop lung inflammation or respiratory signs upon inhalation of LPS-contaminated dust in concentrations commonly found in swine buildings. Possibly, PRRSV or PRCV infection could be the trigger that renders the lungs of pigs susceptible to the detrimental effects of LPS in swine dust.

LPS is not only inhaled with dust, but it is also released locally in the lungs during an infection with Gram-negative bacteria. It was shown that at least part of the lung lesions and clinical signs of an infection with Gram-negative bacteria, such as *Actinobacillus pleuropneumoniae*, are caused by the release of LPS from the bacterial cell wall (Udeze *et al.*, 1987; Idris *et al.*, 1993). The LPS release from 10^5 colony forming units (CFU) of *E. coli* during a 6 hours growth period *in vitro* is 16.8 μg (Van Den berg *et al.*, 1992). Theoretically, $10^{4.1}$ CFU/g lung tissue would thus be sufficient to produce 200 μg of LPS in the lungs during the same period (assuming a lung weight of 100 g). Lung infection with Gram-negative bacteria often results in higher titers (Haesebrouck F., personal communication). It is thus likely that most infections with Gram-negative bacteria will produce high enough amounts of LPS to induce excessive cytokine production in virus-infected lungs. In theory, any infection of the deeper lungs with Gram-negative bacteria has the potential of synergizing with PRRSV or PRCV, if sufficient amounts of LPS are released. We did not perform dual inoculations with virus and whole Gram-negative bacteria, but several groups were unable to demonstrate a clinical synergy between PRRSV and bacteria, like *Haemophilus parasuis* and *Pasteurella multocida* (Cooper *et al.*, 1995; Carvalho *et al.*, 1997; Solano *et al.*, 1997). It should be mentioned, however, that bacteria could not be isolated from the lungs of most dually inoculated pigs in these studies. This means that there was no opportunity for an interaction between PRRSV and locally released LPS. Nevertheless, mixed infections with Gram-negative bacteria are very common under field circumstances. A retrospective study of Zeman (1996) revealed that more than halve of 221 PRRSV-infected lungs were concurrently infected with

bacteria, such as *Pasteurella multocida*, *Haemophilus parasuis* and *Salmonella* species.

The biological activity of LPS, which is determined by the structure of the lipid A component, depends on the species of bacteria (Erridge *et al.*, 2002). We used LPS derived from *E. coli*, which is generally considered to have high endotoxic activity. The endotoxic activity of LPS of Gram-negative bacteria that colonize the airways of pigs has not been studied. It is, however, likely that LPS of several of these bacteria, such as *Bordetella bronchiseptica*, has lower endotoxic activity than that of *E. coli*. The bulk of LPS in swine dust originates most likely from enterobacteriaceae, such as *E. coli*, and can thus be considered highly endotoxic (Zucker *et al.*, 2000; Erridge *et al.*, 2002).

We do not know whether prolonged LPS exposure will lead to chronic respiratory disease or, on the contrary, to LPS tolerance. LPS tolerance has been shown in numerous animal models and is characterized by a decreased sensitivity to LPS after repeated LPS exposure (reviewed in Cavaillon *et al.*, 2003). In unpublished experiments, we have exposed PRRSV-infected pigs up to 3 times to LPS at 3, 6 and 9 days after virus inoculation without a decrease of the clinical response to LPS. This suggests that PRRSV-infected pigs do not become refractory to LPS. Also, recent research demonstrated that 5-day-long and 8-week-long exposure of mice to an aerosol of LPS led to respectively sustained cytokine production and chronic pneumonia (Brass *et al.*, 2003).

An important part of the research in this thesis aspired to explain how virus infections can synergize with LPS in the lungs. LPS recognition and inflammation is initialized by the cooperative interplay between the “LPS-binding protein” (LBP), the membrane-bound or soluble forms of CD14 and the recently identified “Toll-like receptor 4” (TLR4) (for review see Martin, 2000). Together, these proteins form the “LPS receptor complex” which is presented in figure 4 on page 20. The biological effect of LPS in the lungs depends on two antagonistic processes. On the one hand, LPS can bind to scavenger receptors, surfactant protein A or “bactericidal permeability increasing protein”, leading to neutralization and degradation without cytokine production (Hampton *et al.*, 1991; Stamme and Wright, 1999; Iovine *et al.*, 2002). On the other hand, LPS can bind to the LPS receptor complex, leading to intracellular signaling, stimulation of inflammatory genes and cytokine production.

So, the inflammatory effect of LPS in the lungs depends on the balance between scavenger molecules and signaling receptors (Martin, 2000; Jiang *et al.*, 2003). Our working hypothesis was that virus infection in the lungs would lead to increased recognition and subsequent inflammatory signaling of LPS. The early recognition of LPS is mediated by LBP and CD14. We speculated that the amount of both proteins would be limited in "healthy" uninfected lungs and that infection with PRRSV or PRCV would significantly increase their levels. This hypothesis was inspired by earlier work on the pathogenesis of inflammatory bowel disease. Resident macrophages of healthy intestines express no or low CD14 and are unresponsive to LPS (Smith *et al.*, 2001; Smythies *et al.*, 2005). The gut naturally contains high amounts of LPS and the absence of CD14 is seemingly important to prevent chronic inflammation. In contrast, intestines affected with inflammatory bowel disease are typically infiltrated with CD14-positive monocytes (Rugtveit *et al.,* 1997). These highly LPS-responsive cells are assumed to contribute significantly to the chronic inflammation of the intestinal wall.

We found that lungs of uninfected pigs contained relatively low amounts of CD14 and LBP (chapters 4.1 and 4.2). Most resident macrophages expressed little CD14 and occasionally a highly CD14-positive monocyte was found in the lung tissue. Accordingly, LBP levels varied from 0 to 100 ng/ml BAL fluid, which is 20 times less than normal serum levels. The low amount of CD14 and LBP may protect the lungs from an excessive response to common LPS exposure. This "quiescent" state alters radically during PRRSV or PRCV infection. Both virus infections caused a massive increase of the LPS recognition molecules in the lungs. LBP levels in BAL fluids increased up to 14 and 35 times during PRRSV and PRCV infection respectively. Similarly, we found a 40 and 15 times increase of cell-associated CD14 in the lung tissue. Moreover, PRCV-infection caused a significant increase (× 4) of soluble CD14 in the BAL fluids. Soluble CD14 can render CD14-negative cells, such as airway epithelial cells, sensitive to LPS (Pugin *et al.*, 1993). This parameter was not studied in BAL fluids of PRRSV-infected lungs. It is tempting to speculate that the increase of LPS and CD14 is responsible for the increased LPS response during infection. We did not prove a causal relationship in our studies, but many other researchers found a correlation between the amount of CD14 and LBP in the lungs

and the sensitivity of the lungs to LPS (Dubin *et al.*, 1996; Martin *et al.*, 1997; Alexis *et al.*, 2001; Strohmeier *et al.*, 2001).

We now have indications that both PRCV and PRRSV sensitize the lungs to LPS similarly through the increase of CD14 and LBP. This is remarkable as the pathogenesis of both viruses differs explicitly (Van Reeth *et al.*, 1999). PRRSV has a strict tropism for macrophages and causes a slow and persistent lung infection. Maximum virus replication occurs exceptionally late at 7 to 14 days after inoculation and low amounts of virus are able to persist for several weeks thereafter. In contrast, PRCV has a tropism for epithelial cells and causes a typical acute lung infection. Replication lasts one week and the virus is then fully cleared by an effective immune response. It is thus not surprising that we found some major differences in the profile of CD14 expression and LBP levels between both virus infections.

Firstly, CD14-positive cell types in the lungs are different for both virus infections. PRRSV-infected lungs are typically infiltrated with massive amounts of highly CD14-positive monocytes, whereas PRCV infection also triggers a progressive increase of highly CD14-positive macrophages and hyperplastic type 2 pneumocytes towards the end of infection.

Secondly, the amounts of CD14 and LBP in the lungs are tightly correlated with the kinetic profile of PRRSV replication, but not with that of PRCV replication. Highest amounts of CD14 and LBP are found at the peak of PRRSV replication and levels decrease strongly as virus titers decline towards the end of infection. This is clearly different for PRCV-infected lungs, where high amounts of CD14 and LBP are found at the end of infection, when a specific immune response is mounted and most virus has been cleared from the lungs. The reasons for these differences are thus far unclear.

LBP and CD14 are also involved in the recognition of conserved molecules of other types of pathogens, such as peptidoglycan and lipoteichoic acid, which are components of the cell wall of Gram-positive bacteria (Muhvic *et al.*, 2001; Schroder *et al.*, 2003). It is therefore possible that PRRSV and PRCV also sensitize the lungs for other bacterial cell wall components. Moreover, research indicates that LPS, peptidoglycan and lipoteichoic acid synergize in the induction of cytokines (De Kimpe *et al.*, 1995; Wray *et al.*, 2001). Stable dust contains a mixture of these

molecules and the effect of LPS may thus be enhanced by other cell wall components (Zhiping *et al.*, 1996).

To our knowledge, PRRSV and PRCV are the first respiratory viruses that are shown to synergize with LPS in the induction of respiratory disease. The question arises whether the sensitisation to LPS is a typical feature of PRRSV and PRCV infections or may also occur during infection with other respiratory viruses. PRRSV and PRCV are both enveloped RNA viruses that belong to the order *Nidovirales*, based on a common genomic organisation and cellular replication strategy (reviewed by Cavanagh, 1997). Despite their classification in the same order of viruses and some structural resemblances, the pathogenesis of both virus infections differs strikingly as outlined on pages 4 to 5 and 10 to 11. Importantly, the target cells for replication are unrelated and the interferon-inducing capacity differs completely for both viruses. It is thus tempting to speculate that the increase of LPS recognition proteins and sensitisation to LPS is an unspecific response of the lungs to viral infection and possibly also occurs during infection with other, unrelated respiratory viruses. Moreover, mechanical and thermal insults have been reported to increase CD14 and LBP expression in the lungs (Fang *et al.*, 2002). Recent experiments in rabbits, for example, demonstrated that mechanical ventilation with a large tidal volume causes up-regulation of CD14 in the lungs, which is followed by increased LPS sensitivity (Moriyama *et al.*, 2004). It is thus likely that a wide variety of agents can prime the lungs to LPS under experimental conditions. Nevertheless, respiratory viruses like PRRSV and PRCV may play a unique pioneer role in sensitizing the lungs to LPS or other environmental agents in the field. Unlike most bacteria, PRRSV and PRCV are fully capable of infecting healthy "unspoiled" lungs. In this regard, respiratory viruses may be the first pieces of the puzzle that is multifactorial respiratory disease.

The synergy between virus and LPS is remarkably potent. Virus infection potentiates LPS-induced TNF-α production up to 100-fold in the lungs, which often results in TNF-α levels exceeding 1000 U/ml of BAL fluid (Van Reeth *et al.*, 2000; chapters 3.1, 3.2 and 4.2). These levels are many-fold higher than induced by any single respiratory virus infection, including influenza virus infection, or combination of virus infections ever examined in our laboratory. Moreover, careful scrutiny of the literature learns that a cytokine synergy of this magnitude in the lungs is undocumented for any other combination of agents. Eileen Thacker and co-workers,

for example, studied the interactions between PRRSV and *mycoplasma hyopneumoniae* infections and reported a mere 1.5-fold increase of TNF-α production in the lungs of dually infected pigs, compared to pigs infected with each agent alone (Thanawongnuwech *et al.*, 2004). Important in this regard is that the cell wall of *mycoplasma* species contains no LPS.

Most pigs become infected with PRRSV, PRCV and potentially many other viruses during the first weeks after weaning. In a very short time frame, lungs of growing pigs are thus loaded with LPS recognition proteins and LPS-responsive cells in an environment burdened with LPS and other bacterial compounds. This potentially explosive situation might lead to excessive cytokine production in the lungs of some pigs and tilt primarily subclinical virus infections above a clinical threshold.

Our findings throw a new light upon the role of virus infections in exacerbations of inflammatory lung diseases such as asthma and the "acute respiratory distress syndrome" (ARDS). Respiratory viruses, such as rhinovirus and respiratory syncytial virus, are known to trigger acute asthma attacks (Gern, 2004; Tan, 2005). Johnston *et al.* (2005), for example, found that the "September epidemic" of acute asthma attacks occurs mainly in children with underlying rhinovirus infections, which thrive abundantly during that time of the year. The mechanisms hereof are largely unknown, but our work suggests that virus-induced sensitisation to LPS might be a factor. This hypothesis is fed by the knowledge that humans are particularly sensitive to environmental LPS, which is also a known risk factor for acute asthma exacerbations (Michel *et al.*, 1996).

Recently, a new coronavirus with a tropism for pneumocytes, named "severe acute respiratory syndrome" coronavirus (SARS CoV), emerged in humans (Ksiazek *et al.*, 2003). Although the infection sets off during the first week as a mild viral pneumonia, with fever as the main symptom, a minority of patients develop the lethal ARDS during the second week of infection (Peiris *et al.*, 2003, Van Bever *et al.*, 2004). Typically, the clinical worsening of SARS CoV infection occurs when the virus load in the lungs has decreased and virus-specific antibodies appear in the circulation (Wang *et al.*, 2004). This led several authors to assume that SARS is an "immunopathological" disease, but the true pathogenesis of the syndrome remains enigmatic. Our work with PRCV provides a new way of thinking regarding clinical exacerbations of coronavirus infections. Indeed, we demonstrated that the lungs are

bulked with CD14 and LBP at the end of infection and preliminary work demonstrates that this may be associated with an increased LPS response. Interestingly, SARS-affected lungs are typically loaded with "activated" macrophages and the syndrome is associated with excessive production of cytokines (Huang *et al.*, 2005; Salto-Tellez *et al.*, 2005). LPS recognition proteins have not been quantified in SARS-CoV infected lungs, but an exaggerated response to LPS or other bacterial compounds might contribute to the development of SARS.

It is commonly accepted that viruses cooperate with secondary bacteria in the induction of severe respiratory disease, yet little is known about the mechanisms of virus-bacterium cooperation. Most proposed mechanisms emanate from the assumption that viruses damage barriers, decrease innate and/or specific immune responses and that this opens the gate for secondary invaders. The latter are in turn responsible for clinical worsening (reviewed by Brockmeier *et al.*, 2002). Indeed, mixed infections with viruses and bacteria are very common in pigs, but a true decrease of antibacterial lung defences has not been proven for most respiratory virus infections of swine or other species. This thesis describes a new kind of interaction between respiratory viruses and bacteria in the induction of severe respiratory disease. We found that infection with PRRSV or PRCV sensitizes the lungs of pigs to LPS, a cell wall component of Gram-negative bacteria. Virus-infected pigs that are exposed to an as such "harmless" dose of LPS develop excessive amounts of proinflammatory cytokines in their lungs, together with acute respiratory distress. Moreover, we found that infection with PRRSV or PRCV increases the amount of LBP and CD14, two components of the LPS receptor complex, in the lungs. We propose that the increase of both proteins in the lungs during virus infection enhances the early recognition and inflammatory effects of LPS. However, the exact role of CD14 and LBP in the clinical synergy between virus and LPS requires further study.

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6.

CONCLUSION AND FUTURE PROSPECTS

Conclusion and future prospects

As a general conclusion, it can be stated that infection with PRRSV or PRCV sensitizes the lungs of pigs to LPS, which is a major inflammatory component of the cell wall of Gram-negative bacteria. Virus-infected pigs that are exposed to an as such "harmless" dose of LPS develop excessive amounts of proinflammatory cytokines in their lungs, together with acute respiratory distress. Prior treatment of these pigs with a phosphodiesterase inhibitor reduces the severity of clinical signs, but it is unclear through which mechanisms this drug manifests its effects. Further work revealed that infection with PRRSV or PRCV increases the amount of LBP and CD14 in the lungs. We propose that the increase of both components of the LPS receptor complex enhances the early recognition and inflammatory effects of LPS. Possibly, the interaction between virus infection and bacterial LPS represents one of the catalysts of multifactorial lung disease.

Several issues discussed in this thesis require further research. Importantly, we still need to prove that the increased amounts of LBP and CD14 in the lungs contribute to the enhanced response to LPS during virus infection. A plausible approach to achieve this is neutralization of LBP and CD14 by use of specific antibodies. Two strategies can be followed here. One consists of *in vivo* treatment with antibodies to elucidate whether neutralization of LBP and/or CD14 attenuates disease signs and cytokine production in the lungs of virus-infected pigs after instillation of LPS. This approach requires large amounts of antibodies, but proved to be successful in other species, such a mice and rabbits. An *in vitro* model with LPS-responsive cells may represent a less elaborative alternative. BAL fluids of virus-infected pigs, containing elevated levels of LBP and soluble CD14, are likely to enhance cytokine production in cultured cells after LPS challenge. If neutralization of LBP and/or soluble CD14 abolishes these effects, the biological significance of elevated levels of LBP and soluble CD14 in BAL fluids of virus-infected lungs is confirmed.

In addition, the effect of virus infection in the lungs on the expression of two remaining components of the LPS receptor complex, namely TLR4 and MD-2, merits attention. The biology of Toll-like receptors represents an exciting and promising new chapter in the "book" of infectious animal diseases. The nucleotide and amino acid

sequences of porcine TLR4 were recently published (GenBank®: accession numbers AB188301 and BAD36843). Although no specific antibodies against porcine Toll-like receptors are available at this moment, it is likely that they will be in the near future. This would enable us to study the impact of virus infection on the expression of these signaling receptors that reside at the interface of the extra- and intracellular milieu.

The general discussion of this thesis formulates the hypothesis that sensitisation to LPS might be a common feature of different types of respiratory viruses. Indeed PRRSV and PRCV cause different types of infections in the lungs, still both infections prime the lungs similarly for an increased LPS response. This hypothesis can be challenged by infecting pigs with a third non-related respiratory virus, such as swine influenza virus, and subsequently assessing their response to LPS. Moreover, it is worth to examine whether virus infection sensitizes the lungs to cell wall components of other types of micro-organisms, such as lipoteichoic acid and peptidoglycan of Gram-positive bacteria or β-glucan of fungi. Organic dust contains a mixture of these molecules and the lungs are thus naturally exposed to a combination of different cell wall toxins.

The combination of virus and LPS in the lungs provides a unique model to study the role of different proinflammatory cytokines in multifactorial respiratory disease. Impressive amounts of proinflammatory cytokines are produced in the lungs at a highly predictable time point, starting at 1 hour after the LPS inoculation. As illustrated in chapter 3.2, treatment with anti-inflammatory drugs that target enzymes involved in cytokine production or activity is not specific enough to pinpoint the contribution of different cytokines in respiratory disease. The latter goal is better achieved by selective blocking of cytokine effects with neutralizing antibodies or receptor antagonists. This will be a focus of future research in our laboratory.

7.

SUMMARY - SAMENVATTING

Summary

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine respiratory coronavirus (PRCV) are respiratory viruses of swine, that are important primary agents of multifactorial respiratory disease. Little is known about the mechanisms of cooperation between respiratory viruses and other agents in the induction of multifactorial lung disease. In an attempt to study this, we have performed subsequent inoculations of pigs with either PRRSV or PRCV, followed one or several days later by an intratracheal inoculation with bacterial lipopolysaccharide (LPS). LPS is the most important inflammatory component of the cell wall of Gram-negative bacteria and is present in organic dust of swine stables. Virus-infected pigs that are exposed to LPS (20 μg/kg) in the lungs typically develop severe respiratory disease signs, whereas uninfected pigs that are exposed to the same dose of LPS do not. Thus, both respiratory viruses synergize with LPS in the induction of clinical signs.

The main aims of this thesis were to unravel the pathogenesis of virus-LPS induced respiratory disease, with special emphasis on the role of proinflammatory cytokines as mediators of virus-LPS disease, and to explore possible mechanism(s) of virus-induced sensitisation to LPS.

Chapter 1 reviews the pathogenesis of PRRSV and PRCV infections and the biological effects of LPS on the lungs. We explain how LPS is recognized in the lungs and how this results in the production of proinflammatory cytokines. Moreover, we review the current knowledge on virus-LPS interactions both *in vitro* and in different laboratory animals.

Chapter 3 deals with the pathogenesis of the respiratory disease caused by the combination of PRRSV infection and LPS in the lungs. In chapter 3.1, we studied the pathogenesis of the PRRSV-LPS interaction on a cellular and cytokine level. More specifically, we examined whether PRRSV synergizes with LPS in the induction of proinflammatory cytokines in the lungs and whether high cytokine levels are associated with the appearance of respiratory signs. For this purpose, gnotobiotic pigs were inoculated intratracheally with PRRSV followed by LPS (20 μg/kg) at 3, 5, 7, 10 or 14 days of infection, and euthanized 6 hours after the LPS inoculation. Control pigs

were inoculated exclusively with PRRSV, LPS or phosphate-buffered saline (PBS). Virus titers, (histo)pathological changes in the lungs, numbers of inflammatory cells and bioactive tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 levels in the bronchoalveolar lavage (BAL) fluids were examined.

As expected, all PRRSV-LPS inoculated pigs developed severe respiratory disease, in contrast to the singly inoculated control pigs. Moreover, PRRSV infection significantly enhanced cytokine production in response to LPS. Peak TNF-α, IL-1 and IL-6 titers were 10 to 100 times higher in PRRSV-LPS inoculated pigs than in the singly PRRSV- or LPS-inoculated pigs and the titers correlated with the respiratory signs. Neutrophil infiltration and pathological changes in the lungs of PRRSV-LPS inoculated pigs resembled the additive effect of the single PRRSV and LPS inoculations without synergy. These data demonstrate a synergy between PRRSV and LPS in the induction of proinflammatory cytokines and an association between these cytokines and disease.

The pathogenesis of the PRRSV-LPS induced disease appears to be similar to the pathogenesis of the PRCV-LPS induced disease. The combination of PRCV and LPS does not markedly enhance neutrophil infiltration or lung lesions, but causes an exaggerated production of proinflammatory cytokines in the lungs, similar to the PRRSV-LPS combination (see chapter 4.2). The profile and quantity of cytokines in the lungs is similar for both combinations, suggesting that both viruses sensitize the lungs to LPS in a similar way.

The study described in chapter 3.2 aimed to confirm the role of proinflammatory cytokines in the induction of respiratory signs. We studied the effect of pentoxifylline, a phosphodiesterase inhibitor, on PRRSV-LPS induced cytokine production and disease. According to the literature, pentoxifylline can suppress the production of TNF-α and other proinflammatory cytokines. The clinical effects of two prostaglandin inhibitors, namely meloxicam and flunixin meglumine, were also examined.

Pentoxifylline, but not the prostaglandin inhibitors, significantly reduced respiratory signs and fever in PRRSV-LPS inoculated pigs from 2 to 6 hours after the LPS inoculation. The levels of TNF-α and IL-1 in the lungs of pentoxifylline-treated PRRSV-LPS inoculated pigs were moderately reduced compared to untreated

PRRSV-LPS inoculated pigs. Still, cytokine levels remained markedly higher than in control pigs inoculated exclusively with PRRSV or LPS. So, the beneficial effect of pentoxifylline on the respiratory disease could not be attributed solely to the limited reduction of proinflammatory cytokines in the lungs. We conclude that pentoxifylline is not a good tool to study the role of proinflammatory cytokines in virus-LPS induced respiratory disease.

The research described in chapter 4 aspired to explain how virus infections can synergize with LPS in the lungs. LPS recognition and inflammation are initialized by the cooperative interplay between the "LPS-binding protein" (LBP), the membrane-bound or soluble forms of CD14 and the recently identified "Toll-like receptor 4" (TLR4). Together, these proteins form the "LPS receptor complex" which is presented in figure 4 on page 20. Our working hypothesis was that virus infection in the lungs leads to LPS sensitisation by increasing the amount of LPS recognition molecules. The earliest recognition of LPS is mediated by LBP and CD14 and binding to these proteins initiates the LPS signaling cascade. We speculated that the amount of both proteins would be limited in "healthy" uninfected lungs and that infection with PRRSV or PRCV would significantly increase their levels.

In the study described in chapter 4.1, we quantified the amount of LBP and cell-associated CD14 in the lungs of pigs throughout a PRRSV infection. Gnotobiotic pigs were inoculated intranasally with PRRSV or PBS (control pigs) and euthanized 1 to 52 days later. The amount of LBP in the BAL fluids was determined with an ELISA and the amount of CD14 expression in lung tissue sections was determined by immunofluorescence microscopy and image analysis.

Infectious virus was detected in the lungs from 1 to 40 days post inoculation (DPI). PRRSV infection caused a clear increase of CD14 expression from 3 to 40 days post inoculation (DPI) and of LBP from 7 to 14 DPI. Both parameters peaked at 9-10 DPI (40 and 14 times higher than in uninfected control pigs, respectively) and were correlated tightly with virus replication in the lungs. Double immunofluorescence labeling demonstrated that resident macrophages expressed little CD14 and that the increase of CD14 expression in the PRRSV-infected lungs was mainly due to infiltration of highly CD14-positive monocytes in the interstitium.

The study of chapter 4.2 aimed to quantify CD14 and LBP in the lungs throughout a PRCV infection and to examine whether increased amounts of CD14 and LBP may be associated with an increased *in vivo* response to LPS. Gnotobiotic pigs were inoculated intratracheally with PRCV or PBS (control pigs) and euthanized 1 to 15 DPI. LBP and cell-associated CD14 were quantified in a similar way as for the PRRSV infection. Moreover, we quantified soluble CD14 in the BAL fluids using a flow cytometric assay. In an additional experiment, pigs were inoculated intratracheally with PRCV and 3 (n = 3) or 7 (n = 4) days later with LPS (20 μg/kg). Control pigs were inoculated exclusively with LPS. Pigs were euthanized 4 hours after the LPS inoculation and levels of TNF-α and IL-6 were determined in the BAL fluids.

Infectious virus was detected in the lungs from 1 to 9 DPI. The amount of cell-associated CD14 in the lungs increased up to 15 times between 1 and 12 DPI. The cell types expressing CD14 varied throughout the infection. High levels of CD14 expression were subsequently found on monocyte-like cells (1-2 DPI), macrophage-like cells (3-12 DPI) and pneumocytes (7-9 DPI). Moreover, soluble CD14 and LBP levels in the BAL fluids increased up to 4 and 35 times between 1 and 12 DPI. In correspondence, we found that part of the PRCV-inoculated pigs developed acute respiratory distress and high cytokine titers (× 6) in the BAL fluids upon LPS exposure at 3 (n = 2) and 7 (n = 2) DPI, which was not seen after LPS exposure of uninfected control pigs. This adds to an earlier study in which we demonstrated an enhanced LPS response at 1 day after PRCV inoculation.

The increase of LBP and CD14 in the lungs during the PRRSV and PRCV infections could thus account for the increased LPS response. This implies that both viruses sensitize the lungs in a similar way. Still, the evolution of LBP levels and CD14 expression during both virus infections differs in two important aspects. Firstly, the amount of LBP and CD14 in the lungs is tightly correlated with the kinetic profile of PRRSV replication, but not with that of PRCV replication. At the late stage of the PRRSV infection (20-40 DPI), virus titers are strongly reduced and at the same time the amount of LBP and CD14 in the lungs is significantly decreased. In contrast, high amounts of LBP and CD14 are found in the lungs at the end of the PRCV infection (7-9 DPI), when most virus has been cleared from the lungs. Secondly, PRRSV and PRCV infections cause an increase of different types of CD14-positive cells in the

lung tissue. Both infections induce infiltration of CD14-positive monocytes, but highly CD14-positive macrophages and type 2 pneumocytes are only found during the PRCV infection.

In conclusion, we found that infection with PRRSV or PRCV sensitizes the lungs of pigs to LPS. Virus-infected pigs that are exposed to an as such "harmless" dose of LPS develop excessive amounts of proinflammatory cytokines in their lungs, together with acute respiratory distress. Further work revealed that infection with PRRSV or PRCV increases the amount of LBP and CD14 in the lungs. We propose that the increase of both components of the LPS receptor complex enhances the early recognition and inflammatory effects of LPS. However, the exact role of LBP and CD14 in the clinical synergy between virus and LPS remains to be proven. Possibly, the interaction between virus infection and bacterial LPS represents one of the catalysts of multifactorial lung disease.

Samenvatting

Het porcien reproductief en respiratoir syndroom virus (PRRSV) en het porcien respiratoir coronavirus (PRCV) zijn respiratoire virussen van het varken die een rol spelen als gangmakers van multifactoriële ademhalingsziekte. Het is grotendeels onduidelijk hoe deze virussen samenwerken met andere agentia bij het tot stand komen van multifactoriële ademhalingsziekte. Om dit te bestuderen, hebben we een experimenteel model op punt gesteld waarbij varkens eerst geïnoculeerd worden met PRRSV of PRCV en vervolgens, na een interval van één of meerdere dagen, een intratracheale toediening van bacterieel lipopolysaccharide (LPS) krijgen. LPS is de belangrijkste ontstekingsverwekkende component van de celwand van Gram-negatieve bacteriën en komt voor in organisch stof van varkensstallen. Virus-geïnfecteerde varkens waarvan de longen blootgesteld worden aan LPS (20 μg/kg) ontwikkelen typisch ernstige ademhalingsziekte. Dit is niet het geval bij niet-geïnfecteerde varkens die aan dezelfde dosis LPS worden blootgesteld. Er is dus een duidelijk synergisme tussen deze virusinfecties en bacterieel LPS bij de inductie van acute ademhalingsziekte.

De doelstellingen van deze thesis waren om meer inzicht te krijgen in de pathogenese van virus-LPS geïnduceerde ademhalingsziekte, met de nadruk op de mogelijke rol van pro-inflammatoire cytokinen als ziektemediatoren, en om na te gaan welke mechanismen aan de basis liggen van de verhoogde LPS gevoeligheid van de longen tijdens virusinfectie.

Hoofdstuk 1 bespreekt de pathogenese van PRRSV en PRCV infecties en de biologische effecten van LPS op de longen. We verklaren hoe LPS wordt herkend in de longen en hoe dit aanleiding geeft tot de lokale productie van pro-inflammatoire cytokinen. Daarenboven bieden we een overzicht van wat gekend is over de interacties tussen virussen en LPS en dit zowel *in vitro* als bij proefdieren.

Hoofdstuk 3 gaat over de pathogenese van de ademhalingsziekte veroorzaakt door de combinatie van een PRRSV infectie en LPS in de longen. De studie in hoofdstuk 3.1 beschrijft de pathogenese van de PRRSV-LPS geïnduceerde ziekte op het niveau van cellen en cytokinen. We hebben ondermeer onderzocht of er een synergisme

bestaat tussen PRRSV en LPS in de inductie van pro-inflammatoire cytokinen in de longen en of hoge cytokinengehalten geassocieerd zijn met het optreden van ademhalingsziekte. Daartoe hebben we gnotobiotische biggen intratracheaal geïnoculeerd met PRRSV gevolgd door LPS (20 μg/kg) 3, 5, 7, 10 of 14 dagen later. Deze varkens werden 6 uren na de LPS toediening geëuthanaseerd. Controlevarkens werden uitsluitend geïnoculeerd met PRRSV, LPS of fosfaat-gebufferde zoutoplossing (FGZ). De longletsels werden macroscopisch en microscopisch beoordeeld. Het aantal ontstekingscellen en de gehalten van bioactief tumor necrosis factor-α (TNF-α), interleukine-1 (IL-1) en IL-6 werden bepaald in de bronchoalveolaire lavage (BAL) vochten.

Zoals verwacht, ontwikkelden de PRRSV-LPS geïnoculeerde varkens duidelijke ademhalingssymptomen in tegenstelling tot de controlevarkens die enkel met PRRSV of LPS geïnoculeerd werden. Daarenboven veroorzaakte de LPS toediening bij de PRRSV-geïnfecteerde varkens een opvallend verhoogde productie van pro-inflammatoire cytokinen in de longen. De piekgehaltes van TNF-α, IL-1 en IL-6 waren 10 tot 100 keer hoger bij PRRSV-LPS geïnoculeerde varkens dan bij varkens die enkel geïnoculeerd werden met PRRSV of LPS. Meer nog, het gehalte van deze cytokinen was gecorreleerd met de ernst van de ademhalingssymptomen. Het aantal neutrofielen in de BAL vochten en de longletsels van PRRSV-LPS geïnoculeerde varkens waren het additief effect van de enkelvoudige PRRSV en LPS inoculaties, zonder een synergistische interactie. Uit deze studie blijkt dat er een synergisme bestaat tussen PRRSV en LPS in de inductie van pro-inflammatoire cytokinen en dat de overmatige productie van deze cytokinen geassocieerd is met het optreden van ademhalingssymptomen.

De pathogenese van de PRRSV-LPS geïnduceerde ziekte vertoont sterke gelijkenissen met deze van de PRCV-LPS geïnduceerde ziekte. De combinatie van PRCV en LPS veroorzaakt eveneens geen opvallende toename van de neutrofieleninfiltratie of longletsels, maar veroorzaakt wel een overmatige productie van pro-inflammatoire cytokinen in de longen, net zoals bij de combinatie van PRRSV en LPS (zie hoofdstuk 4.2). Het cytokinenprofiel in de longen is gelijkaardig voor beide combinaties, wat doet vermoeden dat PRRSV en PRCV de longen op een gelijkaardige manier sensitiseren voor LPS.

De studie in hoofdstuk 3.2 had als doelstelling om het belang van pro-inflammatoire cytokinen als mediatoren van de virus-LPS geïnduceerde ademhalingsziekte te bevestigen. Zo hebben we het effect bestudeerd van pentoxifylline, een fosfodiesterase inhibitor, op de PRRSV-LPS geïnduceerde cytokinen en symptomen. Volgens verschillende literatuurbronnen remt pentoxifylline de productie van TNF-α en mogelijk ook van andere pro-inflammatoire cytokinen. Daarenboven hebben we nagegaan of behandeling met twee verschillende prostaglandineninhibitoren, namelijk meloxicam en flunixine meglumine, een effect heeft op de symptomen.

Pentoxifylline, maar niet de prostaglandineninhibitoren, veroorzaakte een significante onderdrukking van zowel de respiratoire symptomen als de koorts bij de PRRSV-LPS geïnoculeerde varkens in de periode van 2 tot 6 uren na de LPS inoculatie. De titers van TNF-α en IL-1 in de longen van pentoxifylline-behandelde PRRSV-LPS geïnoculeerde varkens waren matig onderdrukt in vergelijking met die van onbehandelde PRRSV-LPS geïnoculeerde varkens. De cytokinentiters waren echter nog steeds significant hoger dan deze van controlevarkens die enkel geïnoculeerd werden met PRRSV of LPS. Het gunstig klinisch effect van pentoxifylline kan dus niet uitsluitend verklaard worden door de beperkte onderdrukking van de cytokinenproductie. Daarom besluiten we dat behandeling met pentoxifylline géén goede strategie vormt om de rol van pro-inflammatoire cytokinen in de virus-LPS geïnduceerde ademhalingsziekte te ontleden.

Het onderzoek dat geschetst wordt in hoofdstuk 4 ambieerde om een verklaring te vinden voor het synergisme tussen virus en LPS in de longen. De herkenning van LPS en het daarmee-geassocieerde ontstekingssignaal worden geïnitieerd door de binding met het “LPS-bindend proteïne” (LBP), CD14 en de recent ontdekte “Toll-like receptor 4” (TLR4). Tezamen vormen deze eiwitten het “LPS receptorcomplex” dat schematisch weergegeven wordt in figuur 4 op pagina 20. Het was onze werkhypothese dat een virusinfectie de longen sensitiseert voor LPS door toename van verschillende “LPS herkenningsmoleculen”. LBP en CD14 zijn verantwoordelijk voor de allervroegste herkenning van LPS en vormen de eerste schakels in de signalisatieketting van LPS. We veronderstelden dat het gehalte van beide eiwitten in

"gezonde" niet-geïnfecteerde longen beperkt zou zijn en dat infectie met PRRSV of PRCV een significante toename van beide LPS herkenningsmoleculen zou induceren.

In de studie beschreven in hoofdstuk 4.1 hebben we de hoeveelheden LBP en cel-geassocieerd CD14 gekwantificeerd in de longen tijdens het verloop van een PRRSV infectie. Gnotobiotische varkens werden intranasaal geïnoculeerd met PRRSV of FGZ (controlevarkens) en 1 tot 52 dagen later geëuthanaseerd. De hoeveelheid LBP in het BAL vocht werd bepaald met behulp van ELISA en de hoeveelheid CD14 in longweefselcoupes werd bepaald met behulp van immunofluorescentiemicroscopie en beeldanalyse.

PRRSV vermeerderde in de longen van 1 tot 40 dagen post inoculatie (DPI). De infectie veroorzaakte een significante toename van de hoeveelheid CD14 van 3 tot 40 DPI en van LBP van 7 tot 14 DPI. Beide parameters piekten op 9-10 DPI (respectievelijk 40 en 14 keren hoger dan bij niet-geïnfecteerde controlevarkens) en waren goed gecorreleerd met de hoeveelheid virus in de longen. Dubbelkleuringen toonden aan dat de longmacrofagen weinig CD14 tot expressie brachten. De toename van CD14 in PRRSV-geïnfecteerde longen werd voornamelijk veroorzaakt door massale infiltratie van sterk CD14-positieve monocyten in het longweefsel.

We hebben eveneens de evolutie van LBP en CD14 bestudeerd in de longen gedurende een PRCV infectie en nagegaan in hoeverre een eventuele toename van beide eiwitten geassocieerd is met verhoogde LPS gevoeligheid. Deze studie wordt beschreven in hoofdstuk 4.2. Gnotobiotische varkens werden intratracheaal geïnoculeerd met PRCV of FGZ (controlevarkens) en 1 tot 15 dagen later geëuthanaseerd. De hoeveelheden LBP en cel-geassocieerd CD14 werden op dezelfde manier bepaald als bij de PRRSV infectie. Daarenboven hebben we ook de hoeveelheid opgelost CD14 in het BAL vocht bepaald met behulp van een flow-cytometrische test. In een bijkomend experiment werden varkens intratracheaal geïnoculeerd met PRCV en 3 (n = 3) of 7 (n = 4) dagen later met LPS (20 μg/kg). Controlevarkens werden uitsluitend geïnoculeerd met LPS. Deze varkens werden 4 uren na de LPS toediening geëuthanaseerd en de gehalten van TNF-α en IL-6 werden bepaald in de BAL vochten.

PRCV vermeerderde in de longen van 1 tot 9 DPI. De hoeveelheid cel-geassocieerd CD14 in het longweefsel nam tot 15 maal toe in de periode van 1 tot 12 DPI. De celtypes die CD14 tot expressie brachten, varieerden naargelang het stadium van de infectie. In het beging van de infectie was er een uitgesproken accumulatie van sterk CD14-positieve monocyten (1-2 DPI) en op latere stadia werden vooral sterk CD14-positive macrofagen (3-12 DPI) en type 2 pneumocyten (7-9 DPI) waargenomen. Daarenboven was er een 4 en 35-voudige toename van de hoeveelheden opgelost CD14 en LBP in de BAL vochten tussen 1 en 12 DPI. In overeenstemming met deze resultaten, vertoonde een deel van de PRCV-geïnfecteerde varkens een sterk verhoogde LPS respons op 3 (n = 2) en 7 (n = 2) DPI. Deze resultaten vullen een vroegere studie aan waarin we reeds een verhoogde LPS gevoeligheid vaststelden bij PRCV-geïnfecteerde varkens op 1 DPI.

De toename van LBP en CD14 in de longen is dus mogelijk verantwoordelijk voor de verhoogde LPS respons tijdens beide virusinfecties. Deze stelling impliceert dat beide virussen de longen op een gelijkaardige manier gevoelig maken voor LPS. Toch zijn er twee belangrijke verschilpunten op het gebied van LBP en CD14 evolutie in de longen. Ten eerste zijn de hoeveelheden LBP en CD14 strak gecorreleerd met het kinetisch profiel van de PRRSV vermeerdering, maar niet met dat van de PRCV vermeerdering. Tijdens de late fase van de PRRSV infectie (20-40 DPI) is de hoeveelheid virus sterk gereduceerd en is tegelijkertijd de hoeveelheid LBP en CD14 in de longen beduidend afgenomen. Daarentegen zijn grote hoeveelheden LBP en CD14 aanwezig in de longen op het einde van de PRCV infectie (7-9 DPI), ondanks dat het meeste virus reeds verdwenen is uit de longen tijdens deze late fase van de infectie. Ten tweede is er een duidelijk verschil in het type cellen dat CD14 tot expressie brengt. Tijdens beide virusinfecties is er een toename van CD14-positieve monocyten, maar alleen tijdens de PRCV infectie worden ook sterk CD14-positieve macrofagen en type 2 pneumocyten waargenomen.

Als <u>besluit</u> kan gesteld worden dat infectie met PRRSV of PRCV de longen sensitiseert voor LPS. Virus-geïnfecteerde varkens die blootgesteld worden aan een op zich “onschadelijke” dosis LPS produceren overmatig veel pro-inflammatoire cytokinen in de longen, wat gepaard gaat met het plots optreden van acute ademhalingssymptomen. Verder onderzoek toonde aan dat infectie met PRRSV of

PRCV een toename van de hoeveelheden LBP en CD14 induceert in de longen. Wij stellen voor dat de toename van beide LPS receptorcomponenten een vroege herkenning van LPS bespoedigt met een verhoogde biologische respons tegenover LPS tot gevolg. De exacte rol van LBP en CD14 in het klinisch synergisme tussen virus en LPS moet echter nog bewezen worden. Mogelijk is de interactie tussen virusinfectie en bacterieel LPS één van de katalysatoren van multifactoriële ademhalingsziekte.

Curriculum vitae

PERSONALIA

Steven Van Gucht werd geboren op 21 april 1976 te Asse. In 1994 beëindigde hij zijn secundaire opleiding aan het Vrij Katholiek Onderwijs (VKO) te Opwijk in de richting Latijn-Wiskunde. In 2000 behaalde hij het diploma dierenarts met grote onderscheiding aan de Faculteit Diergeneeskunde van de Universiteit Gent. Zijn eindwerk ging over de prevalentie van het caniene herpesvirus in Vlaamse kennels en werd bekroond met de Waltham Award 2000. Sinds juli 2000 is hij werkzaam op het Laboratorium voor Virologie, Faculteit Diergeneeskunde, Universiteit Gent. Gedurende het eerste jaar was hij wetenschappelijk medewerker op een project van het Ministerie van Middenstand en Landbouw, getiteld "Porcien reproductief en respiratoir syndroom virusinfectie als gangmaker voor ademhalingsstoornissen bij biggen". In oktober 2001 kreeg hij een onderzoeksmandaat van het Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen. In het kader daarvan voerde hij onderzoek uit naar de interacties tussen virale infecties en bacterieel endotoxine in de longen van het varken en dit werk heeft geleid tot deze doctoraatsthesis. Steven Van Gucht is auteur van 13 wetenschappelijke publicaties, waarvan 5 als eerste auteur. Hij nam actief deel aan verschillende wetenschappelijke congressen en gaf 14 voordrachten over virale ademhalingsziekten aan dierenartsen in diverse landen.

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